EFFECTS OF L-DOPA ON LYSINE COMPARTMENTATION AND PROTEIN SYNTHESIS IN RAT BRAIN

A Thesis submitted by

MARTIN DAVID KING

a candidate for the Degree of

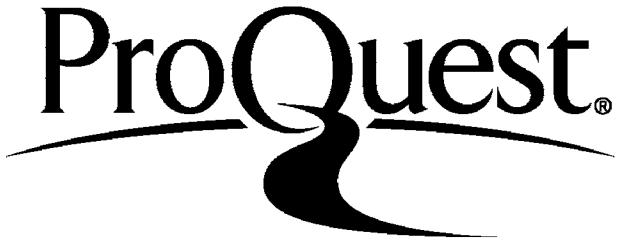
Doctor of Philosophy

in

BIOCHEMISTRY

Department of Biochemistry
Royal Holloway College
University of London
Egham Hill
Egham
Surrey

January 1980



ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr P. W. Beesley for his unending enthusiasm and encouragement while this work was in progress. I am also indebted to Professor J. B. Pridham for his continued interest and support. In addition I owe much to other staff and postgraduate students in the Biochemistry Department at Royal Holloway College for their helpful discussions. My thanks are also due to Mr G. P. Beaumont for his guidance in connection with the statistical treatment of data and who, in collaboration with Mr J. Anderson, provided the results given in Tables A2 and A17. I would like to add a special note of thanks to Dr L. Lim of the Miriam Marks Department of Neurochemistry, The Institute of Neurology, for giving generously of his time and for his invaluable criticisms. I am also grateful to Miss A. Bickel who expertly typed this thesis. Finally, I would like to take this opportunity to thank the Postgraduate Awards Committee of Royal Holloway College for providing financial support.

ABSTRACT

The effect of L-dopa on rat brain protein synthesis was examined by studying the effect of the drug on the incorporation of radioactive amino acids into TCA-precipitable material. In the majority of experiments trace quantities of either L-[3,4(n)-^{3}H] valine or L-[4,5-^{3}H] lysine were administered subcutaneously. Incorporation was expressed in terms of 1) the relative incorporation (I_{rel}) defined as the final TCA-insoluble radioactivity divided by the final TCA-soluble radioactivity, and 2) the apparent incorporation (I_{app}) defined as the final TCA-insoluble radioactivity divided by the final specific activity of the soluble amino acid pool. It is shown that I_{rel} is not, in general, a reliable index of rates of protein synthesis.

L-dopa (500mg/kg,ip), administered 45 min previously, had no significant effect on either the apparent incorporation of L-[^{3}H] valine, measured after a 15 min incorporation period, or that of L-[^{3}H] lysine, measured after a 7½ min incorporation period. The drug did, however, cause a significant 55-59% decrease in the apparent incorporation of L-[^{3}H] lysine measured after a 15 min labelling period. Computer simulation was used to demonstrate that these results can be quantitatively accounted for if it is assumed that L-dopa has no effect on brain protein synthesis but does affect precursor lysine compartmentation. Temperature controlled experiments were performed in order to rule out the possibility that the difference between the effect of L-dopa on lysine and valine incorporation arose artifactually through the failure to control ambient temperature. Further, in order to obtain more reliable estimates of rates of brain protein synthesis, in two of these experiments, rats were given a quantity of L-[U-^{14}C] valine sufficient to maintain a constant precursor specific activity throughout the incorporation period. The results confirmed those obtained at room temperature using trace quantities of labelled amino acids.

While the possibility that L-dopa has a small inhibitory effect on brain protein synthesis could not be ruled out, it was concluded that a drug-induced effect on precursor lysine compartmentation was mainly

responsible for the gross inhibition of lysine incorporation observed in some experiments. Preliminary experiments indicated that a similar effect may occur in rat liver.

CONTENTS

Page No.

TABLE OF FIGURES

LIST OF TABLES

Page No.

Page No.

INDEX OF SYMBOLS AND ABBREVIATIONS

a	Duration of the incorporation period
$b(a)$	Proportionality factor defined by equations (3.2.12) and (4.16)
$C(a)$	Proportionality factor defined by equations (3.2.6) and (4.13)
D	General solution matrix (see equation B.13, Appendix B)
ESCR	External standard channels ratio
F_{ij}	Amino acid flow rate from the ith to the jth compartment
g_{av}	Centrifugal force at r_{av}
GABA	δ-Aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
I_{app}	Apparent incorporation defined by equation (1.13)
I_{rel}	Relative incorporation defined by equation (1.14)
ip	Intraperitoneal
k_{ij}	Rate constant defined by equation (1.3)
λ_j	Eigenvalue (satisfies the secular equation (B.12))
MAO	Monoamine oxidase
NH_4OAc	Ammonium acetate
NS	Not statistically significant
PPO	2,5-Diphenyloxazole
q_{app}	Apparent incorporation quotient defined by equation (3.2.16)
q_{rel}	Relative incorporation quotient defined by equation (3.2.11)
Q_i	Amino acid content of the ith compartment
R_i	Radioactive content of the ith compartment
R_{rel}	Relative incorporation rate defined by equation (3.1.1)
RCP	Radiochemical purity
$\overline{S}(a)$	Time-average specific activity in the time interval 0 to a
S_{ec}	Specific activity of the extracellular amino acid pool
S_{ic}	Specific activity of the intracellular amino acid pool
S_i	Specific activity of the ith amino acid compartment
S_p	Precursor amino acid specific activity
sc	Subcutaneous
s.d.	Standard deviation

t	Time (except in connection with Student's t test)
$t_{\frac{1}{2}}$	Half-life
TCA	Trichloroacetic acid
u	Micro
U_{ij}	Fractional uptake rate defined by equation (4.11)

With the exceptions listed above the biochemical abbreviations used in this thesis are those recognised by the Biochemical Journal (1976) 153, 1-21. Also listed are those mathematical symbols which are used repeatedly throughout the text. The remaining symbols are defined where they appear. Lists of the variable names used in Appendix B are given in Tables B1 and B2.

1. INTRODUCTION

1.1 THE COUPLING OF IMPULSE ACTIVITY AND CELLULAR METABOLISM IN NERVOUS TISSUE

During recent years it has become apparent that maintained synaptic input is essential for the integrity of the functional properties of neurons, and that the dynamic state of each nerve cell within a network is somehow linked with the metabolic state of the neurons with which it makes synaptic contact. It has been suggested that this may be an essential feature of the mechanism by which neurons are integrated to form functional networks (ECCLES, 1973). The importance of neuronal activity in the maintenance of the structure and function of nervous tissue has been demonstrated in both the central nervous system (see for example HUBEL and WIESEL, 1970, and references therein) and at the neuromuscular junction (GUTH, 1968; GUTMANN, 1973, 1976). This implies a coupling of nerve impulse activity with the regulation of the synthesis and degradation of macromolecules. Consequently one of the major goals facing neurobiologists today is the elucidation of the mechanisms involved in the control of macromolecular metabolism in nervous tissue in order to gain some insight into the manner in which this coupling is mediated.

Numerous studies have been made of the metabolic changes that accompany neuronal excitation and inhibition (for reviews see PALLADIN et al., 1977; JAKOUBEK and SEMIGINOVSKÝ, 1970; JAKOUBEK, 1974). Excitation has been achieved mainly through electrical or pharmacological stimulation, although 'physiological stimulation' (acoustic and photic stimulation etc.) has also been employed. Inhibition of neuronal activity is usually achieved pharmacologically. In general, the results of these studies support the hypothesis that impulse activity and cellular metabolism are linked.

The neuromuscular junction has been extensively used in the study of the trans-synaptic regulation of macromolecular metabolism (for reviews see GUTH, 1968; GUTMANN, 1973, 1976), and while there is increasing evidence for the involvement of axonally transported trophic factors in the maintenance of muscle structure (APPELTAUER and KORR, 1975; FERNANDEZ and RAMIREZ, 1974; LENTZ, 1974a, 1974b; OH, 1975, 1976; KUROMI and HASEGAWA, 1975) the hypothesis that acetylcholine itself is the trophic factor as

well as the mediator of conductance changes remains controversial. A dual role for the neurotransmitter substance at other sites has been suggested. For example, GISIGER (1971) found that preganglionic stimulation of the excised rat superior cervical ganglion had a biphasic effect on the incorporation of [5-^{3}H] uridine into RNA, and evidence was provided to support the conclusion that the metabolic signal was given by either the interaction of acetylcholine with its receptor, or the resulting transmembrane flux. GEINISMANN (1971) found that electrical stimulation of the central ends of sectioned dorsal roots resulted in a decreased RNA content of motorneurons and surrounding glia in spinal rats. Antidromic stimulation via the ventral roots was without effect indicating that the synaptic event itself, rather than some later event, was responsible.

In a particularly elegant study, GAINER and BARKER (1974) found that electrical stimulation of the branchial nerve resulted in a selective inhibition of protein synthesis in an identified neuron of the isolated abdominal ganglion of Aplysia californica. A similar response was observed when dopamine was added to the incubation medium, which is particularly significant, since the hyperpolarizing component of the biphasic response to nerve stimulation is possibly mediated by dopamine.

Sandoval and co-workers have studied the metabolism of GABA in relation to cerebral protein synthesis in mice (SANDOVAL and TAPIA, 1975; SANDOVAL et al., 1976) and have suggested that GABA synthesis may be important with respect to the control of brain protein metabolism. GABA was reported to stimulate the incorporation of radioactive amino acids into protein in a cell-free system prepared from rat brain (CAMPBELL et al., 1966), although HEMMINKI (1972) was not able to confirm this. GABA also had no effect on the rate of protein synthesis in isolated immature brain cells (HEMMINKI, 1973). 5-Hydroxytryptamine (5-HT) inhibits protein synthesis in rat brain cell-free systems (CAMPBELL et al., 1966; HEMMINKI, 1972; WIDELITZ et al., 1976) and in isolated rat brain cells (HEMMINKI, 1973). This appears to be consistent with reports that 5-hydroxytryptophan (5-HTP) disaggregates cerebral polysomes via the formation of 5-HT (WEISS et al., 1973, 1975).

The present study of the effect of L-dopa on cerebral protein synthesis was initiated by reports that L-dopa disaggregates brain polysomes, and the suggestion that this effect is mediated via the formation of dopamine and its binding to cytoplasmic receptors (WEISS et al., 1971, 1972, 1974a, 1974b, 1975). On the basis of their results Weiss and co-workers suggested that biogenic amines may affect the integrity of cerebral polysomes and play a direct role in the regulation of brain protein synthesis (WEISS et al., 1972, 1973). In the following review of the effect of L-dopa on cerebral protein synthesis the work of Weiss, Munro, Wurtman and co-workers is discussed in relation to the pharmacology of the central dopaminergic system. In many instances one or two papers are cited as being illustrative of a number of publications supporting a statement, and no attempt is made to provide a comprehensive list of references. The failure to make reference to a particular study signifies nothing more than the difficulty in covering the vast literature that exists on some of the topics discussed. A brief review of recent work on the effect of phenylalanine and tryptophan on brain protein metabolism is included for comparative purposes.

1.1.1 THE EFFECT OF AROMATIC AMINO ACIDS ON CEREBRAL PROTEIN METABOLISM

1.1.1.1 Phenylalanine

The developing brain is affected by a chronic excess of phenylalanine (WAISMAN et al., 1960; CLARKE and LOWDEN, 1969; CHASE and O'BRIEN, 1970). Plasma phenylalanine levels are abnormally high in phenylketonuria, a genetic disorder invariably characterised by a reduction in the activity of hepatic phenylalanine hydroxylase, and untreated phenylketonuria is, in the majority of cases, accompanied by mental retardation. (For brief reviews on phenylketonuria see HSIA, 1976; GAULL et al., 1975).

A deficiency in brain myelin has been associated with a long term excess of phenylalanine, both in phenylketonuria and in experimentally induced hyperphenylalaninaemia (see MORELL et al., 1976; WOOLF, 1970). Whether a defect in myelin protein synthesis is involved, possibly resulting from an impairment of protein synthesis in general, remains a matter for debate. This problem is relevant to the present study not only because phenylalanine is structurally similar to dopa, but also because the painstaking work carried out by some researchers in this field has

clearly demonstrated the problems involved in the study of the effect of drugs on brain protein metabolism. In particular, it has become clear that extreme caution must be exercised in the interpretation of data obtained from experiments in which polysome disaggregation is used as an index of drug-induced inhibition of protein synthesis. In the following pages particular attention is paid to the work of those who have concerned themselves with such problems of methodology.

Following the acute administration of phenylalanine, polysomes isolated from immature rat brain are disaggregated and show a reduced capacity for *in vitro* protein synthesis. Polysomes isolated from older animals are relatively unaffected by the treatment (AOKI and SIEGEL, 1970; SIEGEL *et al.*, 1971; MACINNES and SCHLESINGER, 1971; WONG *et al.*, 1972; TAUB and JOHNSON, 1975).

Polysome disaggregation is accompanied by a decrease in brain tryptophan levels (AOKI and SIEGEL, 1970; SIEGEL *et al.*, 1971; ROBERTS and MORELOS, 1976), which is considered significant because it has been demonstrated that in the liver, tryptophan might be rate-limiting with respect to protein synthesis and therefore affect polysome aggregation (BALIGA *et al.*, 1968 and references therein; SIDRANSKY *et al.*, 1967). AOKI and SIEGEL (1970) found that phenylalanine had no effect on either liver polysomes or liver trytophan levels, which is consistent with the hypothesis that a depletion of tryptophan may be the cause of polysome disaggregation in the brain. A depletion of brain tryptophan was also seen in 28-day-old phenylalanine-treated rats, but this was not accompanied by polysome disaggregation (AOKI and SIEGEL, 1970). Free polysomes are reported to be more sensitive to disaggregation than bound polysomes (TAUB and JOHNSON, 1975), and consequently, AOKI and SIEGEL (1970) suggested that the greater sensitivity to phenylalanine of 7-day rats compared with older animals might be explained on the basis of the observation that free polysomes predominate in immature rat brain, while bound polysomes predominate at 4 weeks (their unpublished observation).

A number of investigators have reported that phenylalanine inhibits the *in vivo* incorporation of amino acids into cerebral protein (MACINNES and

SCHLESINGER, 1971; LINDROOS and OJA, 1971; SWAIMAN et al., 1968). On the other hand, ROBERTS and MORELOS (1976) found that phenylalanine had no effect on the relative incorporation (see Section 1.2 for definition) of intracisternally administered [^{3}H] lysine and [^{14}C] lysine. AGRAWAL et al. (1970) reported that although phenylalanine inhibited the incorporation of [^{35}S] methionine and [^{14}C] leucine into total brain protein, this was accounted for by a parallel reduction in the uptake of these amino acids into the acid-soluble pool. Consistent with this is the observation that phenylalanine had no effect on the entry of [^{14}C] glycine into brain tissue and its subsequent incorporation into protein. Although these results indicate that the synthesis of brain protein is, in general, insensitive to inhibition by phenylalanine, the incorporation of [^{35}S] methionine into myelin protein was inhibited to a greater degree than its incorporation into a myelin-free protein fraction. Agrawal and co-workers therefore suggested that hyperphenylalaninaemia may specifically affect the synthesis of myelin protein.

In a study of the effect of phenylalanine (14mM) on protein synthesis in isolated brain cells, HUGHES and JOHNSON (1976) observed a drug-induced decrease in the incorporation of radioactive amino acids into protein. The magnitude of this decrease was dependent upon the labelled amino acid used. They also found that phenylalanine caused a reduction in the rate of uptake of several amino acids, and a decrease in their intracellular concentrations. A rapid phenylalanine-induced efflux of amino acids was also observed. They suggested that a drug-induced decrease in precursor specific activity accounted for the greater inhibition of incorporation observed with some amino acids compared with others. When a correction was made for differences in precursor specific activity, amino acid incorporation was inhibited by about 20%, which, they suggest, represents a real inhibition of protein synthesis. These results, and those of AGRAWAL and co-workers (1970) clearly demonstrate that drug-induced changes in precursor specific activity can make a very significant contribution to observed changes in protein specific activity. The desirability of using several labelled amino acids in this type of study, preferably belonging to different transport classes is also demonstrated.

ROBERTS and MORELOS (1976) have made a detailed study of the effect of phenylalanine on cerebral polysomes. They found that phenylalanine-induced disaggregation of immature rat brain polysomes was accompanied by a particularly large increase in the size of the disome peak, which they attributed to mild ribonuclease disaggregation. In agreement with this conclusion, they found little or no evidence of drug-induced polysome disaggregation in postmitochondrial supernatants prepared in the presence of rat liver ribonuclease inhibitor. On the basis of these results it was concluded that phenylalanine-induced disaggregation of purified polysomes is an artifact, and occurs after their isolation. Consistent with their assertion is their observation that not only was the ribonuclease activity of postmitochondrial supernatant prepared from immature rat brain greater than that in adult preparations, but that phenylalanine treatment caused an activation of the enzyme in samples prepared from immature but not adult rats. They also provided evidence indicating that polysomes isolated from phenylalanine-treated immature rats are sensitized to ribonuclease degradation. Activation of ribonuclease by phenylalanine was probably a secondary effect, since no change in enzyme activity was observed when the amino acid was added directly to postmitochondrial supernatants. The authors suggested that developmental changes in the stability or concentration of lysosomes (VERITY and BROWN, 1968; SELLINGER and NORDRUM, 1969) might account for the decreasing sensitivity of rats to phenylalanine during the first few weeks after birth. WEINER has suggested (see ROBERTS, 1974) that since lysosomal membranes are stabilized by catecholamines, the ribosome fraction isolated from phenylalanine-treated rats may be more heavily contaminated with lysosomes, and that subsequent lysis of this organelle might account for the observed disaggregation of purified polysomes.

The results of Roberts and Morelos are at variance with those of JOHNSON et al. (1975) who observed no difference in the state of purified polysomes prepared from phenylalanine-treated and control 17-day-old rats, while phenylalanine-induced polysome disaggregation was observed in postmitochondrial supernatants. These authors point out that when

postmitochondrial supernatants are analysed on sucrose gradients, small changes in the size of the monosome peak may be obscured as a result of its incomplete separation from other material in the sample. Consequently they proposed that changes in polysome aggregation be determined by measuring the quantity of active polysomes present in a given amount of postmitochondrial supernatant (expressed as ug of ribosomal protein per mg of postmitochondrial supernatant protein). Using this technique they observed a significant 9% decrease in the quantity of active polysomes present in samples prepared from phenylalanine-treated rats, as compared with their controls.

TAUB and JOHNSON (1975) have argued that ribonuclease degradation is not the cause of phenylalanine-induced disaggregation, since they observed no difference between profiles of freshly prepared and stored samples, whether or not a ribonuclease inhibitor was present. Further, in their study with mice, they observed a phenylalanine-induced increase in the size of the monosome peak only, the quantity of material present as small aggregates remaining relatively unchanged. To provide additional evidence against the involvement of ribonuclease, polysome preparations from control and phenylalanine-treated mice were subjected to KCl dissociation. Monoribosomes present in samples prepared from phenylalanine-treated mice were dissociated into ribosomal subunits, indicating that the phenylalanine treatment caused an increase in the proportion of 'vacant couples' and not an increase in the concentration of 'active monomers'. On the basis of their results TAUB and JOHNSON (1975) postulated that disaggregation of polysomes results from a phenylalanine-induced imbalance between rates of initiation and elongation, such that initiation becomes rate limiting. It is interesting to note therefore, that when HUGHES and JOHNSON (1977) studied the effect of phenylalanine on various mouse brain aminoacyl-tRNAs, they found that phenylalanine caused a significant decrease in the percentage of met-tRNA present in the acylated form. Because met-tRNA$_f^{Met}$ plays an important role in initiation (WEISSBACH and OCHOA, 1976), they suggested that this may be the mechanism by which phenylalanine inhibits protein synthesis in nervous tissue. They separated met-tRNA into three different species and identified the initiator species. Acylation of all three species was inhibited by phenylalanine, the concentration of the acylated initiator species being reduced by about 10%. It is interesting to

note that phenylalanine resulted in an increase in the concentrations of alanyl-tRNA, lysyl-tRNA and leucyl-tRNA, all by about 15%, (although only the first of these increases was statistically significant). The authors suggested that this effect may have been secondary to an inhibition of protein synthesis. Although tryptophan has often been implicated in phenylalanine-induced polysome disaggregation, cerebral tryptophan-tRNA was found to be fully acylated in extracts of both control and phenylalanine-treated mice.

In a later study, HUGHES and JOHNSON (1978) found that brain polysomes recovered naturally 2-4h after phenylalanine administration, although the amino acid remained elevated in the brain, and met-tRNA levels remained depressed. The incorporation of [^{3}H]lysine into protein in brain postmitochondrial supernatants prepared 30 and 120min after phenylalanine administration was lower than in control supernatants, which, because re-initiation does not occur to a significant extent in this system, was attributed to a reduced rate of elongation. On the basis of these results, Hughes and Johnson suggested that the apparent recovery of polysomes 2-4h after drug administration was not the result of a recovery of the protein synthetic machinery. They postulated that phenylalanine inhibits both initiation and elongation, but that with the passage of time, elongation becomes more limiting and thus polysomes reaggregate. According to this hypothesis, the degree of polysome aggregation does not reflect *in vivo* protein synthetic activity. Hughes and Johnson also observed that the administration of a balanced mixture of valine, methionine, tryptophan, leucine, isoleucine and threonine to phenylalanine-treated mice restored the level of acylated met-tRNA and caused polysomes to reaggregate, although brain phenylalanine levels remained high. They therefore suggested that an imbalance of these amino acids may be responsible for the disaggregation of polysomes.

1.1.1.2 L-Dopa and L-5-hydroxytryptophan

WEISS *et al.* (1971) carried out a series of experiments to determine whether L-dopa produces a disaggregation of cerebral polysomes and a concomitant decrease in brain tryptophan similar to that observed after

the administration of phenylalanine. This might be expected because of the structural similarity of the two amino acids. Rat brain polysomes from 15-20g rats (7-9 day-old), were prepared by centrifugation through discontinuous sucrose gradients, analyzed on continuous sucrose gradients and the extent of polysome disruption estimated from the dopa-induced change in the percentage of the total absorbance attributable to trisomes and larger aggregates. L-dopa had no significant effect on the integrity of brain polysomes at a dose of 20 mg /kg (ip), but resulted in a significant disaggregation at 50, 100, 200 and 300 mg/kg. Similarly, brain tryptophan levels were not significantly affected by L-dopa at a dose of 20 mg/kg, but were elevated in rats given 50, 200 and 300 mg/kg. When the amino acid was administered at a dose of 300 mg/kg a significant disaggregation of polysomes was observed 40 and 60min later but not at 20 and 120min, while a significant elevation of brain tryptophan was observed at 20, 40 and 120min. At doses ranging from 100 to 500 mg/kg, L-dopa resulted in a significant increase in brain tryptophan when administered to 100-120g rats, while a substantial disaggregation of polysomes was observed only at 500 mg/kg (WEISS et al., 1971). It can be seen from Table 1.1 that a dose of 500mg/kg is of the same order as that reported to elicit behavioural responses.

TABLE 1.1 BEHAVIOURAL EFFECTS OF L-DOPA IN RATS AND OTHER RODENTS

DOSE	TIME OF INJECTION	RESPONSE	REFERENCE
200mg/kg (approx)	ip	Compulsive gnawing, starting 60-75 min after drug administration and lasting 30-45min. (animal weights 90-100g)	ERNST, 1967
400mg/kg 1200mg/kg	sc sc	Little effect. Hyperactivity and stereotype behaviour.	RANDRUP and MUNKVAD, 1966a
1200mg/kg	sc	Stereotype behaviour starting 75-90min after drug administration and lasting 1-3h. Stereotypy was accompanied by increased locomotor activity. Rage was observed in 2 out of 6 rats.	RANDRUP and MUNKVAD, 1966b
100-200mg/kg	ip	No change in locomotor activity. (animal weights 180-250g)	BUTCHER et al., 1972
≧500mg/kg Mice	ip	Locomotor stimulation.	BLASCHKO and CHRUŚCIEL 1960

All experiments were performed using rats except where indicated otherwise.

In a subsequent series of experiments, WEISS and co-workers (1972) set out to identify the substance responsible for polysome disaggregation. L-dopa gives rise to increased levels of brain dopamine, noradrenaline and 3-O-methyldopa (in addition to dopa itself), and depletes the brain of S-adenosylmethionine. Each one of these changes was considered in turn as the possible cause of polysome disaggregation. The involvement of 3-O-methyldopa was eliminated by the observation that when this material was given intraperitoneally (500mg/kg to 50g rats), its brain concentration rose to a level greater than that observed after the administration of L-dopa itself, but brain polysomes were relatively unaffected. To rule out the possibility that depletion of brain S-adenosylmethionine was the cause of polysome disruption, the effect of D-dopa (500mg/kg) was compared with that of L-dopa. D-dopa, which also undergoes O-methylation, caused a decrease in brain S-adenosylmethionine similar to that observed in L-dopa-treated rats, but the D-isomer had no significant effect on brain polysomes. When L-dopa was administered 30min after the aromatic acid decarboxylase inhibitor RO4-4602, cerebral polysomes isolated 60min after dopa administration were not disaggregated, although brain dopa, 3-O-methyldopa and S-adenosylmethionine levels were similar to those observed in rats given L-dopa alone. This result confirmed that 3-O-methyldopa and depletion of S-adenosylmethionine were not directly responsible for polysome disruption in L-dopa-treated rats, and indicated that the active substance was probably a decarboxylation product. The MAO inhibitor pheniprazine potentiated the L-dopa-induced dissociation of polysomes suggesting that either dopamine or noradrenaline was involved. However, when dopamine or noradrenaline (100ug) was given intracisternally, no polysome disruption was observed although brain amine levels were higher than in those animals given L-dopa (500mg/kg). WEISS and co-workers (1972) point out that the failure of intracisternally administered catecholamines to cause polysome disruption does not exclude the possible involvement of one of these materials as the active substance, since, when injected into the cisterna magna, they may fail to penetrate the brain, leaving the majority of brain cells unaffected.

WEISS et al. (1973) report that L-5-HTP (200-500mg/kg in 50-75g rats) disrupts brain polysomes via a mechanism that parallels that observed in L-dopa treated rats. The effect was not observed in rats given RO4-4602 (800mg/kg) 30min before 5-HTP, and pargyline (10mg/kg) given 120min before the amino acid potentiated disaggregation. It is clear that the mechanism by which L-dopa and 5-HTP cause cerebral polysomes to disaggregate is different from that involved in phenylalanine-induced disaggregation. In the later case, phenylalanine itself is probably the active material, while L-dopa and 5-HTP require decarboxylation. Further, phenylalanine is reported to act via a decrease in brain tryptophan, while tryptophan levels were elevated in dopa-treated rats.

ROEL et al. (1974) studied the effect of L-dopa on the incorporation of L-[U-^{14}C]leucine and L-[U-^{14}C]lysine into brain protein. Rats (40g) were injected intracisternally with radioactive amino acid 45min after the intraperitoneal administration of L-dopa (500mg/kg) or injection medium, and both total homogenate and TCA-insoluble radioactivity measured after a 7, 15 or 30min incorporation period. From the data given in Table 2 of ROEL et al. (1974), calculation shows that the incorporation of [^{14}C]lysine into brain TCA-insoluble material of L-dopa-treated animals was reduced to 26% and 37% of the control level at 7 and 15min, respectively. Roel and co-workers point out that a reduction in precursor specific activity probably contributed to this reduction in TCA-insoluble radioactivity in dopa-treated rats since, in these animals, the size of the soluble lysine pool was 34% greater than in control rats (although the difference was not statistically significant). L-dopa resulted in a decrease in the incorporation of [^{14}C]leucine to 65%, 46% and 38% of the control level at 7, 15 and 30min, respectively. An L-dopa-induced decrease of [U-^{14}C]lysine incorporation into brain soluble protein and tubulin was also demonstrated. BONE (1975) has also studied the effect of L-dopa on the incorporation of radioactive leucine into rat brain protein. Weight-matched 20-day-old rats were injected with L-dopa (150mg/kg) or injection medium, followed 15min later by the injection of L-[U-^{14}C]leucine. The animals were killed after a 15, 30, 45 or 60min incorporation period and the radioactive content of TCA-

precipitable material determined together with the specific activity of the soluble leucine pool. There was no significant difference between the [^{14}C]leucine incorporation levels into the cerebral protein of control and dopa-treated rats at 30, 45 and 60min after dopa administration. At 75min, however, the difference was significant, the incorporation in dopa-treated rats being 88% of the control level. It should be noted that WEISS et al. (1971) failed to observe polysome disaggregation in 19-day-old rats given 200mg/kg of L-dopa.

In their later study, WEISS et al. (1975) set out to determine whether the disaggregation of brain polysomes seen after the administration of L-dopa and L-5-HTP is mediated via central dopamine and 5-HT receptors, respectively. Polysomes were prepared 60min after injecting 50-75g rats with L-dopa (500mg/kg) or L-5-HTP (500mg/kg). Pretreatment with the dopamine antagonist pimozide (25mg/kg,ip) blocked the dopa-induced dissociation of cerebral polysomes, but failed to abolish the 5-HTP effect, while haloperidol (20mg/kg,ip) caused a significant reduction in the disruption of polysomes in both L-dopa and L-5-HTP-treated rats, although the effect was less marked in the latter case. While these observations may indicate that disaggregation is mediated via a receptor that has structural requirements similar to that of the synaptic dopamine receptor, the doses of haloperidol and pimozide used in this study are at least an order of magnitude greater than those required to block the behavioural response to dopaminergic agents (see Section 1.1.2). The 5-HT antagonists methysergide (2mg/kg,ip) and cyproheptadine (10mg/kg,ip) failed to affect the dopa response, but blocked the 5-HTP-induced disaggregation of polysomes. These doses are of the same order as those required to attenuate the behavioural effects of 5-HTP. (see eg. VAN RIEZEN, 1972; KÄRJÄ et al., 1961). The peripheral aromatic amino acid decarboxylase inhibitor MK-486 was used in experiments designed to determine whether the disruption of brain polysomes in dopa-treated rats is mediated centrally or via some peripheral mechanism. On the basis of the observation that brain polysomes were disaggregated in rats given MK-486 plus L-dopa, WEISS et al. (1975) concluded that the effect must be mediated centrally. Inspection of the data given in Table 2 of WEISS et al. (1975) indicates, however, that although brain polysomes isolated from animals given MK-486 plus

L-dopa exhibited a significant disaggregation when compared with polysomes isolated from rats receiving neither drug, no significant difference was observed between the groups receiving MK-486 plus L-dopa and those receiving MK-486 alone. Consequently, the conclusion that a central mechanism is involved must be viewed with caution.

Weiss and co-workers assume that the disaggregation of brain polysomes in L-dopa-treated rats reflects an altered rate of brain protein synthesis (ROEL et al., 1974; WEISS et al., 1975) and have suggested that this may involve either a synaptic dopamine receptor system or some dopamine receptor that is directly coupled with the protein synthesizing machinery. In order to distinguish between these two possibilities apomorphine was administered to rats in doses ranging from 25 to 500 mg/kg. The drug was without effect. They also administered L-dopa (100mg/kg) to 21-day-old rats that had previously received two intracisternal injections of 6-hydroxydopamine (100ug), once on the day of birth and again 2 days later. The 6-hydroxydopamine pretreatment failed to abolish the dopa response. On the basis of these results the authors argue in favour of the soluble receptor mechanism. Further, they suggest that the insensitivity of cerebral polysomes to dissociation by intracisternally administered dopamine is not consistent with the involvement of a synaptic dopamine receptor system. They also point out that the magnitude of the dopa response indicates that the total population of cerebral polysomes is affected, ie., that both neuronal and glial polysomes are disrupted, and that the effect is not limited to those neurons receiving catecholaminergic input. The problems associated with using polysome disaggregation as an index of impaired protein synthesis have been mentioned previously in relation to studies on phenylalanine. Accepting the limitations of this method, the arguments put forward by Weiss and co-workers are now discussed in relation to the pharmacology of the central dopaminergic system.

1) Effect of 6-hydroxydopamine

WEISS et al. (1975) have suggested that according to a scheme involving the synaptic dopamine receptor, "exogenous dopa (or 5-HTP) would be taken up within dopaminergic, noradrenergic or serotoninergic nerve terminals,

and decarboxylated to form dopamine (or serotonin). This amine would then be released into the synaptic cleft, where it might combine with postsynaptic dopamine (or serotonin) receptors...." If this scheme is correct, "it would be expected that pretreatment with intracisternal 6-hydroxydopamine would attenuate the dopa-induced polysome disaggregation." This reasoning is questionable for three reasons.

a) 6-Hydroxydopamine has been extensively used in the study of the behavioural pharmacology of dopaminergic drugs, and the results obtained by some researchers raise doubt concerning the effectiveness of the procedure adopted by Weiss and co-workers. Amphetamine-induced behavioural changes are mediated principally through the release of catecholamines from presynaptic terminals and, at high doses (5-10mg/kg), by a blockade of their reuptake (see for example FUXE and UNGERSTEDT, 1970). EVETTS et al. (1970) found that 6-hydroxydopamine, when administered alone, failed to attenuate the behavioural effects of amphetamine despite a reduction in brain catecholamine levels of 75-80%. On the other hand, FIBIGER (1973) found that when the oxidative deamination of 6-hydroxydopamine was inhibited by the prior administration of a MAO inhibitor, the behavioural response to amphetamine was drastically reduced, and endogenous catecholamine levels fell by as much as 90%. WEISS et al. (1975) report that in their experiments dopamine levels fell from 512ng/g in control rats to 168ng/g in 6-hydroxydopamine-treated rats, a decrease of only 67%. This indicates that a significant proportion of dopamine neurons had survived the treatment.

b) The capacity of the brain to accumulate dopamine following the administration of L-dopa is not impaired after 6-hydroxydopamine (LYTLE et al., 1972, although this work is subject to the above criticism since dopamine levels in 6-hydroxydopamine rats were reduced by only 62%,), which is consistent with reports that the uptake of exogenous dopa and its conversion to dopamine is not restricted to those neurons that normally synthesize dopamine. Dopa and 5-HTP are probably decarboxylated by the same enzyme (KUNTZMAN et al., 1961; BERTLER and ROSENGREN, 1959; GOLDSTEIN et al., 1971) and it has been suggested that dopa is taken up and decarboxylated within 5-HT neurons, especially at high concentrations of the amino acid (BUTCHER et al., 1970, 1972; NG et al., 1970, 1971). In addition brain

capillary wall endothelial cells and pericytes are important sites of decarboxylation (BERTLER et al., 1966; CONSTANTINIDIS et al., 1968 and references therein). ANDÉN et al. (1972) have suggested that dopamine formed in the capillary wall in the brain may diffuse to its effector sites and that capillary wall decarboxylation may therefore be a source of active dopamine.

c) Behavioural responses to L-dopa, which are mediated principally via the synaptic dopamine receptor, are potentiated rather than attenuated by 6-hydroxydopamine pretreatment (UNGERSTEDT, 1971b; CREESE and IVERSEN, 1975). It is generally assumed that potentiation of the response to dopaminergic agents after denervation results from a supersensitivity of the receptor (LANGER, 1975) although alternative explanations have been offered (see for example COOLS and VAN ROSSUM, 1976). BUTCHER et al. (1972) have suggested that because moncaminergic neurons have a large capacity for storing biogenic amines, it may be necessary to saturate these stores before a sufficient extracellular dopamine concentration can be achieved to bring about receptor activation. This explains the high dose of L-dopa required to produce a behavioural response in the rat. At high dopa concentrations leakage of moncamines from neurons can be seen as an increase in extracellular fluorescence. On the basis of these observations Butcher and co-workers postulated that a reduced uptake of catecholamines into dopamine neurons augments receptor sensitization and contributes to the supersensitivity to L-dopa observed in 6-hydroxydopamine-treated rats. This is contrary to the argument put forward by WEISS et al. (1975). Although the mechanism by which the behavioural response to L-dopa is potentiated by 6-hydroxydopamine may not be established, the observation itself suggests that if the dopa-induced disruption of polysomes is mediated via the dopamine synapse, then one should not necessarily expect to observe an attenuation of the response as suggested by Weiss and co-workers.

2) Effect of intracisternal dopamine

WEISS et al. (1972) reported that intracisternally administered dopamine (100ug in 50g rats) had no effect on the state of cerebral polysomes 15 and 45min after drug administration, although at 15min the concentration of dopamine in the brain was greater (6020ng/g) than in those rats given 500mg/kg L-dopa (2230ng/g). Forty-five minutes after administration

brain dopamine levels were not significantly different from the control level. WEISS and co-workers (1972) pointed out that the failure of intracisternal catecholamines to cause disaggregation does not exclude the possibility that these compounds are effector substances in dopa-treated rats since catecholamines injected into the cisterna magna may be distributed only within surface brain areas. In their later paper (WEISS et al., 1975), however, they argue that if the synaptic dopamine receptor is involved in the L-dopa-induced disruption of polysomes, then intracisternal dopamine would be expected to mimic the response.

The distribution of intracranially administered catecholamines has been investigated by a number of researchers. CHALMERS and WURTMAN (1971) studied the fate of intracisternally administered [^{3}H] noradrenaline in the rabbit and found that the regional uptake of radioactivity did not parallel the endogenous distribution of noradrenaline. It was concluded that the regional distribution of [^{3}H] noradrenaline was largely determined by the location of different brain regions relative to the injection site. This is in contrast to the observation that following the injection of [^{3}H] dopamine or [^{3}H] noradrenaline into the lateral ventricle of the rat the greatest accumulation of radioactivity occurred in catecholamine rich brain regions (GLOWINSKI and IVERSEN, 1966). Although the difference between these results may be attributable to the different modes of administration adopted, Chalmers and Wurtman suggested that distance is an important factor in the rabbit because of its size. Distance may not therefore be a significant factor in the rat. Nevertheless, the possibility remains that the failure of intracisternally administered dopamine to disaggregate rat brain polysomes may have arisen because a sufficient concentration of dopamine was not achieved at some distant site.

3) Effect of apomorphine

WEISS et al. (1975) have argued that if the synaptic dopamine receptor is involved in the disaggregation of brain polysomes by L-dopa, then apomorphine would be expected to mimic the response. Experimental data

indicate , however, that the dopamine receptor associated with different brain regions may have different structural requirements for activation and that some receptors are insensitive to apomorphine. Consequently, the observed insensitivity of brain polysomes to disaggregation by apomorphine may not necessarily rule out the involvement of the synaptic dopamine receptor in dopa-induced polysome disruption.

There are two major telencephalic dopamine systems, the nigrostriatal and the mesolimbic systems (ANDÉN et al., 1966; UNGERSTEDT, 1971a; LINDVALL and BJÖRKLUND, 1974). The nigrostriatal pathway originates from the A9 dopamine cell group (see DAHLSTRÖM and FUXE, 1964) situated in the substantia nigra (mainly within the zona compacta, but also in the zona reticulata and the pars lateralis) and the area ventralis tegmenti, and innervates the caudate nucleus, putamen and globus pallidus. Axons of the mesolimbic system originate from the A10 dopamine cell bodies in the ventral mesencephalic tegmentum (situated dorsal to the nucleus interpenduncularis) and innervate the nucleus accumbens, the tuberculum olfactorium and the nucleus interstitialis striae terminalis. Pharmacological stimulation of central dopaminergic mechanisms induces characteristic behavioural changes consisting of stereotyped behaviour (repetitive limb and head movements, sniffing, gnawing, biting and licking) and /or hyperactivity. Attempts have been made to dissociate the hyperactive and stereotyped behavioural responses, and to determine which dopaminergic mechanism mediates which effect. On the basis of an abundance of pharmacological data it has been suggested that the extrapyramidal (ie., nigrostriatal) system is principally involved in stereotypy while the nucleus accumbens is of major importance with respect to hyperactivity (see for example KELLY, 1975). The following observations support this view.

1) The locomotor response to peripherally administered amphetamine was abolished in rats with bilateral 6-hydroxydopamine lesions of the nucleus accumbens, but was not abolished after bilateral lesions of the caudate nucleus. In contrast, lesions of the caudate nucleus but not the nucleus accumbens attenuated the stereotyped behavioural response to amphetamine (KELLY et al., 1975b.)

2) Bilateral injections of amphetamine into the nucleus accumbens caused locomotor stimulation (PIJNENBURG et al., 1976).

3) The bilateral administration of dopamine into the nucleus accumbens of nialimide pretreated rats caused mainly locomotor stimulation, while bilateral administration into the caudate nucleus resulted in a predominantly stereotypic response with less intense hyperactivity (PIJNENBURG and VAN ROSSUM, 1973; JACKSON et al., 1975; PIJNENBURG et al., 1975; COSTALL et al., 1977b). Similar responses were seen in non-pretreated rats (PIJNENBURG et al., 1976)

4) A long lasting locomotor stimulation was observed in rats after receiving bilateral injections of ergometrine into the nucleus accumbens. This was antagonized by haloperidol and pimozide (PIJNENBURG et al., 1973).

5) The dopamine analogue 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (ADTN) produced a strong and long lasting motor stimulation when injected bilaterally into the nucleus accumbens but had no significant effect on motor activity when injected into the caudate nucleus (ELKHAWAD and WOODRUFF, 1975). In contrast, however, COSTALL et al. (1977b) observed both stereotypic and hyperactive responses following the injection of ADTN into both the nucleus accumbens and the neostriatum of nialimide-pretreated rats.

Although some of these results indicate that both the neostriatum and the nucleus accumbens have the potential to mediate both hyperactive and stereotyped behavioural responses to dopamine agonists, they confirm that the hyperactive response is mediated predominantly via the mesolimbic system, and stereotypy principally via the extrapyramidal system. It is of interest to note therefore, that apomorphine (administered both peripherally and intracerebrally) and other aporphine derivatives have repeatedly been shown to lack the ability to induce hyperactivity in normal rats. Only after systemic 6-hydroxydopamine treatment or specific lesions of the nucleus accumbens is hyperactivity normally observed in response to these materials (IVERSEN et al., 1975; KELLY et al., 1975a, 1975b, 1976; COSTALL et al., 1975b, 1977b). This has been attributed to a denervation-induced

change in the sensitivity and/or specificity of the limbic dopamine receptor (COSTALL et al., 1975b). Consequently, the observation that polysome integrity is not affected by peripherally administered apomorphine is not inconsistent with the hypothesis that dopa inhibits cerebral protein synthesis through the activation of a synaptic dopamine receptor, possibly within the nucleus accumbens. However, it is not possible to rule out the involvement of a cytoplasmic dopamine receptor having structural requirements similar to that of the limbic receptor. It would be of interest to determine whether apomorphine disaggregates cerebral polysomes in rats with 6-hydroxydopamine lesions of the nucleus accumbens.

Recent work indicates that the simple concept that stereotypy and hyperactivity are mediated by distinct, homogeneous dopaminergic mechanisms is probably an oversimplification. COSTALL et al. (1977a) have suggested that stereotypy should not be regarded as a single behavioural syndrome caused by the activation of a single mechanism, but that it consists of behavioural components probably involving separate dopamine systems. Further, a few researchers have observed motor stimulation following the peripheral and central administration of apomorphine to normal rats (MAJ, et al., 1972; JACKSON et al., 1975; GRABOWSKA and ANDÉN, 1976), while PIJNENBURG et al. (1976) report inconsistent effects consisting of depressed motor activity in some animals and stimulation in others. Nevertheless, it is clear that when given peripherally, apomorphine may fail to activate those dopamine mechanisms responsible for hyperactivity, and consequently the possibility that the dopa effect on cerebral polysomes is mediated via this apomorphine insensitive mechanism should be considered, since it is not ruled out by the available experimental data.

4) Magnitude of the dopa effect

While the concept that neurotransmitters are directly involved in the regulation of metabolic processes in nervous tissue is not new (see Section 1.1), physiologically this effect must, by definition, be restricted to those neurons receiving the synaptic input in question. WEISS et al. (1975) pointed out, however, that in order to account for the

gross polysome disaggregation observed in dopa-treated rats, it must be assumed that the total population of cerebral polysomes is affected by L-dopa. They therefore argued against the involvement of a synaptic dopamine receptor system. This argument is, in part, valid. The highest concentration of central dopamine receptors is found in the corpus striatum where it has been estimated that only about 15% of striatal synapses are dopaminergic (see SYNDER 1975). Consequently, the extensive polysome disaggregation seen after dopa administration is not compatible with hypotheses implicating a direct coupling of synaptic dopamine receptors with the protein synthesizing machinery. WEISS and co-workers (1971) suggested that the entry of L-dopa into brain cells probably occurs via the neutral amino acid carrier. Accordingly, since there is a ubiquitous requirement for neutral amino acids as precursors of protein, L-dopa would be expected to accumulate within the total population of brain cells. It was further suggested (WEISS et al., 1974a) that intracellular dopa is decarboxylated and that polysome disaggregation is mediated through the interaction of dopamine with an intracellular receptor. If this cellular receptor mechanism is to be implicated in the disruption of cerebral polysomes by L-dopa, one would have to postulate that the soluble dopamine receptor is not restricted to those neurons receiving dopaminergic input, but is common to the majority of neurons and glial cells. In this connection it is interesting to note that SMYTHIES (1970 and references therein) has suggested that biogenic amines (including dopamine and 5-HT) may intercalate between the base pairs of nucleic acids. The cellular receptor hypothesis is not, however, consistent with the observation that dopa decarboxylase is not generally distributed but is mainly localized within the capillary cell wall and monoaminergic neurons (BERTLER et al., 1966; ANDÉN et al., 1972). Consequently, while there appears to be little regional heterogeneity with respect to dopa uptake into the brain following its peripheral administration, the distribution of the resulting exogenous dopamine parallels that of the endogenous amine (BERTLER and ROSENGREN, 1959; PLETSCHER and GEY, 1962).

An alternative possibility that might be considered is that a synaptic dopamine receptor system is indirectly involved in the disruption of polysomes seen after dopa-administration. A number of researchers have

demonstrated the presence of a wide-spread dopaminergic innervation of the telencephalon originating from the mesencephalic dopamine cell group (LINDVALL and BJÖRKLUND, 1974; LINDVALL et al., 1974; FUXE et al., 1974; BERGER et al., 1974, 1976). Although the function of this mesocortical dopamine system remains a matter for speculation, it is interesting to note that dopaminergic transmission appears to play an important part in the regulation of cerebral activity. This is indicated by the following.

1) On the basis of a study of the effect of lesions of catecholaminergic systems on EEG and behavioural waking in the cat, JONES et al. (1973) concluded that two separate systems may be involved in the maintenance of waking. Firstly dopaminergic neurons of the ventral tegmentum, which may be essential for the maintenance of behavioural arousal, and secondly, noradrenergic neurons of the pons and possibly the medulla, which may mediate tonic cortical activity.

2) The neuroleptics chlorpromazine and haloperidol are reported to attenuate the EEG desynchronisation seen after apomorphine and amphetamine administration (VOTAVA and DYNTAROVA, 1970; BRADLEY and HANCE, 1957; CONSROE and WHITE, 1972).

3) FLORIO and LONGO (1971) found that mesencephalic stimulation caused EEG activation in the rabbit. This was inhibited by chlorpromazine, haloperidol and spiroperidol. Further, L-dopa restored the EEG response to mesencephalic stimulation in haloperidol or spiroperidol pretreated animals.

These results indicate that a dopamine synaptic system is an integral part of the diffuse mechanisms involved in the regulation of gross cerebral activity. The hypothesis might therefore be considered that the protein synthesizing machinery is linked in some way to neuronal impulse activity. Thus, in dopa-treated rats, dopamine may act at a well defined site of dopaminergic transmission, causing a change in gross cerebral electrical activity and a concomitant change in brain protein metabolism. MCILWAIN (1970) has presented evidence consistent with the hypothesis that electrical activity and general cellular metabolism in central nervous tissue are linked. The coupling of protein metabolism with nerve impulse activity

might represent just one aspect of this more general regulation.

Intracranial self-stimulation (ICSS) is a phenomenon that is relevant to the above hypothesis since ICSS sites have been shown to be associated with highly diffuse catecholamine systems which project to wide-spread brain regions. Through these diffuse systems a few brain cells have the capacity to control neuronal activity throughout the entire brain. GERMAN and BOWDEN (1974) made a detailed study of the wealth of published data on ICSS and concluded that three of the four major catecholaminergic systems (the mesolimbic and nigrostriatal dopamine systems and the dorsal noradrenaline system) are capable of supporting self-stimulation. Additional evidence for the involvement of dopaminergic mechanisms in ICSS is provided by a number of other researchers (STINUS et al., 1976; PHILLIPS et al., 1976; ROLLS et al., 1974; BROEKKAMP et al., 1975; FOURIEZOS and WISE, 1976). WISE (1978) suggests that a critical dopamine link exists in the systems mediating all rewarding stimulation, but that this link need not be activated directly for stimulation to be rewarding.

In a study of the contrasting effects of hypothalamic and nucleus accumbens self-stimulation on brain stem unit activity and cortical arousal, ROLLS (1971) found that a large population of brain stem units were excited through self-stimulation electrodes placed in the hypothalamus, and that trains of hypothalamic stimuli caused cortical desynchronization. Further, the firing rate of brain stem units driven (probably via polysynaptic pathways) by these trains of stimuli, correlated with the accompanying cortical activation. In contrast, very few brain stem units were excited through self-stimulation electrodes in the nucleus accumbens, and self-stimulation in this region did not cause cortical desynchronization. It is suggested above that the observed insensitivity of brain polysomes to disruption by apomorphine is consistent with the hypothesis that the nucleus accumbens is involved in the disaggregation of polysomes seen in L-dopa-treated rats. It is clear, however, that any hypothesis implicating the nucleus accumbens as a primary site of dopamine action in the disaggregation of brain polysomes by L-dopa is inconsistent with the results of ROLLS (1971) if, according to the hypothesis, inhibition of protein synthesis is the consequence of altered cerebral electrical activity mediated indirectly via the activation of brain stem units.

It is not possible, on the basis of the available experimental data on the

dissociation of cerebral polysomes by L-dopa, to rule out the involvement of either the intracellular mechanism proposed by Weiss and co-workers, or the indirect synaptic mechanism outlined above. According to the hypothesis that a synaptic dopamine system is involved, D-amphetamine is expected to mimic the response. Thus it might be possible to distinguish between the two hypotheses through a study of the effect of this drug.

1.1.2 THE EFFECT OF D-AMPHETAMINE ON CEREBRAL PROTEIN SYNTHESIS

A number of researchers have studied the effect of amphetamine on cerebral protein synthesis but their results are conflicting. MAGOUR et al. (1976) allowed rats free access to a solution of 0.02% D-amphetamine for up to 23 days in order to study the effect of its chronic self-administration on a number of parameters including motor activity and the rate of [^{14}C] leucine incorporation into protein. The daily amphetamine intake increased from 16 to 47mg/kg/day during the 23-day experiment. Locomotor stimulation was observed throughout the experimental period, but at no time did the animals exhibit stereotyped behaviour. The incorporation of [^{14}C] leucine into cerebral slices prepared from amphetamine-treated rats was increased by 28%, 43% and 37% on the second, fifth and tenth days, respectively, but returned to the control level by the sixteenth day. The acute administration of amphetamine (2-10 mg/kg) had no effect on [^{14}C] leucine incorporation in brain slices. Further, the drug was without effect when added directly to the incubation mixture in the concentration range 10^{-4} - 1.0 mM.

DEWAR and WINTERBURN (1973) failed to detect any change in a number of parameters following the acute or chronic administration of amphetamine (0.1 - 25 mg/kg) to 3-month-old rats. No change in RNA content or its rate of degradation, in the rate of RNA transfer from nucleus to cytoplasm, or the rate of [^{14}C]leucine incorporation into cerebral protein was observed. In contrast to these results a number of researchers have reported that the acute administration of amphetamine (8-16mg/kg) causes a disruption of cerebral polysomes and a reduction in their capacity for protein synthesis in cell-free systems (MOSKOWITZ et al., 1975; WIDELITZ et al., 1975, 1976, 1977; BALIGA et al., 1976). MOSKOWITZ et al. (1975) reported that while 15mg/kg D-amphetamine caused a disaggregation of cerebral polysomes in rats aged 26 days or more, larger doses (50mg/kg) were required to cause a similar effect in 7-9-day-old rats. This is not

necessarily inconsistent with the report that the amount of L-dopa required to disrupt rat brain polysomes increases with increasing age (WEISS et al., 1971). Firstly, on the basis of the preliminary observation that, within a group of rats of various ages, polysome disaggregation correlated with brain dopa concentrations, WEISS et al. (1971) suggested that a lower central drug concentration might account for the reduced sensitivity of older rats to dopa-induced polysome disruption. Further, the apparent insensitivity of cerebral polysomes of 7-day-old rats to disaggregation by amphetamine may result from a comparative immaturity of the presynaptic component of the dopamine synapse at that stage in their development. Thus, LAL and SOURKES (1973) report that although both amphetamine and apomorphine-induced stereotyped behaviour in 2-day-old rats, the response was rudimentary. The behavioural response in 18-day-old rats resembled that of the adult, excepting for the prominence of locomotor activity, while the response in 35-day-old animals was indistinguishable from that of the adult rat. LOIZOU (1972) has shown histochemically that while axon proliferation of 5-HT and noradrenergic neurons is greatest during the first three postnatal weeks, terminal proliferation of dopamine neurons occurs gradually over the first four weeks. A lower brain drug level may also contribute to the insensitivity of young rats to peripherally administered amphetamine, since peak brain levels in young adult rats are reported to be twice the level found in immature rats given the same intraperitoneal dose (LAL and FELDMÜLLER, 1975). It is possible, therefore, that a lower drug level coupled with an incomplete development of the dopamine synapse might be the cause of the relative insensitivity of immature rat brain polysomes to amphetamine-induced dissociation.

WIDELITZ et al. (1975) commented on their failure to see polysome disaggregation in rats given 3.5 mg/kg amphetamine, while doses as low as 1.5 and 3.0 mg/kg are reported to induce stereotyped behavioural responses (RANDRUP et al., 1963). They attributed this discrepancy to differences in either the strains of rats used or the injection methods employed, since they failed to observe any significant behavioural changes following the administration of amphetamine in doses of up to 3.5 mg/kg. It should be noted that RANDRUP and MUNKVAD (1970) used a dose of 10 mg/kg to induce a definite behavioural response.

Amphetamine-induced polysome disaggregation is attenuated by the intraperitoneal administration of the neuroleptic drugs pimozide (25mg/kg), haloperidol (15-20mg/kg), chlorpromazine (10-12mg/kg) and thioridazine (25mg/kg) (MOSKOWITZ et al., 1975; WIDELITZ et al., 1977). WIDELITZ et al. (1977) have commented on the observation that, at their maximum effective doses, chlorpromazine is a more potent inhibitor of amphetamine-induced polysome disaggregation than haloperidol, while haloperidol is the more potent neuroleptic. They discussed this in relation to the observed discrepancy between the clinical potency of these materials, their ability to inhibit dopamine-sensitive adenyl cyclase (MILLER and IVERSEN, 1974) and their affinity for dopamine binding sites (CREESE et al., 1975, 1976). CREESE et al. (1975) have explained these inconsistencies in terms of a two-state model of the dopamine receptor. According to this model the potency of a neuroleptic drug is determined by its affinity for the antagonist state of the dopamine receptor, as determined by its ability to compete for [^{3}H]haloperidol binding sites. On this basis, haloperidol is a more potent dopamine antagonist than chlorpromazine. This is consistent with reports that smaller doses of haloperidol are required to inhibit amphetamine-induced stereotyped behaviour as well as other in vivo responses to dopaminergic agents (Table 7 of HORN, 1975). Consequently, the observation that haloperidol is a less powerful inhibitor of amphetamine-induced polysome disruption may indicate that this effect is not mediated via a synaptic dopamine receptor system. Further, MOSKOWITZ et al. (1975) and WIDELITZ et al. (1977) employed doses of neuroleptic drugs at least an order of magnitude greater than that required to inhibit behavioural responses to dopaminergic agents. For example, the ED_{50} (50% inhibitory dose) for haloperidol in amphetamine-treated stereotypic rats is 0.02 mg/kg (HORN, 1975) compared with the 15-20mg/kg dose used by Moskowitz and co-workers. If large quantities were administered because smaller doses were without effect, then this may again indicate that a synaptic dopamine receptor is not involved in the response. Finally, according to the indirect receptor mechanism outlined above, the dissociation of polysomes by dopaminergic drugs is expected to be brain specific, which is consistent with the report that liver polysomes are unaffected by amphetamine (8mg/kg) (WIDELITZ et al., 1975). In contrast, however, BALIGA et al. (1976)report that according to their unpublished results, liver polysomes are disrupted in amphetamine-treated rats.

In an attempt to elucidate the mechanisms involved in the disruption of polysomes by amphetamine a comparison has been made of its effect on initiation-dependent and initiation-independent cell-free protein synthesis. In one such study NOWAK and MUNRO (1977) used homologous initiating and non-initiating systems prepared from rabbit reticulocytes. BALIGA et al. (1976) carried out a similar series of experiments using an initiation-dependent system comprising natural or synthetic mRNA plus wheat germ ribosomal material and soluble factors, and a non-initiating system consisting of a rat liver pH5 enzyme fraction and rat brain or liver polysomes. Elongation was unaffected by amphetamine (4mM in the case of the heterologous brain-liver system and 10mM in the case of the homologous reticulocyte system). This is not necessarily inconsistent with reports that cerebral polysomes isolated from amphetamine-treated rats (3-16mg/kg) exhibit a reduced capacity for cell-free protein synthesis (WIDELITZ et al., 1975, 1976) since polysome disaggregation with no reduction in elongation rates would result in a reduction in protein synthetic capacity. In contrast, however, no significant reduction was observed in the activity of a mouse brain microsomal protein synthesizing system as a result of amphetamine pretreatment in doses of up to 30mg/kg (LOH et al., 1973). One possible explanation for the apparent discrepancy between these two sets of results is that polysomes exhibited a reduced activity because of their degradation during the purification process (see Section 1.1.1.1). Amphetamine ($\geq$ 4mM) inhibits initiation-dependent cell-free protein synthesis (BALIGA et al., 1976; NOWAK and MUNRO 1977). Assuming that in vivo elongation rates remain unchanged, an inhibition of initiation could also account for polysome disruption. If, however, this is the mechanism by which amphetamine causes disaggregation, then drug-pretreatment would be expected to cause a reduction in the activity of the microsomal system of LOH et al. (1973). NOWAK and MUNRO (1977) found that amphetamine inhibited leucyl-tRNA aminoacylation, and, since their elongation system was not dependent upon added tRNA for maximum activity while the initiation-dependent system required added tRNA for maximum activity, they suggested that this might be the cause of the inhibition of initiation-dependent protein synthesis.

BALIGA and co-workers (1976) reported that [^{3}H] amphetamine incubated in the presence of mRNA plus wheat germ extracts became bound to a component

that sedimented near to the 80S ribosome peak on sucrose density gradients. In the absence of messenger no binding was observed. No binding was observed when poly(U) was substituted for the natural messenger, which is consistent with their observation that poly(U)-dependent phenylalanine polymerization was not affected by amphetamine. This is of particular interest since the binding of poly(U) to ribosomes in the presence of a high concentration of Mg^{++} is independent of initiation factors, and differs therefore from the binding of natural messenger (WEISSBACH and OCHOA, 1976). These results are to some extent at variance with those of WIDELITZ and co-workers (1976) who reported that protein synthesis was inhibited in a system consisting of poly(U), rat brain ribosomes prepared from amphetamine-pretreated rats (8mg/kg), and a rat brain ribosome-free enzyme fraction. They also found that, when added directly to the incubation medium amphetamine (final concentration range 2.5-12mM) inhibited polyphenylalanine synthesis. When [^{3}H]poly(U) was added to the amphetamine containing mixture and the incubation products analysed on sucrose gradients radioactivity was localized in the monosome region. In the absence of the drug radioactivity migrated into the polysome region after a 15min incubation. This indicated that, in this system, although ribosomes bound to poly(U) in the presence of amphetamine, they failed to form polysomes.

It should be noted that the concentrations of amphetamine used in these *in vitro* studies were orders of magnitude greater than that required to disrupt polysomes *in vivo*. Thus, a disaggregation of cerebral polysomes was observed 30min after the peripheral administration of 8mg/kg (MOSKOWITZ *et al.*, 1975; WIDELITZ *et al.*, 1975). According to MAICKEL *et al.* (1969) the brain concentration of amphetamine 30min after the peripheral administration of 8mg/kg is about 25ug/g falling to about 13ug/g by one hour. BALIGA and co-workers (1976) argued that the concentration of amphetamine required to inhibit cell-free protein synthesis may not be unphysiological, since a similar concentration may be attained within selected neurons. WEISS and co-workers (1975) have pointed out, however, that if a drug brings about a gross disruption of brain polysomes, then it must affect the majority of brain cells. Consequently, the gross polysome disaggregation seen in amphetamine-treated rats cannot be explained

on the basis of the accumulation of large quantities of the drug within a few selected neurons if it is also assumed that the drug acts directly on the protein synthesizing machinery.

To summarise, amphetamine causes a disaggregation of cerebral polysomes when administered at a dose level similar to that required to elicit behavioural responses. This is consistent with the hypothesis that a synaptic system is indirectly involved in the disruption of cerebral polysomes by dopaminergic agents. Large doses of various neuroleptic drugs inhibit the response. Amphetamine inhibits initiation-dependent cell-free protein synthesis, but the concentrations required are not physiological.

The original purpose of the present study was to gain some insight into the mechanism by which L-dopa disrupts brain polysomes. In particular, it was proposed that standard pharmacological procedures be used to test the hypothesis of WEISS et al. (1975) implicating the involvement of a soluble dopamine receptor and the alternative proposal that the effect is mediated indirectly via a synaptic dopaminergic system. The majority of the conclusions of Weiss and co-workers were based on results obtained from a study of the effect of dopaminergic agents on cerebral polysomes, while the accompanying effects on amino acid incorporation have received relatively little attention. Consequently, a more detailed study of the effect of L-dopa and related materials on the incorporation of amino acids into brain and liver protein was planned. While this work was in progress it was reported that LSD (10-100ug/kg,iv) causes a disruption of rabbit brain polysomes (HOLBROOK and BROWN, 1976, 1977b). The effect was not seen in rabbit spleen and kidney, and rat brain polysomes were unaffected by LSD in doses of up to 1.3mg/kg (HOLBROOK and BROWN, 1976, 1977a). Evidence was presented indicating that the effect was caused by an inhibition of initiation rather than an activation of RNase or premature termination.

These observations are particularly interesting since LSD is reported to stimulate central dopaminergic mechanisms (SPANO et al., 1975; PIERI et al., 1974; VON HUNGEN et al., 1974, 1975). Consistent with the hypothesis that dopaminergic agents cause polysome disaggregation indirectly via the stimulation of a dopamine receptor system and a subsequent change in gross cerebral electrical activity is the report that doses of LSD that caused polysome disruption also resulted in cortical activation (HOLBROOK and BROWN, 1977b). A requirement for increased neuronal activity was also suggested by the observation that the response was inhibited by ethanol or pentobarbital pretreatment. Polysome disaggregation was attenuated by haloperidol (100-500ug/kg) and chlorpromazine (500ug/kg). The low doses of these materials required to inhibit the response and the observed greater sensitivity of haloperidol compared with chlorpromazine are compatible with the involvement of a synaptic dopamine system. The 5-HT antagonists methysergide (2mg/kg) and cyproheptadine (2mg/kg) potentiated the response while pizotyline (500ug/kg) inhibited polysome disruption. The adrenergic antagonists phentolamine (2mg/kg) and propranolol (800ug/kg) completely blocked polysome disaggregation in LSD-treated animals. Holbrook and Brown suggested that these materials blocked synaptic transmission along catecholamine pathways activated by the binding of LSD to some receptor system. The possible involvement of corticosteroids released through stress was also discussed (HOLBROOK and BROWN, 1977a; compare with Section 1.1.3).

1.1.3 THE EFFECT OF HANDLING AND OTHER STRESSFUL STIMULI ON CEREBRAL PROTEIN SYNTHESIS

On the basis of the observation that so many different types of treatments affect the incorporation of radioactive amino acids into brain protein, (for reviews see JAKOUBEK and SEMIGINOVSKÝ, 1970; JAKOUBEK, 1974; PALLADIN et al., 1977) ENTINGH and DAMSTRA (1976) suggested that some common or non-specific mechanism might be involved. In order to determine whether trivial experimental manipulations might trigger these changes, they examined the effect of handling on the incorporation of [^{3}H]lysine into mouse brain protein. Handling consisted of lifting an animal by its tail, immediately placing it in a beaker, and 20sec later gently returning it to its cage. They found that this handling experience caused increases of up to 58% in the incorporation of [^{3}H]lysine into brain protein.

Similarly, REES et al. (1974) found that a number of environmental stimuli, including handling, caused an increase in the relative incorporation (see Section 1.2) of lysine in mouse brain. The importance of these results with respect to any study of brain protein metabolism is obvious. ENTINGH and DAMSTRA (1976) suggested that the effect of various stimuli on brain protein metabolism may combine in a non-linear manner, and that apparently trivial experimental manipulations may push metabolic changes to some ceiling and thus obscure the otherwise positive effect of the treatment being examined.

In the light of the above observations, the administration of radioactive precursors might be expected to have a significant effect on brain protein metabolism. This is substantiated by the report that intracranial puncture inhibits the incorporation into mouse brain protein of peripherally administered [^{3}H]lysine (DUNN, 1975). Furthermore, consistent with the hypothesis that the response to experimental manipulations may, in some cases, obscure the positive effects of the treatment under investigation, Dunn found that whereas electroconvulsive shock caused a profound inhibition of the incorporation into brain protein of peripherally administered radioactive amino acids, no such effect was seen when the amino acids were injected directly into the brain (for references see DUNN, 1975).

A number of researchers have suggested that stress may be a factor that is common to the multitude of treatments reported to affect brain protein metabolism. In particular, stress-induced increases in ACTH output and plasma corticosteroid levels have been implicated in these changes (JAKOUBEK, 1974; JAKOUBEK and SEMIGINOVSKÝ, 1970; see also references cited by SCHOTMAN et al., 1977). If this is so, then the observation that handling affects brain protein synthesis is not surprising, for although gentle handling may ordinarily be regarded as a mild stimulus, it represents a novel and probably stressful experience in the case of laboratory animals not previously subjected to the treatment. In the majority of experiments in the present study, labelled amino acids were administered by subcutaneous injection, mainly because this was regarded as being the least stressful mode of administration. Subcutaneous injections are reported, however, to elevate plasma corticosterone levels

(SCHOTMAN et al., 1977; HODGES and MITCHLEY, 1970). Clearly, the problem of stress-induced artifacts requires consideration in any in vivo study of brain protein metabolism.

1.2 MEASUREMENT OF RATES OF PROTEIN SYNTHESIS

In this section a brief review is made of some of the procedures that have been adopted in the measurement of rates of amino acid incorporation into protein. Precursors labelled with radioisotopes are extensively used in the determination of protein turnover rates, but it has become evident that a meaningful interpretation of the data obtained is often difficult. OJA (1973) has defined very clearly the problems involved, and these are discussed in the following pages making particular reference to the measurement of rates of protein synthesis in central nervous tissue.

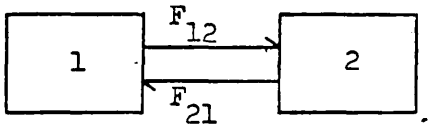


FIG. 1.1 A CLOSED, TWO-COMPARTMENT EXCHANGING SYSTEM

Consider the closed two-compartment system shown in Fig. 1.1. Pool 1 represents the precursor amino acid compartment and 2 represents the protein-bound amino acid pool. Further, assume that the steady-state approximation is applicable. If a trace quantity of radioactive amino acid is introduced into compartment 1 (without perturbing the system) then the rate of appearance of radioactivity in compartment 2 (R_2) is given by

$$\frac{dR_2}{dt} = F_{12}S_1 - F_{21}S_2 \qquad (1.1)$$

where F_{ij} is the amino acid flow rate from pool i to pool j, expressed, for example, in umole/min/g tissue, and S_i is the specific activity of the ith pool. The steady state requirement together with the specification that the system is closed fixes

$$F_{12} = F_{21}. \qquad (1.2)$$

Rate constants can be defined by

$$k_{ij} = F_{ij}/Q_i \qquad (1.3)$$

where Q_i is the size of the ith pool, expressed, for example, in umole/g tissue. Substitution of (1.3) into (1.1) yields

$$\frac{dR_2}{dt} = k_{12}Q_1S_1 - k_{21}Q_2S_2 \qquad (1.4a)$$

$$= k_{12}R_1 - k_{21}R_2. \qquad (1.4b)$$

The rate of change of the specific activity of pool 2 is given by

$$\frac{dS_2}{dt} = \frac{1}{Q_2}\frac{dR_2}{dt} \qquad (1.5a)$$

$$= \frac{Q_1}{Q_2}k_{12}S_1 - k_{21}S_2 \qquad (1.5b)$$

(compare with equation 1 of OJA, 1973).

It is important to emphasize that because the system is in a steady state with respect to the tracee amino acid (ie., because the quantity of tracee in each compartment and its flow rate between the compartments are both constant) these first-order rate equations are valid irrespective of the kinetic behaviour of the system with respect to the tracee. That is, the order of the exchange 'reaction' is unimportant.

Integration of (1.5b) yields

$$S_2(t=a) = \frac{Q_1}{Q_2}k_{12}\int_o^a S_1dt - k_{21}\int_o^a S_2dt. \qquad (1.6)$$

When the incorporation time (a) is sufficiently short that $S_2 \ll S_1$ the second term can be neglected so that

$$S_2(t=a) = \frac{Q_1}{Q_2}k_{12}\int_o^a S_1dt. \qquad (1.7)$$

The mean-value theorem (see pages 51-53 of STEPHENSON, 1966) states that

$$\int_o^a S_1(t)dt = aS_1(e) \qquad (1.8)$$

where $0 \leq e \leq a$. For convenience $S_1(e)$ is written $\overline{S_1}$, and is called the time-average specific activity. Thus (1.7) becomes

$$S_2(t=a) = \frac{Q_1}{Q_2}k_{12}a\overline{S_1(t=a)}. \qquad (1.9)$$

An examination of (1.9) and the assumptions made in its derivation gives a very clear indication of the problems involved in the measurement of rates of protein synthesis. Although, in the following discussion particular reference is made to the study of protein metabolism in the brain, many of the comments are applicable to kinetic tracer experiments in general.

The steady state assumption

While the analysis of results obtained from nonsteady-state, multi-compartment systems is feasible, the equations defining these systems are relatively complicated, and a considerable simplification is achieved if a steady state can be assumed. Although a true steady state cannot be attained experimentally, it should be possible to approach this condition with suitable experimental design. For example, the validity of the steady-state approximation is doubtful when applied to experiments of long duration, and it is therefore desirable to keep incorporation periods short.

It is well established that brain soluble amino acid pool sizes undergo significant changes during the development of the central nervous system (MAKER et al., 1976). In a study of brain protein metabolism during post-natal development, OJA (1967) found that the rate of entry of tyrosine into the brain soluble and protein-bound pools of young, growing rats was not matched by the corresponding efflux rates. This situation persisted until about 14 days after birth. During this period there was a significant deposition of new protein. These results indicate that the steady-state treatment should not be applied to the analysis of results obtained from in vivo experiments using growing animals unless the incorporation period is sufficiently short that these time-dependent changes can be neglected. The free amino acid composition of adult rat brain is reported to remain relatively constant under most conditions (MAKER et al., 1976). Consequently, the assumption that amino acid pool sizes are invariant should be more reasonable when applied to the adult rat brain.

The need to assume constant pool sizes also represents a problem in the analysis of results obtained from brain slice experiments, since the rate at which protein is degraded exceeds that of protein synthesis. As a result of this imbalance there is a net release of amino acids from protein during incubation (JONES and MCILWAIN, 1971; DUNLOP et al., 1974;

NEIDLE et al., 1975). This problem may be effectively overcome by the addition of a sufficient quantity of tracee amino acid that the percentage change in the concentration of the free amino acid in the medium and tissue becomes insignificant.

Precursor specific activity

Absolute rates of protein synthesis can be calculated using equations (1.7) and (1.9) only if the amino acid precursor specific activity is known throughout the incorporation period. Precursor specific activity is expected to be a complex function of time, dependent upon rates of amino acid transport across numerous membranes in the brain and peripheral tissue, as well as the fate of the amino acid at various sites of metabolism. Amino acid metabolism can present an additional problem if the radioactive precursor amino acid is labelled in a position such that significant quantities of other amino acids become radioactive as the original amino acid is metabolised, since various parameters assumed to be time independent will change as significant amounts of radioactivity appear in new amino acids.

Further complication arises from cellular compartmentation, since it is established that structural heterogeneity in brain is accompanied by metabolic heterogeneity. A number of researchers have demonstrated that the incorporation of labelled amino acids into the protein of neuronal cell bodies is rapid compared with the labelling of neuropil protein (for references see ROSE, 1973; HAMBERGER and HENN, 1973). Subcellular compartmentation may add yet another level of complexity since according to ILAN and SINGER (1975) tRNA and aminoacyl-tRNA synthetases may be compartmentalized. This was demonstrated in a series of in vivo experiments using newts in which they measured the specific activity of leucine in nascent polypeptide chains and found that following the administration of [^{3}H] leucine the specific activity of growing polypeptide chains isolated from free polysomes was twice that of polypeptides isolated from membrane-bound polysomes. Clearly, apparent rates of amino acid incorporation into the protein of nervous tissue must be an average of different rates associated with different compartments, and must also be based on some average precursor specific activity.

Having accepted the problems of interpretation that inevitably accompany metabolic compartmentation, the direct measurement of even a tissue-average precursor specific activity is not usually feasible, since this must involve a separation and purification of aminoacyl-tRNA and the measurement of minute quantities of amino acids. Consequently, whilst a few researchers have calculated absolute rates of protein synthesis based on the specific activity of aminoacyl-tRNA, this work has usually been carried out by those whose primary interest is amino acid compartmentation in relation to protein synthesis (see Section 1.3). Various alternative procedures have been adopted, and these are now discussed. Some of these procedures, together with their underlying assumptions, are summarised in Table 1.2.

Although a number of researchers have demonstrated in a variety of tissues that the specific activity of the tRNA-bound amino acid pool may differ from that of the intracellular and extracellular compartments (see Section 1.3), it is common to assume that the specific activity of the total soluble pool is a good approximation to that of aminoacyl-tRNA. As stated above, absolute rates of synthesis can be calculated using equations (1.7) and (1.9) only if the precursor specific activity is known as a function of time. Following the peripheral administration of a trace quantity of labelled amino acid, the specific activity of the brain soluble pool initially increases rapidly and reaches a maximum at a time dependent upon a number of variables, the site of administration being a particularly important factor. The precise measurement of precursor specific activity during this early part of the incorporation period is difficult and DUNLOP *et al.* (1975a) have therefore suggested that measurements be made in an interval of time during which the specific activity of the soluble amino acid decreases monotonically. They showed that rates of protein synthesis determined in this manner are in good agreement with those obtained from flooding experiments (see below). This indicates that it is probably reasonable to assume that the specific activity of the total soluble amino acid pool and the specific activity of aminoacyl-tRNA are the same in the case of a control group of rats. It is important, however, to consider the possibility that various treatments may invalidate this equality.

TABLE 1.2 PROCEDURES ADOPTED FOR THE MEASUREMENT OF ABSOLUTE AND COMPARATIVE RATES OF PROTEIN SYNTHESIS

	Method	Assumptions
1	Injection of a trace quantity of radioactive amino acid. Measurement of the specific activity of aminoacyl-tRNA (S_p) and calculation of a time-average precursor specific activity using equation (1.8). Measurement of TCA-insoluble radioactivity at the end of the incorporation period and calculation of the absolute incorporation rate using (1.9).	Aminoacyl-tRNA is not compartmentalized.
2	As in (1) except that aminoacyl-tRNA specific activity measurements are not made. Instead the specific activity of either the intracellular or the total tissue soluble amino acid pool (S_1) is determined.	As in (1) and that $S_1 = S_p$.
3	Injection of a trace quantity of radioactive amino acid. Measurement of TCA-insoluble radioactivity and the specific activity of either the intracellular or total tissue amino acid pool at the end of the incorporation period. Calculation of the apparent incorporation using equation (1.13).	As in (1) and that within the group of experimental animals $S_1(a) = g\int_0^a S_p(t)dt$ where $S_1(a)$ is the specific activity of the soluble amino acid pool at the end of the incorporation period (ie., when t=a) and g is constant for a given amino acid and incorporation time.

Table 1.2 Continued.....(2)

	Method	Assumptions
4	Injection of a trace quantity of radioactive amino acid. Measurement of TCA-soluble and TCA-insoluble radioactivity at the end of the incorporation period. Calculation of the relative incorporation using equation (1.14).	As in (1) and that within the group of experimental animals $R_1(a) = hS_1(a) = hg\int_o^a S_p(t)\,dt$ where $R_1(a)$ is the final TCA-soluble radioactivity and h is constant for a given amino acid and incorporation period. It is also assumed that tissue extracts do not contain significant amounts of radioactive amino acid metabolites.
5	Injection of a quantity of carrier amino acid with the radioactive tracer sufficient to flood endogenous amino acid pools. Measurement of TCA-insoluble radioactivity and calculation of an absolute incorporation rate assuming the specific activity of the precursor amino acid pool and that of the administered amino acid to be equal.	As in (2) and that high concentrations of amino acids do not affect rates of protein synthesis.
6	Measurement of TCA-insoluble radioactivity after maintaining tissue amino acid pools at a constant specific activity by the continuous intravenous infusion of a radioactive amino acid or by pellet implantation.	As in (2).

In the preceding discussion it is assumed that precursor specific activity is known as a function of time and that the radioactive content of protein is measured only at one point in time. Rearrangement of equation (1.1) yields

$$F = (\frac{dR_2}{dt})/(S_1 - S_2) \qquad (1.10)$$

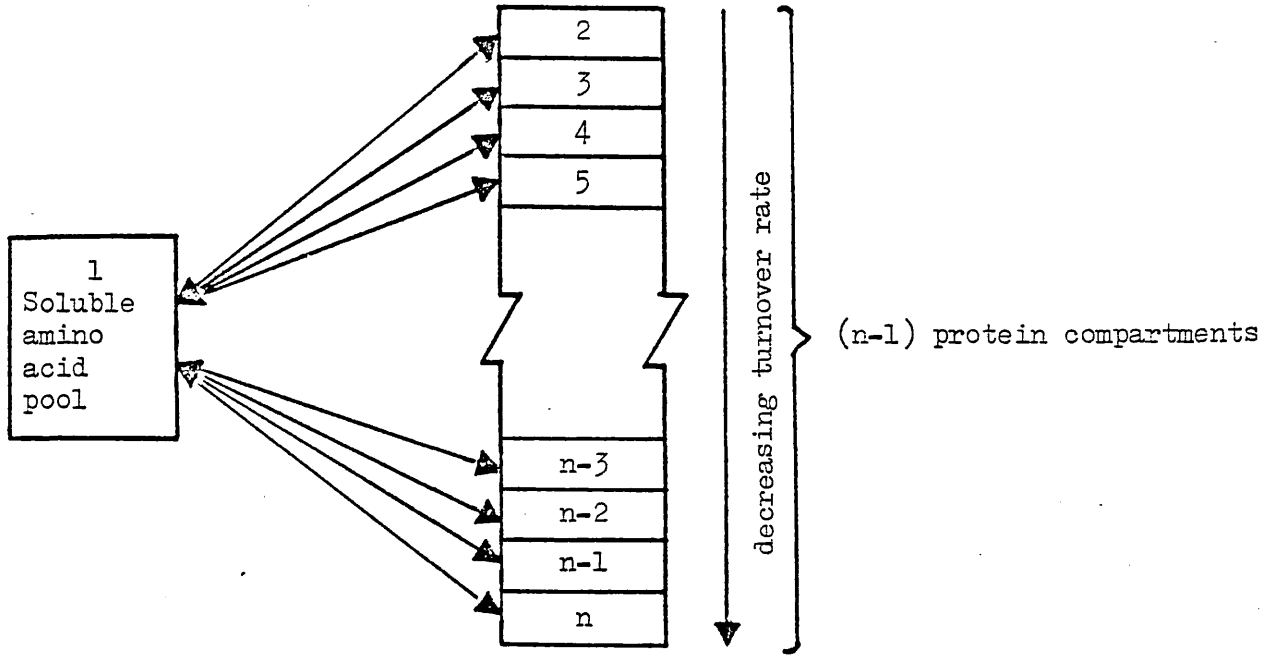
where $F = F_{12} = F_{21}$ (closed steady-state system). Equation (1.10) suggests an alternative approach. The radioactive content of protein may be measured at intervals during an incorporation period and this data used to evaluate (dR_2/dt) at a given point in time. This may be done graphically. Rates of protein synthesis can then be determined on the basis of precursor specific activity measured at that point in time. The advantage of this approach is that the acquisition of the data required for substitution into (1.10) is less time consuming than the laborious task of accumulating sufficient data to accurately define precursor specific activity-time curves. It should be noted, however, that the error associated with (dR_2/dt) will, in general, be much larger than the error in R_2 itself. Consequently, ZILVERSMIT et al. (1943) has recommended that the integral equation (ie., equation (1.7)) be used in preference to the differential form (ie., equation (1.10)) (also see Table 4.2).

In an early study of cerebral protein metabolism, LAJTHA et al. (1957) adopted the approach based on equation (1.10). They found that protein turnover rates apparently became slower as the duration of the incorporation period increased. They interpreted this as resulting from heterogeneity of protein turnover rates. The theoretical basis for this interpretation is as follows. Consider a closed system of n amino acid compartments, one precursor pool (compartment 1) and (n-1) protein pools (compartments 2,3,..,n) of different turnover rates (Fig. 1.2). The rate of change of the total protein-bound radioactivity $(\frac{dR}{dt}\text{prot})$ is given by

$$\frac{dR}{dt}\text{prot} = \sum_{i=2}^{n} \frac{dR_i}{dt} = \sum_{i=2}^{n} (F_{1i}S_1 - F_{i1}S_i) \qquad (1.11a)$$

$$= (\sum_{i=2}^{n} F_{1i})S_1 - \sum_{i=2}^{n} F_{i1}S_i . \qquad (1.11b)$$

FIG. 1.2 A CLOSED n-COMPARTMENT SYSTEM CONSISTING OF (n-1) PROTEIN COMPARTMENTS AND A SINGLE SOLUBLE AMINO ACID POOL



In the mathematical treatment outlined in the main text protein fractions are categorized according to their turnover rates, ie., a given protein is allocated to a compartment in accordance with its turnover rate.

Providing the incorporation period is sufficiently short that $S_i \ll S_1$, i=2,3,..,n, the second term on the right-hand side of (1.11b) can be neglected (NB. $F_{1i} = F_{i1}$), and hence

$$\frac{dR_{prot}}{dt} = \left(\sum_{i=2}^{n} F_{1i}\right) S_1 \qquad (1.12a)$$

$$= FS_1 \qquad (1.12b)$$

where $F = \sum_{i=2}^{n} F_{1i}$ (compare with equation (1.1) with $S_2 \ll S_1$). As the incorporation time is increased, $\sum_{i=2}^{n} F_{i1} S_i$ will become significant. In a situation in which a protein compartment representing only a small percentage of total protein turns over very rapidly compared with the average rate, this term may become significant at a time when the average protein specific activity ($\overline{S}_{prot}$) remains insignificant compared with that of the precursor compartment (S_1). Thus equation (1.10b), which may appear to be a reasonable approximation since $\overline{S}_{prot} \ll S_1$, yields values of F which decrease with increasing time. An alternative explanation for the observed time dependency of F is that it arises, at least in part, as a result of the problem of defining the precursor pool. LAJTHA et al. (1957) calculated protein turnover rates on the basis of the specific activity of the total soluble lysine pool. If precursor lysine and total soluble lysine specific activities are different functions of time, then rates of synthesis based on the later will be time dependent (see Section 4.1). As mentioned above the possibility that different protein pools are linked to separate precursor compartments adds yet another level of complexity. SETA et al. (1973) have pointed out that if a protein compartment exchanges with a very small precursor pool, then the specific activity of the precursor may be maintained at a level significantly lower than that of the large intracellular compartment as a result of its dilution by amino acid released from degraded protein. If the size of this protein compartment or its turnover rate are such that it makes a significant contribution to the total insoluble radioactivity, then turnover rates calculated on the basis of the specific activity of the total soluble amino acid pool will be in error. (Several of these points are examined in some detail in Section 4.1.) It is interesting to note, however, that rates of protein synthesis determined from both intravenous infusion experiments (SETA et al., 1973; GARLICK and MARSHALL, 1972) and flooding experiments (DUNLOP et al., 1975a; GAITONDE and RICHTER, 1956) are not significantly time dependent. This indicates that the major cause of the time dependence observed by LAJTHA and co-workers (1957) was the problem of defining the precursor pool, and not protein heterogeneity.

The construction of amino acid specific activity-time curves is a laborious task that involves the use of relatively large numbers of animals. Consequently, many researchers choose to make specific activity measurements

only at the end of the incorporation period. Calculation of absolute rates of protein synthesis is not then possible, but an "apparent" incorporation (I_{app}) may be calculated using

$$I_{app}(t=a) = R_2(t=a)/S_1(t=a) \qquad (1.13)$$

where $R_2(t=a)$ and $S_1(t=a)$ are the TCA-insoluble radioactivity and the specific activity of the total soluble amino acid pool, respectively, both measured at the end of the incorporation period (ie., when t=a). The use of equation (1.13) is meaningful only if the incorporation period is sufficiently short that loss of protein-bound radioactivity through degradation can be neglected. Further, it is shown in Section 3.2.2.4 that I_{app} is an accurate index of the absolute incorporation rate only if the final specific activity of the acid-soluble amino acid pool and the time-average specific activity of this pool are linearly correlated within the group of experimental animals. Thus, although the procedure based on the use of equation (1.13) may be useful as a means of correcting for differences in the amounts of labelled precursor administered to different animals, when comparing results obtained from groups of animals which have been subjected to different treatments, the possibility should be considered that the treatment may affect the relationship between the final specific activity and the time-average specific activity of the acid-soluble amino acid pool. It is shown in Section 4.3 that, for example, a change in the size of the soluble amino acid pool or an altered rate of precursor uptake into the tissue in question can affect this relationship and thus give rise to misleading results when I_{app} is used as an index of absolute rates of protein synthesis.

A number of researchers have used acid-soluble radioactivity as an index of precursor specific activity (REES et al., 1974; TIPLADY 1972). When this procedure is adopted it is important that a radioactive precursor is chosen which does not produce significant amounts of labelled metabolites. BANKER and COTMAN (1971) recommended that carboxyl-labelled amino acids be used since the radioactive carbon is lost as $^{14}CO_2$ during their metabolism. Alternatively, L-[4,5-^{3}H]lysine and L-[2,3-^{3}H]valine may be employed since ^3H_2O is their only radioactive metabolite that accumulates in significant amounts, and this may be removed by evaporation (BANKER and COTMAN, 1971; TIPLADY, 1972; also see Section 3.1.). A relative incorporation may be calculated using

$$I_{rel}(t=a) = R_2(t=a)/R_1(t=a) \qquad (1.14)$$

where $R_1(t=a)$ is the TCA-soluble radioactivity (after removal of volatile radioactive metabolites). It is shown in Section 3.2.2.3 that I_{rel} is a reliable index of protein synthetic rates only if the time-average precursor specific activity and acid-soluble radioactivity are linearly correlated within the group of experimental animals. The relationship between these two parameters is particularly sensitive to changes in amino acid pool sizes. Consequently, the reliability of I_{rel} determinations made, for example, in the study of drug or environmental effects on protein metabolism is questionable, unless shown experimentally to be otherwise.

Recognition of the problems intrinsic to the measurement of precursor specific activity led to their circumvention through the design of new experimental methods. Continuous intravenous amino acid infusion has been adopted in order to maintain plasma and tissue pools at a constant specific activity (GARLICK and MARSHALL, 1972; SETA et al., 1973). Protein turnover rates were calculated assuming that the specific activity of the total soluble amino acid pool was a good approximation to that of the precursor. A number of other procedures have been suggested. LAJTHA et al. (1976) gave intraperitoneal injections of [^{14}C]tyrosine combined with subcutaneous implantations of [^{14}C] tyrosine pellets in order to maintain the specific activity of plasma and brain tyrosine at a constant level over a period of days. GAITONDE and RICHTER (1956) measured rates of [^{35}S]methionine incorporation into cerebral protein following the intracisternal or subarachnoid administration of large doses (1mg/animal) of the amino acid. Turnover rates were calculated on the basis of the assumption that this dose was sufficient to completely swamp the endogenous brain soluble methionine pool (12ug total) and that precursor specific activity was therefore the same as that of the administered amino acid. In a detailed study of this flooding technique, DUNLOP et al. (1975a) found that in the rat, although the brain valine concentration increased gradually during a 2h period following the intraperitoneal administration of L- [1-^{14}C]valine (10-15 umole/g body wt), its specific activity remained at a constant level of about 90% that of the injected amino acid during the time interval 30 to 120 min. Both Dunlop and co-workers and Gaitonde and Richter found that rates of protein synthesis calculated on the basis of the specific activity of the administered amino acid were time independent, which is consistent with the assumption that brain precursor specific activity is maintained

at a constant level by the injection of flooding quantities of amino acids.

Relatively few researchers have concerned themselves with the measurement of rates of protein degradation in the brain although there is no reason for assuming that control of degradation is unimportant. Rates of degradation are usually determined from the rate of loss of radioactive amino acid from previously labelled protein although the calculation of accurate turnover rates is often not possible because an unknown percentage of labelled amino acid released from degraded protein is reutilized. A number of researchers have considered the possibility that a small percentage of cerebral protein is stable and after deposition remains throughout the lifetime of the brain. In one such study LAJTHA and TOTH (1966) administered a [^{14}C]lysine containing diet to breeding pairs of mice and continued to give this diet to the mothers throughout pregnancy and lactation. Thus, since the offspring had had access to lysine of a constant specific activity throughout their development, the specific activity of brain protein-bound lysine was the same as that of the diet. At 60 days after birth, carrier amino acid was substituted for [^{14}C]lysine and the decrease of radioactivity in protein-bound and soluble lysine determined during the following 150-day period. Lajtha and Toth calculated that 90% or more of cerebral protein had an average half-life of between 10 and 20 days, but because the specific activity of the soluble lysine pool was significant for many days after the label was withdrawn recycling was inevitable. Consequently, the true average turnover rate must have been more rapid than the calculated rate. DAVISON (1961) found that radioactivity was retained in proteolipid protein for up to 250 days after the administration of [1-^{14}C]glycine to neonatal rats, consistent with proteolipid protein being metabolically inert. The author argued that it is unlikely that the apparent stability of proteolipid protein arose artifactually from reutilization of labelled glycine, since this requires that recyclisation was restricted to this protein. Further, relatively little radioactivity was incorporated into proteolipid protein when [1-^{14}C]glycine was administered to adult rats. In contrast, other CNS proteins, with the possible exception of collagen, were shown to be metabolically active in both young and adult rats. This early work has been challenged, however, (see SABRI *et al.*, 1974 for references) and the problem was therefore re-examined (SABRI *et al.*, 1974). In this later study, in order to investigate the possible involvement

of reutilization of label, developing rats were injected with either [^{3}H]lysine or [^{14}C]glucose. Following the administration of [^{14}C]glucose radioactivity is incorporated into protein subsequent to the formation of TCA-cycle amino acids. In contrast to lysine, amino acids derived from the TCA cycle are rapidly metabolised and consequently the problem of recyclization is circumvented. Total brain protein turnover rates calculated on the basis of the rate of loss of protein-bound radioactivity following the administration of [^{14}C] glucose was more rapid ($T_{1/2}$ = 4 days) than that calculated from lysine incorporation data ($T_{1/2}$ = 27 days), indicating that a substantial percentage of [^{3}H] lysine was recycled. The results of this study did, however, confirm the earlier observation that proteolipid protein as well as some myelin basic proteins are relatively stable.

In an early but elegant study of hepatic protein metabolism, SWICK (1958) demonstrated that lysine recyclization also occurs in the liver. In this study, rats were continuously exposed to $^{14}CO_2$ and protein turnover rates were calculated from arginine incorporation data. The author emphasized the advantages of this method. The guanidinium group of free arginine turns over extremely rapidly, CO_2 being swiftly incorporated and then released as urea. According to the estimate that 98% of the guanidinium carbon in free arginine was newly synthesized, the reincorporation of carbon-14 into protein after the release of [guanido-^{14}C]arginine through proteolysis was expected to be minimal. When this work was carried out, the direct measurement of arginine specific activity was not practicable, but maximal and minimal estimates of the rate of labelling of the quanidinium carbon were obtained from the rate of labelling of intracellular CO_2 and urea, respectively. Apparent rates of protein turnover were also calculated using data obtained from experiments in which rats were fed a diet containing [1-^{14}C]lysine. The apparent percentage rate of lysine replacement (ie., the apparent rate of lysine replacement relative to the size of the protein-bound lysine pool) was of the order of three times less than the percentage rate of replacement of the arginine quanidinium carbon. Assuming that the absolute percentage replacement rate was the same for all amino acids Swick estimated that the probability of lysine reutilization was 46-58%. It should be noted, however, that in a situation in which protein pools of a significant size and rich in a particular amino acid turn over at a rate significantly different from the average rate, the absolute percentage replacement rate would not be the same for all amino acids.

In this brief review of some of the methods used in the measurement of rates of protein synthesis particular attention has been paid to problems of methodology and interpretation. Numerous publications continue to appear concerning the effects of a plethora of treatments on protein metabolism in the brain and other tissue. Although the problems associated with this kind of work are now well documented, a few researchers continue to ignor them and assume that rates of radioactive amino acid incorporation into protein are reliable indices of protein synthetic activity. Such an approach is clearly unacceptable unless this assumption is shown to be valid. OJA (1973) has made the following comment in relation to this kind of study. Analysing "rates of brain protein synthesis correctly is a formidable task, and hardly feasible at the present..... It is fairly easy with a very simple experimental design to label cerebral proteins with radioactive amino acids. A meaningful interpretation of the results calls, however, for a laborious investigation which is planned with sober judgement."

1.3 AMINO ACID COMPARTMENTATION AND PROTEIN SYNTHESIS

Amino acid compartmentation is mentioned in Section 1.2 in relation to problems of interpretation of data obtained from tracer experiments. Whilst the compartmentation of the amino acid neurotransmitters has been extensively studied (for reviews see BERL et al., 1975; BALÁZS and CREMER, 1973), other cerebral amino acids have received relatively little attention in this context. There has, however, been considerable interest in amino acid compartmentation in non-nervous tissue in relation to protein metabolism. Because some of the results obtained in the present study are explained in terms of models of compartmentation, several aspects of this subject are discussed in some detail in the following pages.

When animals are infused with carbon-14 labelled amino acids the specific activity of the plasma pool becomes constant but at a level lower than that of the infused material. Similarly, the specific activity of the intracellular amino acid pool of brain and other tissue also becomes constant, but at a level lower than that of the plasma (GAN and JEFFAY, 1967; GARLICK and MARSHALL, 1972; SETA et al., 1973). This phenomenon is observed with essential amino acids, and consequently dilution of the

intracellular pool cannot be attributed to de novo synthesis. Instead, it is generally assumed that [^{12}C] amino acid derived from degraded protein is responsible and that the intracellular pool: plasma specific activity ratio is an index of the contribution that amino acid released from degraded protein makes to the intracellular pool compared with that derived from the plasma. According to this hypothesis only about 40-45% of liver soluble lysine and 77-86% of the brain soluble lysine pool is supplied directly from the plasma (GAN and JEFFAY, 1967; SETA et al., 1973). GARLICK and MARSHALL (1972) carried out a series of infusion experiments in mice using [^{14}C] tyrosine and found that after a 30min infusion period the specific activity of the brain soluble tyrosine pool was 60% that of the plasma pool. During the time interval 30 to 120 min, the brain: plasma specific activity ratio showed little change, although the absolute specific activity of each pool continued to increase exponentially. On the basis of these results the authors concluded that 40% of cerebral intracellular tyrosine was derived from degraded protein.

An alternative explanation for the observed dilution of intracellular amino acid pools is that this phenomenon arises wholly or partly as a result of the presence of nonexchanging intracellular amino acid compartments. Experiments have been performed in order to investigate this possibility. MORTIMORE et al. (1972) reported that when rat liver was perfused with [1-^{14}C]valine (ca. 0.6 mM) the specific activity of the intracellular pool (S_{ic}) became constant after about 6 to 8min at a level approximately 50% that of the extracellular pool (S_{ec}). As the concentration of [^{14}C]valine in the perfusate was increased the ratio S_{ic}:S_{ec} approached unity. Further, the intracellular valine concentration (C_{ic}) was found to be a linear function of the extracellular concentration (C_{ec}) defined by

$$C_{ic} = C_{ec} + 0.4\text{mM}. \qquad (1.15)$$

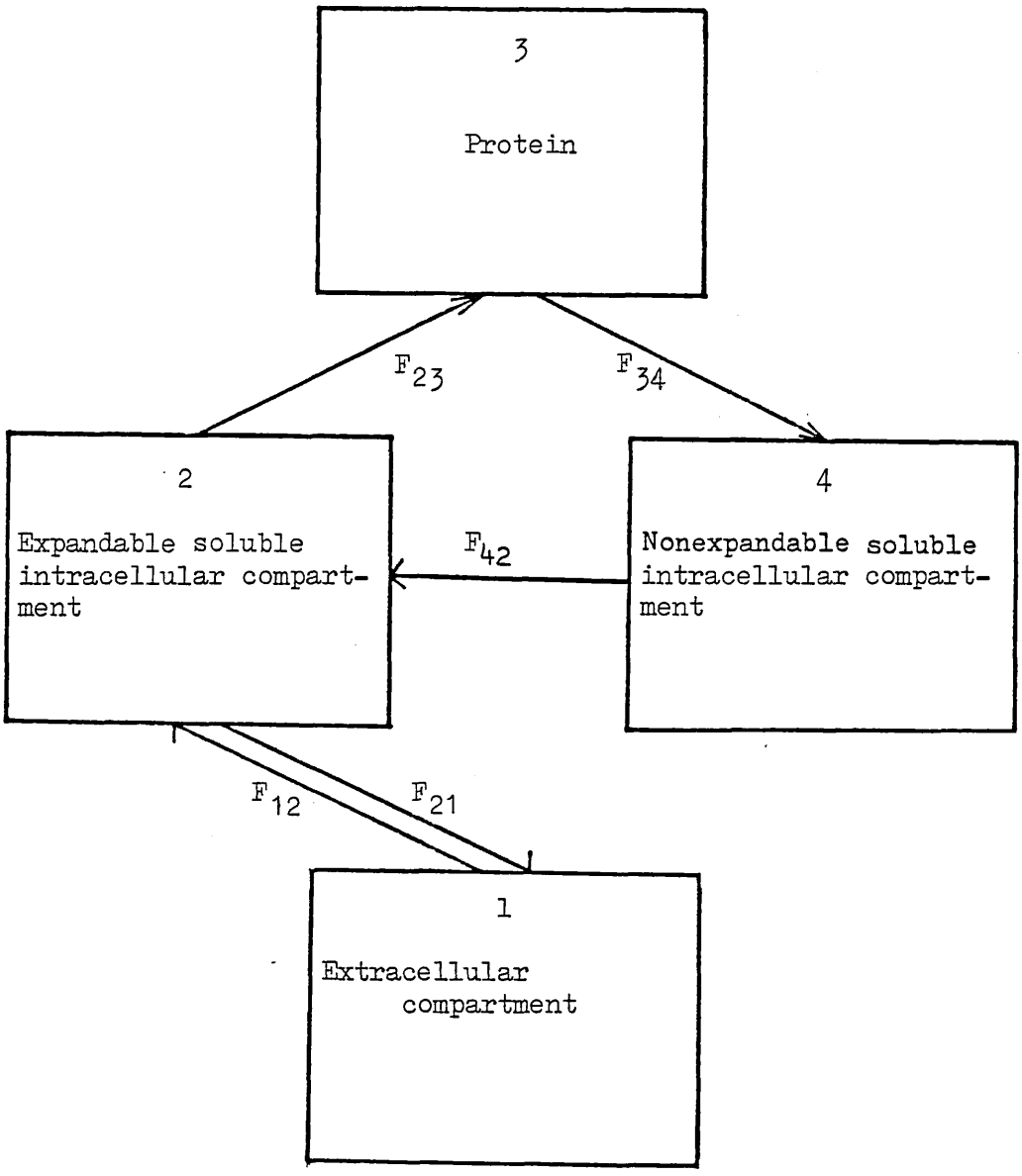
These results are compatible with the hypothesis that the intracellular valine pool is heterogeneous and contains at least one nonexpandable compartment. Apparent rates of valine incorporation were calculated assuming the precursor pool to have a specific activity equal to either that of the intracellular or extracellular pools. With a valine perfusate concentration greater than 5mM there was a good agreement between the two incorporation rates, but as the concentration was reduced apparent rates based on S_{ic}

increased while those based on S_{ec} decreased. On the basis of the assumption that rates of protein synthesis were independent of the valine concentration, this indicated that at concentrations less than 5 mM precursor specific activity was intermediate to that of the intracellular and extracellular pools. The premise that protein synthesis in perfused rat liver is unaffected by increases in free amino acid concentrations has been verified. Thus KHAIRALLAH and MORTIMORE (1976) showed that a valine load had no significant effect on perfused liver protein turnover rates, while PEAVY and HANSEN (1976) reported that the addition to the perfusate of amino acids other than valine at concentrations of up to ten times their plasma levels failed to affect the rate of valine incorporation. It is interesting to note that WOODSIDE and MORTIMORE (1972) found that while the addition of high concentrations of amino acids to the perfusate failed to affect rates of protein synthesis in rat liver, some amino acids suppressed hepatic proteolysis.

MORTIMORE and co-workers (1972) discussed their results in terms of a model in which a nonexpandable intracellular pool receives valine released from degraded protein (Fig. 1.3). Theoretical rates of protein synthesis were calculated assuming the precursor specific activity to be equal to that of the expandable intracellular compartment, while the size of the non-exchanging compartment was varied empirically. With the size of the non-expanding pool set at 0.34umole per ml of intracellular water apparent rates were found to be independent of valine concentration. This result is in good agreement with that given by equation (1.15).

In a later study of valine compartmentation in rat liver, AIRHART et al. (1974) measured the specific activity of intracellular, extracellular and tRNA-bound valine at various times following the intraperitoneal administration of [^{3}H]valine, and found that during the time interval 2 to 15min after injection, the specific activity of the intracellular pool did not exceed 16% that of the extracellular pool. Contrary to the model shown in Fig. 1.3, they attributed this dilution to the continual supply of amino acid from degraded protein. They also suggested, however, that the intracellular pool was heterogeneous, since they found that the specific activity of valyl-tRNA rose more rapidly than that of the soluble intracellular pool. For example, at 2min the specific activity of valyl-tRNA

FIG. 1.3 VALINE COMPARTMENTATION IN PERFUSED RAT LIVER. MODEL 1 (ADAPTED FROM MORTIMORE et al., 1972).

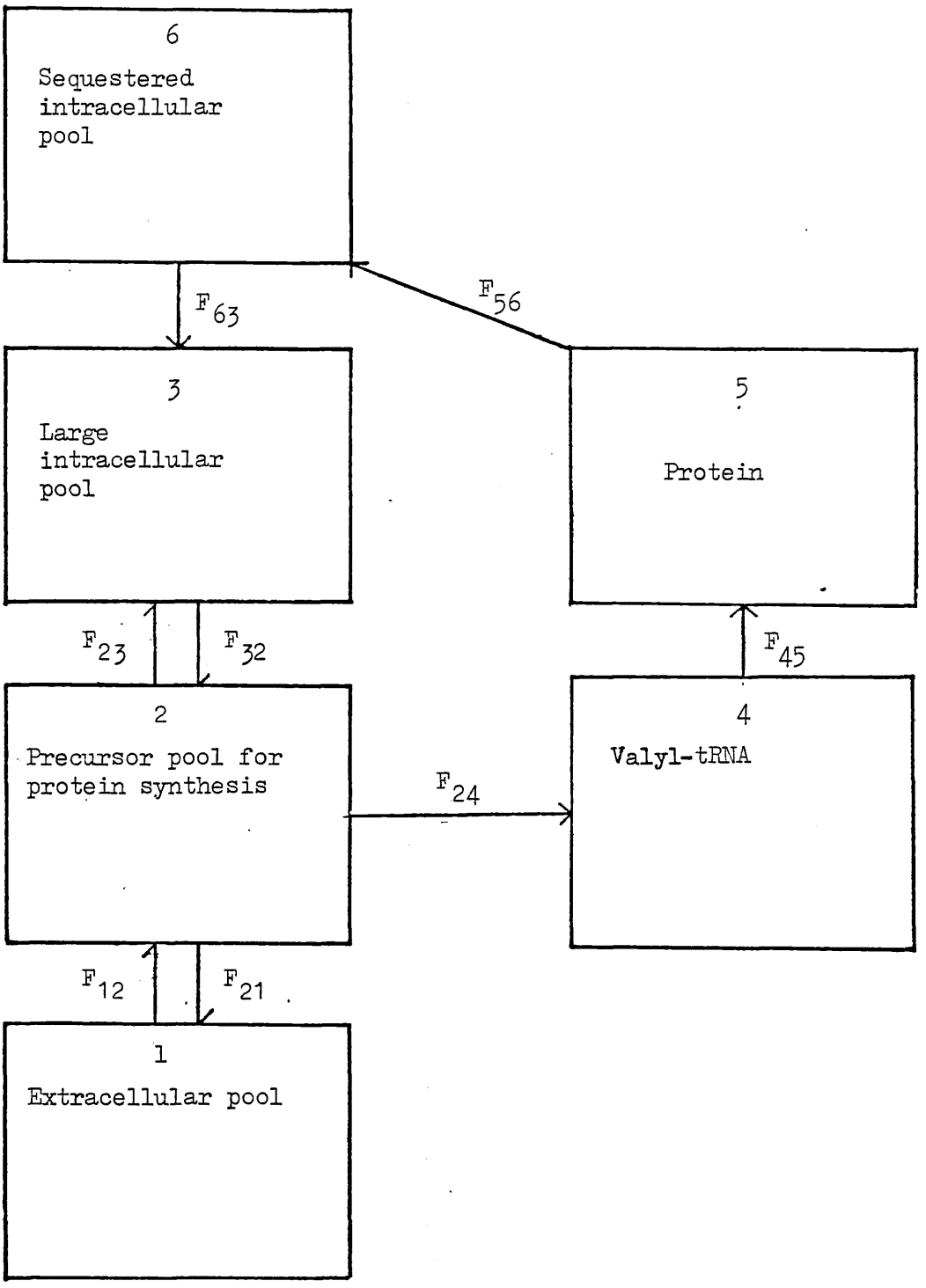


F_{23} and F_{34} are the rates of valine incorporation into and release from protein, respectively. F_{12} and F_{21} are the valine transport rates across the cell membrane.

was approximately twice that of the intracellular pool (and less than 50% that of the extracellular pool). The authors postulated that valyl-tRNA derives its amino acid from a small compartment that is associated with the cell membrane and which receives a supply of valine from both the intracellular and extracellular fluid. AIRHART and co-workers (1974) commented on the compartmental model of MORTIMORE et al. (1972) and pointed out that according to the latter authors' estimate of the size of the non-exchanging compartment a large percentage of intracellular valine is contained within this compartment. The presence of a nonexchanging compartment equivalent to 0.3-0.4 umole per ml of intracellular water is not compatible with the data given in Fig. 1 of VIDRICH et al. (1977), which indicates that the total soluble valine concentration of the liver intracellular pool fluctuates within the range 180 to 325 uM.

To further investigate the mechanism by which amino acids are segregated for protein synthesis KHAIRALLAH and MORTIMORE (1976) performed a series of additional rat liver perfusion experiments. They reported that while rates of valine incorporation calculated on the basis of both extracellular and intracellular specific activities were time dependent, rates based on the specific activity of tRNA were independent of perfusion time. This is consistent with the above hypothesis that neither the intracellular nor the extracellular pools exclusively supply tRNA with amino acid. The authors suggested that the earlier model of MORTIMORE et al. (1972) (see Fig. 1.3) be modified in the light of the observation that the initial rate at which tRNA becomes labelled during perfusion is much faster than the rate of labelling of the intracellular pool (KHAIRALLAH and MORTIMORE, 1976; AIRHART et al., 1974). Their modified model, which combines the hypothesis of AIRHART et al. (1974) with some features of the earlier model of MORTIMORE and co-workers (1972), is shown in Fig. 1.4. In a study of the influence of diurnal changes in hepatic valine concentrations on the specific activity of valyl-tRNA in rats given intraperitoneal injections of [^{3}H]valine, VIDRICH et al. (1977) obtained results which support this modified model. They found that, although the valine concentration of the intracellular and extracellular pools changed continually during a 24h period, the specific activity of valyl-tRNA (S_{tRNA}) was accurately predicted by

FIG. 1.4 VALINE COMPARTMENTATION IN PERFUSED RAT LIVER. MODEL 2 (FROM KHAIRALLAH AND MORTIMORE, 1976).



F_{45} is the rate of valine incorporation into protein and F_{56} the rate of valine release from degraded protein.

$$S_{tRNA} = \left(\frac{[val]_{ec}}{[val]_{ic} + [val]_{ec}}\right)S_{ec} + \left(\frac{[val]_{ic}}{[val]_{ic} + [val]_{ec}}\right)S_{ic} \qquad (1.16)$$

where S_{ec} and S_{ic} are the specific activities of extracellular and intracellular valine, and $[val]_{ec}$ and $[val]_{ic}$ are the concentrations of valine in the extracellular and intracellular fluids, respectively. It was therefore postulated that as amino acids are transported across the cell membrane they are fed into a small membrane compartment and that intracellular and extracellular amino acids enter this compartment in proportion to their concentrations at the membrane surface (hence equation (1.16)). VIDRICH et al. (1977) pointed out that although the transport of branched amino acids in mammalian systems is generally thought to occur by facilitated diffusion, the intracellular valine concentration was found to be greater than that of the extracellular pool for at least 14h a day. This gradient could be maintained through protein degradation only if the rate of proteolysis exceeds the rate of protein synthesis during that period. They dismissed this as unlikely, pointing out that the liver has the additional burden of synthesizing large amounts of protein for secretion. This anomaly is, however, accounted for by their model, according to which the protein synthesizing machinery acts as a metabolic trap through which a concentration gradient may be achieved in the absence of an active transport system.

Amino acid compartmentation in tissue other than liver, and its relationship to the protein synthesizing machinery, has been studied using a variety of preparations (for early references see HIDER et al., 1969). In a series of experiments using the isolated rat extensor digitorum longus muscle (EDL muscle) HIDER et al. (1969, 1971) obtained results indicating that protein-bound amino acids are derived directly from the medium rather than from the intracellular fluid. Thus, they found that when this tissue was incubated in the presence of [^{14}C] leucine or [^{14}C] glycine the incorporation of radioactivity into protein was linear with no apparent lag phase. When the same tissue was transfered to a medium containing [^{3}H] amino acids, the incorporation of carbon-14 stopped abruptly and tritium immediately appeared in protein. The authors concluded that if an intracellular precursor pool does exist in the isolated EDL muscle, it undergoes very rapid exchange with the medium. VAN VENROOIJ et al. (1972) obtained very similar results in a series of experiments using fragments

of rat pancreas. They observed that although the specific activity of the intracellular pool did not reach equilibrium during a 20min incubation in the presence of [^{14}C]leucine or [^{14}C]lysine, the incorporation of radioactivity into protein was linear with no detectable lag phase. Incorporation stopped abruptly when the tissue was transfered to a medium containing carrier amino acid.

The study of the relationship between amino acid compartmentation and protein metabolism in the brain slice preparation has yielded results which are contrary to those just described. DUNLOP et al. (1974) found that the rate of lysine incorporation into the protein of rat brain slices was not linear during a 20min incubation. Further, when slices were transfered to a medium containing carrier lysine, the incorporation of radioactivity did not stop immediately, indicating that in this tissue lysine enters protein via an amino acid pool that does not exchange rapidly with the medium. JONES and MCILWAIN (1971) carried out similar experiments using guinea-pig cerebral cortex slices and reported that precursor leucine specific activity correlated with that of the intracellular soluble leucine pool.

Absolute rates of protein synthesis can be calculated only if the precursor specific activity is known (see Section 1.2), and consequently it is important to define this precursor pool. HIDER and co-workers (1971) showed that in the isolated rat EDL muscle, there exist at least two free amino acid pools, the larger of which exchanges with amino acids in the medium at 37° but not at 2°C, while the smaller pool exchanges with medium amino acids at both temperatures. Using extracellular markers they demonstrated that the smaller pool was equivalent in size to the extracellular space and the larger equivalent to the intracellular space. Similarly, VAN VENROOIJ et al. (1972) reported that the extracellular and intracellular amino acid pools in fragments of rat pancreas could be distinguished by washing the tissue at 0°C. Compatible with the hypothesis that in the rat EDL muscle amino acids incorporated into protein are derived directly from the medium, rates of protein synthesis calculated on the basis of the specific activity of the extracellular glycine were time independent, while those calculated on the basis of the specific activity of intracellular glycine were time dependent (HIDER et al. 1971).

In an attempt to define the precursor amino acid pool for protein synthesis in rat brain slices, DUNLOP et al. (1974) performed a similar series of experiments. They found that if it was assumed that the lysine concentrations of the extracellular space and medium were the same, then there was a discrepancy between the apparent size of the extracellular space calculated on the basis of the amount of lysine extracted at 0°C and that determined using inulin as an extracellular marker. Further, the specific activity of lysine extracted at 0°C was lower than that of the medium. They suggested that this ambiguity might be explained if the pools separated by the cold extraction procedure did not correspond to the extracellular and intracellular pools. Interpretation of their results is further complicated by the observation that the inulin space is heterogeneous (COHEN et al., 1970; LUND-ANDERSEN and MØLLER, 1977). Clearly, it is not possible to define the precursor pool for protein synthesis in the brain slice preparation without a better understanding of the compartmentation of amino acids in this tissue. DUNLOP et al. (1974) demonstrated, however, that when brain slices were incubated in the presence of the labelled amino acid at a concentration sufficient to swamp the endogenous amino acid (ie., 1mM), the precursor specific activity could be assumed to equal that of the added amino acid. Thus rates of protein synthesis could be determined with relative ease.

NEIDLE et al. (1975) have provided evidence for an intracellular lysine compartment in brain that does not rapidly equilibrate with the main intracellular compartment. They found that when mouse brain slices were incubated in the presence of [^{14}C] lysine, after 1h the specific activity of tissue lysine became constant at a level of 58-59% that of lysine in the medium, while the specific activity ratios of other essential amino acids were in the range 73-92% at 1h and 83-94% at 2h. While the authors considered the possibility that this intracellular dilution may have arisen, in part, from the continuous release of [^{12}C] amino acids from degraded protein, they suggested that a nonexchanging sequestered pool was largely responsible. The question as to whether nonexchanging amino acid pools might be associated with different cell types or with subcellular components was also considered, but no definite answer could be given. PORTUGAL et al. (1970) provided direct evidence for an intracellular lysine compartment in

the liver that does not rapidly equilibrate with cytoplasmic lysine. They infused rats with [^{14}C] lysine for up to 180min and measured the specific activities of soluble lysine associated with various subcellular fractions of liver. After a 60min infusion period, the specific activity of intramitochondrial free lysine was approximately 60% that of the cytoplasmic pool. It should be noted, however, that when released by homogenisation, this nonexchanging mitochondrial pool could not cause a large dilution of the total soluble lysine pool, since it accounted for less than 5% of total liver soluble lysine (see Table 1 of PORTUGAL et al., 1970). Lysosomes are believed to be an important site of protein degradation (SEGAL, 1976 ; DEAN and BARRETT, 1976; BALLARD, 1977). Consequently, assuming that the lysosomal and cytoplasmic amino acid pools do not undergo rapid exchange, the specific activity of lysosomal lysine would be expected to be significantly less than that of the cytoplasmic pool. This is consistent with the observations of Portugal and co-workers, since their mitochondrial fraction is expected to have contained lysosomes.

To summarise, a number of researchers have provided evidence for the existence of nonexchanging amino acid compartments in a number of different tissues. These compartments may receive amino acids released from degraded protein. In connection with the present study it is interesting to note that the presence of a particularly large sequestered lysine pool has been demonstrated in the brain. Similarly, the existence of a subcellular lysine compartment that does not readily exchange with the cytoplasmic pool has been demonstrated in the liver. Further, the reutilization of lysine released from degraded liver proteins occurs to an extent greater than that observed with other amino acids. This raises the question as to whether these two phenomena might be related. It is tempting to speculate that lysine released from degraded protein enters a non exchanging compartment, a percentage of which is fed directly back into protein without mixing with the main intracellular pool.

2 MATERIALS AND METHODS

2.1 MATERIALS

Chemicals

Fluram (fluorescamine) was obtained from Roche Products Ltd., Welwyn Garden City, Herts. and radiochemicals from the Radiochemical Centre, Amersham, Bucks. When appropriate the radiochemical purity of labelled amino acids was determined as outlined in Section 2.4.4. Bio-Rad cation-exchange resin was purchased from BIO-RAD Laboratories Ltd., Watford, Herts. and L-dopa from the Sigma Chemical Company Ltd., Kingston-upon-Thames, Surrey. Other chemicals were generally of Analar grade. NCS Solubilizer was purchased from Amersham/Searle, High Wycombe, Bucks.

Animals

All rats used in this study were of the Wistar strain and were either bred in the departmental animal house or purchased from A. Tuck and Son Ltd., Battlesbridge, Essex. During the early part of the study (Section 3.1) rats were fed ad libitum on Dixon .86 and a strict lighting cycle was not in operation. However, because the rats failed to breed and grow well, the diet was changed to Dixon CDD(R) and controlled lighting conditions (dark period 9.00 p.m. - 9.00 a.m.) were introduced. All animals used in the experiments described in Section 3.2 were housed under the latter conditions. Dixon .86 and Dixon CDD(R) were supplied by E. Dixon and Sons Ltd., Ware, Herts. Rats of either sex were used in the experiments described in Section 3.1, while the experiments of Section 3.2 were carried out using only males. Rats were aged between 27 and 42 days (where appropriate the exact ages and body weights are given).

2.2 ANIMAL PROCEDURES AND TISSUE INCUBATION TECHNIQUES

2.2.1 IN VIVO EXPERIMENTS

In vivo L-dopa experiments (Section 3.2)

Rats were weighed on the day previous to the experiment and ranked in order of ascending weight. Those animals with an even rank number were assigned to the control group and those with an odd number to the

L-dopa-group. The animals were then returned to the animal house. All experimental procedures involving live animals were carried out between 11.00 a.m. and 5.00 p.m. L-dopa-treated and control rats were usually housed in separate groups, except in the experiments carried out at a controlled ambient temperature, in which case they were housed individually. L-dopa and control rats were injected alternately in order of ascending rank number using the injection schedule given in Table 2.1 (details of the injection procedure are given below). This routine was adopted in order to facilitate the pairing of L-dopa-treated and control animals (see Section 3.2.2.3). Thus when the animals were allocated pair numbers in order of ascending rank number not only would the two animals of each pair be weight-matched, but they would also have received their injections within a few minutes of each other thus minimising the effect of diurnal rhythms that might otherwise invalidate the pairing procedure. At the end of the incorporation period each animal was stunned by a blow delivered to the back of the neck and killed by cervical dislocation. Brain and liver tissue was removed, washed rapidly in ice-cold water, blotted and frozen in liquid nitrogen. The frozen tissue was stored at -70°C until it was processed as described in Section 2.3.

Injection procedures

L-dopa (usually 500mg/kg) was administered intraperitoneally (21g x 1½ needle) as a suspension (usually 50mg/ml) in 0.05M HCl (WEISS et al., 1975; ROEL et al., 1974). (Details of dose levels etc. are given in the table legends.) Radioactive amino acids were administered in physiological saline by intraperitoneal or subcutaneous injection (25g x 15/16 needle) as indicated. A simple apparatus was constructed for holding rats during the subcutaneous administration of materials. This consisted of a wooden box, open along the top and lined with rubber foam. Rats were placed in this box, leaving both hands free to make the injection. When held in this manner the rats usually lay still, although they were free to move, and showed no signs of stress.

TABLE 2.1 INJECTION SCHEDULES FOR IN VIVO EXPERIMENTS

A. $7\frac{1}{2}$ min incorporation period

Time (min)	Operation
0	inject rat 1 with L-Dopa
15	inject rat 2 with dil. HCl
30	inject rat 3 with L-Dopa
44	inject rat 4 with dil. HCl
45	inject rat 1 with *amino acid
52.5	kill rat 1 and remove tissue
59	inject rat 5 with L-Dopa
60	inject rat 2 with *amino acid
67.5	kill rat 2 and remove tissue
74	inject rat 6 with dil. HCl
75	inject rat 3 with *amino acid
82.5	kill rat 3 and remove tissue
89	inject rat 7 with L-Dopa
90	inject rat 4 with *amino acid
97.5	kill rat 4 and remove tissue etc.

B. 15 min incorporation period

Time (min)	Operation
0	inject rat 1 with L-Dopa
10	inject rat 2 with dil. HCl
20	inject rat 3 with L-Dopa
30	inject rat 4 with dil. HCl
40	inject rat 5 with L-Dopa
45	inject rat 1 with *amino acid
50	inject rat 6 with dil. HCl
55	inject rat 2 with *amino acid
59	inject rat 7 with L-Dopa
60	kill rat 1 and remove tissue
65	inject rat 3 with *amino acid
69	inject rat 8 with dil. HCl
70	kill rat 2 and remove tissue etc.

Table 2.1 Continued.

C. 35 min incorporation period

Time (min)	Operation
0	inject rat 1 with L-Dopa
10	inject rat 2 with dil. HCl
20	inject rat 3 with L-Dopa
30	inject rat 4 with dil. HCl
40	inject rat 5 with L-Dopa
45	inject rat 1 with *amino acid
50	inject rat 6 with dil. HCl
55	inject rat 2 with *amino acid
60	inject rat 7 with L-Dopa
65	inject rat 3 with *amino acid
70	inject rat 8 with dil. HCl
75	inject rat 4 with *amino acid
79	inject rat 9 with L-Dopa
80	kill rat 1 and remove tissue
85	inject rat 5 with *amino acid
89	inject rat 10 with dil. HCl
90	kill rat 2 and remove tissue etc.

Experiments on in vivo amino acid uptake and metabolism (Section 3.1)

The experiments of Section 3.1 were carried out as described above except that no drugs were administered. After removal from the animal, tissue was washed in ice-cold buffer (10mM sodium phosphate pH 7.0 containing 10mM $MgCl_2$) rather than water and stored at -70°C. The tissue was processed as described in Section 2.3.

2.2.2 IN VITRO EXPERIMENTS

Preparation and incubation of chopped brain tissue

Chopped rat brain was prepared essentially as described in detail by MCILWAIN and RODNIGHT (1962). In order to overcome the problems associated with the structural heterogeneity of the brain, chopped tissue was mixed and sampled as described below.

Rats were killed and their brains removed and rinsed in ice-cold buffer (a pre-oxygenated medium containing NaCl (108mM), KCl (4.4mM), $MgSO_4$ (1.3mM), $CaCl_2$ (2.6mM), glucose (12mM), HEPES (25mM), K_3PO_4 (1.2mM) and NaOH (added to bring the final pH to 7.4, final conc. about 12mM) (from Table 1 of DUNLOP et al., 1975b). Two whole rat brains were simultaneously sliced (0.5mm slices) using a McIlwain tissue chopper. The table was then rotated through 90° and a second series of cuts made. The chopped tissue was placed in ice-cold buffer, mixed by gentle agitation and then separated from the buffer by filtration on to filter paper. The filter paper was cut into squares and one square plus its adhering tissue added to each incubation flask containing ice-cold buffer (1ml or 0.8ml if cycloheximide was to be added). In this manner approximately 20mg of tissue was added to each flask. The incubation mixtures were kept on ice and cycloheximide (200ul, 10uM/ml) added to the appropriate flasks. Incubations were started at 1 min intervals by the addition of L-[4,5-^{3}H]lysine (usually 5uCi in 1ml buffer to give a final incubation volume of 2ml) and the transfer of the flasks to a shaking water bath (35°C).

Simple filtration devices were constructed from disposable syringes fitted with porus polyethylene and cut to a length of about 20mm.

A circle of nylon bolting cloth was placed over the porus polyethylene. One filtration apparatus was set up for each incubation. At one minute intervals incubation flasks were removed from the water bath and its contents emptied into a filtration device attached to a water pump. The piece of bolting cloth plus adhering tissue was removed, washed three times in a large volume of ice-cold water (2sec per wash) and dropped into liquid nitrogen. Not more than 15sec elapsed between the removal of each flask from the water bath and the freezing of the tissue. Tissue (plus bolting cloth) was stored at -70°C until it was processed as described in Section 2.3.

In vitro L-dopa experiments

L-dopa experiments were carried out using a modification of the above procedure. Tissue was added to flasks containing ice-cold buffer (0.5ml). L-dopa (various concentrations in 0.5ml of buffer) was added to each flask at 1 min intervals and the flask immediately transfered to a shaking water bath (35°C). Incorporation was started 30min later by the addition of L-[4,5-^{3}H]lysine in buffer (1ml). Incorporation was stopped after a further 30 min as described above.

L-dopa pretreatment experiment

This experiment was carried out according to the schedule given in Table 2.2. Rats were housed at 25°C for one hour before the start of the experiment and were then injected with L-dopa (500mg/kg, 50mg/ml in 0.05M HCl) or with 0.05M HCl (10ml/kg). Forty-one minutes after injection each rat was killed, its brain removed, rinsed in buffer and blotted. One cortical slice was cut from each hemisphere using a cutting guide as described in detail by MCILWAIN and RODNIGHT (1962). Each slice was incubated separately in 2ml of buffer containing 5uCi L-[4,5-^{3}H]lysine. Incubations were stopped after 20 min and the tissue processed as described above.

TABLE 2.2 TIME SCHEDULE FOR THE L-DOPA PRETREATMENT EXPERIMENT

Time (min)	Operation
0	inject rat 1 with dil. HCl
5	inject rat 2 with L-Dopa
10	inject rat 3 with dil. HCl
15	inject rat 4 with L-Dopa
41	kill rat 1 and cut cortical slices
44	start incubation 1a
45	start incubation 1b
46	kill rat 2 and cut cortical slices
49	start incubation 2a
50	start incubation 2b
51	kill rat 3 and cut cortical slices
54	start incubation 3a
55	start incubation 3b
56	kill rat 4 and cut cortical slices
59	start incubation 4a
60	start incubation 4b
64	stop incubation 1a
65	stop incubation 1b
69	stop incubation 2a
70	stop incubation 2b
74	stop incubation 3a
75	stop incubation 3b
79	stop incubation 4a
80	stop incubation 4b

Experimental details are given in the main text. One cortical slice was cut from each cerebral hemisphere of each rat. The two slices were incubated separately (referred to as incubations a and b).

2.3 PREPARATION OF TISSUE EXTRACTS

2.3.1 PREPARATION OF EXTRACTS FOR THE MEASUREMENT OF TCA-PRECIPITABLE AND TCA-SOLUBLE RADIOACTIVITY

In the majority of experiments TCA-insoluble radioactivity was determined using the paper disc method of MANS and NOVELLI (1961) since, using this method, a large number of samples could be processed with ease. In the two experiments in which the "flooding" technique of DUNLOP et al. (1975a) was employed (see Section 3.2.2.9) the specific activity of brain protein was low, and in order to obtain an acceptable count rate it was necessary to process larger amounts of homogenate. For this reason a centrifugation method was used since the size of the disc limited the amount of material that could be processed on paper.

In vivo experiments

Paper disc method

Tissue extracts were kept cool on ice prior to the addition of TCA, after which all procedures were carried out at room temperature. In early experiments (Section 3.1) frozen tissue was added to ice-cold buffer (10mM sodium phosphate pH 7.0 containing 10mM $MgCl_2$, 4ml per brain or 10ml per liver), minced with scissors and sonicated (MSE ultrasonic disintegrator, high power and maximum amplitude, 3 x 1 min pulses separated by 1 min cooling periods). In later experiments frozen tissue was added to water (4ml per brain or 10ml per liver), minced with scissors, disrupted by homogenization and sonicated (1 x 1 min pulse as above). Aliquots were taken and processed as shown in Fig. 2.1.

Centrifugation method

The following procedure is essentially that of DUNLOP et al. (1975a). Tissue was homogenized in sulphosalicylic acid (35ml, 3%, $^{w}/v$) and the precipitate separated by centrifugation (1,000g_{av}, 6min), resuspended in TCA (35ml, 5%, $^{w}/v$) and recentrifuged. The precipitate was again resuspended in TCA (35ml, 5%, $^{w}/v$), heated on a water bath (90°C, 15min)

FIG. 2.1 PAPER DISC METHOD* FOR THE MEASUREMENT OF TCA-PRECIPITABLE AND TCA-SOLUBLE** RADIOACTIVITY

Tissue was processed as described in Section 2.3.1 and samples of the homogenates processed as follows:-

A. Total homogenate	B. Dried homogenate	C. TCA-precipitable material	D. Protein determination*
100ul samples were added to scintillation vials and digested in NCS as described in Section 2.4.1.1.	200ul samples were added to scintillation vials and dried (100°C/3h). Water (100ul/sample) was added and the samples digested in NCS as described in Section 2.4.1.1.	200ul samples were spotted on to paper discs, oven dried (100°C/15min) and treated as follows:- 1) TCA (10%, $^{w}/_{v}$)*** 2) TCA (5%, $^{w}/_{v}$)/90°C/30min. 3) TCA (5%, $^{w}/_{v}$)/room temp. 4) Ethanol/ether (3:1, $^{v}/_{v}$)/37°C/30min 5) Ethanol/ether (3:1, $^{v}/_{v}$) 6) Ether 7) Oven dried (ca. 40°C) 8) The discs were placed in scintillation vials, water (100ul/vial) was added and the protein digested in NCS as described in Section 2.4.1.1.	In vivo experiments**** a. Lowry method 100ul samples of brain homogenates or 50ul samples of liver homogenates were diluted with water (2ml). 100ul aliquots of the diluted samples were treated as described in Section 2.4.3. b. Biuret method 200ul samples of brain homogenates or 100ul samples of liver homogenates were processed as described in Section 2.4.3. In vitro experiments (Lowry method) 100ul samples were processed as described in Section 2.4.3.

* Based on the method of MANS and NOVELLI (1961).

** Involatile TCA-soluble radioactivity = Radioactive content of the dried homogenate (B)-TCA-precipitable radioactivity (C).

*** Discs were stored in 10% ($^{w}/v$) TCA while the remaining tissue homogenates were processed.

**** The Lowry method for the estimation of protein was used in early in vivo experiments while the Biuret method was used in experiments carried out later in the study.

Total homogenate radioactivity (A) was determined only in a few experiments described in Section 3.1. All determinations were made in triplicate.

and again separated by centrifugation. The material was then washed twice with 35ml of each of the following solvents. 1. Methanol 2. Chloroform/methanol (1:1, $^{v}/v$) 3. Ether. After drying under vacuum to constant weight, aliquots (30-40mg) were weighed into scintillation vials and processed as described in Section 2.4.1.1.

In vitro experiments

Tissue (plus bolting cloth) was added to ice-cold water (2ml/sample) and sonicated (MSE ultrasonic disintegrator, maximum amplitude, high power, 2 x 1 min pulses). Aliquots were processed as indicated in Fig. 2.1.

2.3.2 CHROMATOGRAPHIC SEPARATION OF TISSUE ACIDIC AND BASIC MATERIALS

In those experiments in which amino acid measurements were made, care was taken to ensure that hand contamination of samples was kept to a minimum (see HAMILTON, 1965). In general gloves were worn and all apparatus was acid washed.

A separation of tissue acidic and basic materials was performed using Bio-Rad cation-exchange resin AG 50W-X4 100-200 mesh. Prior to use the resin was washed on a Buchner flask, firstly with HCl (4M) until the filtrate was colourless (800-1600ml required per 100g resin) and then with distilled water. The resin was next converted to the sodium form by washing with NaOH (2M) until the filtrate became alkaline and was finally washed with water until the filtrate was approximately pH 8.0. Small columns made from disposable 5ml syringes fitted with porus polyethylene were packed with resin (4.5ml), washed with buffer (10ml, 0.05M sodium phosphate pH6.5 containing 0.1% ($^{w}/v$) EDTA) and then with water (10ml). Tissue homogenates were prepared for application to the columns as follows. A solution of TCA (40%, $^{w}/v$) was added to the tissue homogenates (0.25ml/ml homogenate) and the mixtures heated (80°C for 20min) on a water bath. After removing the precipitates by centrifugation (ca. $1000g_{av}$, 15min) the supernatants were adjusted to pH 2.0 with NaOH (ca. 2M) and applied to the cation-exchange columns. After eluting acidic material with water (a total of 15ml of

eluate collected per column), amino acids and other basic materials were eluted with NH_4OH (0.88 ammonia/water 1:3, $^{v}/_{v}$, 2 x 10ml fractions). In the experiments described in Section 3.1.3 aliquots of the acidic and basic eluates were counted as described in Section 2.4.1. In those experiments in which amino acid purification was carried out (either for the measurement of amino acid specific activity (Section 3.2.) or for the determination of the radiochemical purity of basic extracts (Section 3.1.3)) the basic eluates were evaporated to dryness under nitrogen while heating on a water bath, and the residue redissolved in water (0.5ml) and adjusted to about pH 2.0 with HCl (Ca. 10M).

2.3.3 PURIFICATION OF AMINO ACIDS

Tissue lysine, valine or leucine was purified as required using a Joel amino acid analyzer. The analyzer was operated in a manner that gave resolution of only that part of the amino acid spectrum required. The composition of buffers and the programmes used are given in Table 2.3. Samples were prepared as described above (Section 2.3.2). The analyzer was operated with the detector pump turned off and the appropriate part of the eluate fractionated and assayed for amino acid content and /or radioactivity as described in Sections 2.4.2 and 2.4.1, respectively. Before each run the performance of the analyzer was checked using a standardised mixture of amino acids.

2.4 ASSAY PROCEDURES

2.4.1 LIQUID SCINTILLATION COUNTING TECHNIQUES

Samples were prepared for liquid scintillation counting as described below and were then left overnight in the counter to equilibrate before counting. Instrument settings etc. are given in Section 2.4.1.2.

2.4.1.1 Preparation of samples for liquid scintillation counting

Preparation of samples for the measurement of TCA-precipitable and TCA-soluble radioactivity

Digestion of samples in NCS

NCS (500ul) was added to the scintillation vials containing the samples (100ul) to be digested and the vials capped and incubated overnight at 37°C. In order to reduce chemiluminescence the samples were cooled on ice and brought to a lower pH by the addition of glacial acetic acid

TABLE 2.3 DETAILS OF THE OPERATION OF THE JOEL AMINO ACID ANALYZER

A. Arrangement of buffer lines

Column	Lines	Solution
Column 1 (Short column)	1, 2, 3, 5, 6 and 7	3rd buffer
	4 and 8	Sodium hydroxide
Column 2 (Long column)	1 and 5	1st buffer
	2, 3, 6 and 7	2nd buffer
	4 and 8	Sodium hydroxide

B. Programme for the purification of lysine

Time (min)	Operation	
0	pump 1 off	
1	change to next sampler	
2	pump 1 (3rd buffer) on	
60	change to line 2	Cycle 1
61	change to line 3	
62	change to line 4	
82	change to line 5	
130	pump 1 off	
131	change to next sampler	
132	pump 1 on	
190	change to line 6	Cycle 2
191	change to line 7	
192	change to line 8	
212	change to line 1	
260	pump 1 off	

Lysine was separated on Column 1. Starting conditions: Pump 1/line 1 on, pumping through empty sampler. Pump 2 and detector pump off.

Table 2.3 Continued

C. Programme for the purification of leucine

Time (min)	Operation	
0	pump 2 off	
1	change to next sampler	
2	pump 2 on	
85	change to line 3	Cycle 1
86	change to line 4	
106	change to line 5	
107	change to line 6	
160	pump 2 off	
161	change to next sampler	
162	pump 2 on	
245	change to line 7	Cycle 2
246	change to line 8	
266	change to line 1	
267	change to line 2	
320	pump 2 off	

Leucine was separated on Column 2. Starting conditions: Pump 2/line 2 on, pumping through empty sampler. Pump 1 and detector pump off.

Table 2.3 Continued

D. Programme for the purification of valine

Time (min)	Operation	
0	pump 2 off	
1	change to next sampler	
2	pump 2 on	
152	change to line 2	Cycle 1
153	change to line 3	
154	change to line 4	
174	change to line 5	
220	pump 2 off	
221	change to next sampler	
222	pump 2 on	
372	change to line 6	Cycle 2
373	change to line 7	
374	change to line 8	
394	change to line 1	
440	pump 2 off	

Valine was separated on Column 2. Starting conditions: Pump 2/line 1 on, pumping through empty sampler. Pump 1 and detector pump off.

Table 2.3 Continued.

E. Buffer compositions (see Joel Handbook).

	1st Buffer	2nd Buffer	3rd Buffer
pH	3.25 ± 0.01	4.25 ± 0.02	5.25 ± 0.02
Sodium citrate $2H_2O$	98.5g	98.5g	171 .3g
Conc HCl (12N)	61.5g	42.0g	32.5g
Octoic acid	0.5ml	0.5ml	0.5ml
Thiodiglycol	25ml	25ml	-
BRIJ - 35 (50% aqueous sol^{n})	10.0ml	10.0ml	10.0ml
Propan-1-ol	390-400ml	-	-
Final volume	5.0l	5.0l	5.0l

(17ul/sample) before the addition of toluene (10ml/vial) containing PPO (5g/l).

Preparation of samples for the measurement of radioactivity in TCA-precipitates prepared by the centrifugation method

Dried TCA-insoluble material was prepared as described in Section 2.3.1 and aliquots (30-40mg) weighed into scintillation vials. Sodium hydroxide (2ml, 1M) was added to each vial which was capped and heated (100°C, 90min). (This period of heating was important since it brought about a partial hydrolysis of protein, without which the material reprecipitated on neutralisation.) After neutralisation by the addition of glacial acetic acid (300ul), the samples were cooled on ice and taken up at 0°C in toluene/Triton X-100 (2:1, v/v) containing PPO (3.33g/l) (10ml/sample). The resulting emulsions were stable at 0-5°C but not at room temperature.

Preparation of samples for the measurement of radioactivity in aqueous solution

Neutral and acidic samples

Aliquots (usually 1ml, although in some cases it was necessary to adjust this volume in order to obtain a stable emulsion) were added to scintillation vials, cooled on ice and taken up in toluene/Triton X-100 (2:1, v/v) containing PPO (3.33g/l) (10ml/sample).

Basic samples (Ion-exchange eluates)

Samples (usually 1ml, or 2ml if the samples were to be oven dried before counting, see Tables 3.1.6 - 3.1.11) were pipetted into scintillation vials and the pH reduced by the addition of glacial acetic acid (100ul/ml sample). (In early experiments basic samples were acidified with concentrated HCl (200ul/ml sample) but this procedure was found not to be satisfactory since when the samples were subsequently emulsified with Triton X-100 the resulting acidic emulsions sometimes exhibited chemiluminescence (see Section 3.1.4 for further details).) Samples to be dried were evaporated under nitrogen while heating

on a boiling water bath and then either oven dried (100°C, 3h) or vacuum dried, as indicated.* The residues were redissolved in water (1ml), cooled on ice and taken up in toluene/Triton X-100 (2:1, v/v) containing PPO (3.33g/l) (10ml/sample). Emulsions containing ammonium acetate were unstable at room temperature but formed a stable emulsion at 0-5°C (see Section 3.1.4).

2.4.1.2 Instrumentation

Samples were counted at 4-5°C using a Packard Tri-Carb spectrometer (Model 3390). Counting efficiencies were determined using the external standard channels ratio (ESCR) method. ESCR calibration was performed using toluene/PPO (5g/l) containing various concentrations of chloroform. [^{3}H]Toluene, n-[1,2(n)-^{3}H]hexadecane, and n-[1-^{14}C]hexadecane were used as internal standards. It is shown in Section 3.1.4 that calibration curves obtained using neutral, colourless toluene/chloroform samples yielded reliable estimates of counting efficiencies in both coloured toluene/NCS samples and in acidic toluene/Triton X-100/water emulsions. The majority of samples were counted in preset channels. In some cases in which a high and variable chemiluminescent background was observed, samples were counted in a narrow energy channel selected as outlined below. Channels for the simultaneous counting of two isotopes were selected as follows.

Double isotope counting (^{3}H/^{14}C)

Suitable instrument settings for ^{3}H/^{14}C counting were determined using tritium and carbon-14 standards added to the same scintillation cocktail that was to be used experimentally (i.e., toluene/Triton X-100/water emulsions in the present study). The composition of the cocktail was adjusted to give a counting efficiency equal to the maximum efficiency

* Some liver extracts became so intensely coloured when heated at 100°C that the subsequent measurement of radioactivity by liquid scintillation counting was not practicable. Peroxide decolourization of heated samples was not adopted because this treatment can lead to chemiluminescence (see for example, WINKELMAN and SLATER, 1967) and a loss of radioactivity from labelled amino acids (BENEVENGA et al., 1968). Instead final traces of volatile material were removed at room temperature under vacuum.

that was to be attained experimentally. With the upper energy discriminator of channel II (D) set at 1000 divisions and the gain equal to 10%, the lower discriminator (C) was adjusted to effectively eliminate tritium events from this channel. A tritium counting efficiency of 0.017% (in a toluene/Triton X-100/water emulsion) was obtained with C set at 100 divisions. With the lower energy discriminator of channel I (A) set at 50 divisions and a gain of 100%, the upper discriminator (B) was adjusted to eliminate as much carbon-14 as possible without reducing the tritium counting efficiency to a level that was unacceptably low. A complete list of channel settings and the maximum counting efficiencies attained in these channels is given in Table 2.4. It should be noted, however, that the discriminator/efficiency relationships are not absolute since they depend upon both the level of quenching and the state of the scintillation counter itself. Tritium (d.p.m.(^{3}H)) and carbon-14 (d.p.m.(^{14}C)) disintegration rates in experimental samples were calculated using

$$\text{d.p.m.}\ (^{3}\text{H}) = (\text{c.p.m.}_{I} - (B_{I} + (\text{c.p.m.}_{II} - B_{II})C_{I/II}))E_{I} \qquad (2.1)$$

and

$$\text{d.p.m.}\ (^{14}\text{C}) = (\text{c.p.m.}_{II} - B_{II})E_{II} \qquad (2.2)$$

where c.p.m._{I} is the count rate in channel I, c.p.m._{II} the count rate in channel II, B_{I} and B_{II} the background count rates in channels I and II, respectively, and E_{I} and E_{II} the counting efficiency of tritium in channel I and of carbon-14 in channel II, respectively. $C_{I/II}$ is the fraction $\text{c.p.m.}(^{14}\text{C})_{I}/\text{c.p.m.}(^{14}\text{C})_{II}$. E_{I}, E_{II} and $C_{I/II}$ were all determined by the external standard channels ratio method.

Measurement of radioactivity in chemiluminescent samples

With some batches of Triton X-100, the background count rate was high and variable. This chemiluminescent background was effectively eliminated by counting in a channel in which the lower energy discriminator was raised. Suitable instrument settings were determined as follows. The upper discriminator was set at 1000 divisions and the gain at 10% for carbon-14 or 100% for tritium. A tritium or carbon-14 standard was

TABLE 2.4 INSTRUMENT SETTINGS (PACKARD TRICARB SPECTROMETER) FOR THE SIMULTANEOUS MEASUREMENT OF TRITIUM AND CARBON-14 DISINTEGRATION RATES

		Channel I (Tritium)	Channel II (Carbon-14)
Lower discriminator		- 50	100
Upper discriminator		250	1000
Gain		100%	10%
Maximum counting efficiency	^{3}H	20%	0.017%
	^{14}C	7%	52%

It should be noted that the above discriminator/efficiency relationships are not absolute since the channel settings were chosen for samples exhibiting a particular range of quenching levels. They also depend upon the state of the scintillation counter.

prepared from standardized [^{3}H] or [^{14}C]hexadecane and the scintillation cocktail that was to be used experimentally. Using this standard and a chemiluminescent background vial, a plot of counting efficiency and background count rate versus the lower discriminator setting was obtained. A lower discriminator setting was then selected that yielded a low background count rate without too great a reduction in counting efficiency. A typical set of results for tritium is given in Fig. 3.1.1, on the basis of which a lower discriminator setting of 200 divisions was chosen. Having set the lower discriminator, the upper discriminator was set at the lowest level giving a maximum counting efficiency for the scintillation cocktail in question (about 650 divisions in the example shown in Fig. 3.1.1).

2.4.2 AMINO ACID DETERMINATION

Amino acid estimations were carried out using a fluorometric assay. In order to minimise the hand contamination of samples (see HAMILTON, 1965) all apparatus was acid washed and gloves were worn. All aqueous solutions were made up in glass-distilled water.

Samples (50 or 100ul as appropriate, see below) were taken from fractionated amino acid analyzer eluates and were added to sodium borate buffer (pH 9.0, 0.2M, 1.40 or 1.45 ml as required to obtain a final volume of 1.5ml) and thoroughly mixed on a vortex mixer. A solution of Fluram in acetone (0.5ml, ca. 250ug/ml) was rapidly added with mixing and the solution left to stand for at least 1min. It should be noted that because Fluram undergoes rapid hydrolysis when added to the borate buffer, it was essential that the two solutions were mixed quickly. Fluorescence was measured using a Locarte fluorimeter (primary filter, 390 nm interference filter; secondary filter, 488nm interference filter). Calibration in the concentration range 0-10nmole/assay mixture for lysine and 0.0-1.0nmole/assay mixture for valine, was carried out using standard solutions of these amino acids, the concentrations of which had been determined by polarimetry. (Using these concentration ranges samples taken from the amino acid analyzer eluate could be assayed directly without an adjustment of their concentrations.) Quinine sulphate was used as a stable standard. A stock solution (50ug/ml in 0.2M H_2SO_4)

was stored in the dark at about 5°C and diluted as required with H_2SO_4 (0.2M).

2.4.3 PROTEIN DETERMINATION

Protein estimations were carried out using either the Lowry or the Biuret method essentially as described by LAYNE (1957).

Lowry protein determination

The following stock solutions were prepared.

Reagent A.	$CuSO_4.5H_2O$	(10g/l)
Reagent B.	Potassium sodium tartrate. $4H_2O$	(20g/l)
Reagent C.	NaOH (4g/l)/Na_2CO_3	(20g/l)

Reagent D and E were prepared immediately before use as follows.

Reagent D.	Reagent A/Reagent B (1:1, $^v/_v$)	
Reagent E.	Reagent C/Reagent D (50:1, $^v/_v$)	

Suitably diluted samples of tissue homogenates (100ul containing in the region 20-200ug protein) were dissolved in NaOH (2ml, 0.1M). Protein was precipitated by the addition of TCA (0.5ml, 3M), the samples left to stand for 5-10min and then centrifuged (1000g_{av}, 20min). The supernatants were removed and water (1ml/sample) added. Reagent E (5ml/sample) was then added and the solutions mixed and left to stand (ca 10min) before the addition of Folin-Ciocalteu reagent (0.5ml/sample, 50% v/v aqueous solution). The solutions were immediately mixed and allowed to stand at room temperature for 30min before absorbance was read at 750nm. Bovine serum albumin was used as the standard.

Biuret protein determination

Biuret reagent was prepared as follows. A solution of NaOH (300ml, 100g/l) was added with stirring to a solution of $CuSO_4.5H_2O$ (1.5g) and potassium sodium tartrate.$4H_2O$ (6.0g) in water (500ml). The solution was diluted to a final volume of 1l and potassium iodide (ca. 1g) added. The reagent was discarded if it showed signs of precipitate formation after storage.

Samples of tissue homogenates (100-200ul containing 1-10mg protein) were added to a solution of NaOH (30g/l) to give a final volume of 1ml. Biuret reagent (4ml/sample) was added, the solutions mixed and allowed to stand for 30min. Lipid material was removed by ether extraction (1.5ml ether/sample) and the phases separated by centrifugation ($400g_{av}$, 20min). The lower phase was removed with a pipette and absorbance measured at 550nm. Bovine serum albumin was used as the standard.

2.4.4 PAPER CHROMATOGRAPHIC DETERMINATION OF THE RADIOCHEMICAL PURITY OF LABELLED AMINO ACIDS

The radiochemical purity of radioactive amino acids was determined if a batch of material was still in use 2-3 months after its purchase and within a few days of their use in the case of those L-[U-^{14}C]amino acids employed as internal standards. Radioactive amino acids were diluted with carrier material and spotted on to Whatman no. 1 paper strips (about 10ug of amino acid applied in 10ul). Papers were eluted using the following solvent systems.

Leucine

System 1.(Bush system). A t-amylalcohol/acetic acid/water (40:2:40, v/v/v) mixture was equilibrated overnight at 36-37°C. The two phases were separated and spotted papers equilibrated in an atmosphere saturated with the aqueous phase (at 36-37°C) for about 20h. The papers were then eluted with the organic phase.

System 2. n-butanol/acetic acid/water (12:3:5, v/v/v)

Valine

n-butanol/acetic acid/water (12:3:5, v/v/v)

Lysine

n-butanol/pyridine/water (1:1:1, v/v/v)

Eluted papers were cut into 5mm strips in the region of the amino acid peak. Chromatograms of [^{14}C]amino acids were counted, without elution from the paper, in toluene/PPO (5g/l). In the case of tritiated amino acid chromatograms, the strips were added to scintillation vials

containing water (100ul) and incubated in NCS and processed as described in Section 2.4.1.1. Radiochemical purities were calculated from the radioactive content of the amino acid peaks and the total radioactivity of the chromatograms.

In order to determine which fractions should be included or excluded from an amino acid peak the following calculations were performed. Starting with a peak that clearly included some impurities at its extremities, the accumulative percentage c.p.m. (P) defined by

$$P = \sum_{i=1}^{n} \text{c.p.m.} \; / \; \sum_{i=1}^{N} \text{c.p.m.} \qquad (2.3)$$

was calculated for n=1,2,...,N, where n is the fraction number and N the total number of fractions in the peak. Probit(P) was then plotted against n (Probit tables are given in Table IX of FISHER and YATES, 1963). (Probit(P) is linear for a gaussian peak, while the presence of impurities at the extremities of the peak causes probit(P) to become sigmoid.) Fractions were excluded one at a time from the tails of the peak and the calculation repeated. The true peak was taken as including the maximum number of fractions that yielded a straight line when probit(P) was plotted against n. (NB. This procedure cannot be applied when the amino acid peak itself is not gaussian. In the present study gaussian shaped peaks were obtained using the above solvent systems.)

If the purity of any radiochemical was below 95% the material was either discarded or purified by paper chromatography. In the later case the radioactive amino acid was located autoradiographically, eluted with 2M HCl/ethanol (1:1, v/v) and reduced to dryness under nitrogen at about 40°C. Its radiochemical purity was then redetermined.

2.5 MATHEMATICAL METHODS

Statistical methods

Where indicated levels of significance were determined using Student's t test. t was calculated using

$$t = \frac{|\bar{X}_1 - \bar{X}_2|}{\sqrt{\frac{\sum_{i=1}^{N_1}(x_{1i}-\bar{X}_1)^2 + \sum_{i=1}^{N_2}(x_{2i}-\bar{X}_2)^2}{N_1+N_2-2}\left(\frac{1}{N_1}+\frac{1}{N_2}\right)}} \qquad (2.4)$$

where $\bar{X}_1$ and $\bar{X}_2$ are the two sample means, $\sum_{i=1}^{N_j}(x_{ji}-\bar{X}_j)^2$ the sum of the squared deviations from the mean for the jth group and N_j the number of animals in that group. A table of the distribution of t (2 tailed) is given in FISHER and YATES (1963). (NB. The number of degrees of freedom (ν) is given by $\nu = N_1+N_2-2$.)

Estimated standard errors of the means ($S_{\bar{X}}$)* were calculated using

$$S_{\bar{X}} = s/\sqrt{N} \qquad (2.5)$$

where s is the sample standard deviation based on the unbiased variance estimate, ie., s is given by

$$s = \sqrt{\sum_{i=1}^{N}(x_i - \bar{X})^2/(N-1)}. \qquad (2.6)$$

Combination of (2.5) and (2.6) yields

$$S_{\bar{X}} = \sqrt{\sum_{i=1}^{N}(x_i - \bar{X})^2/N(N-1)}. \qquad (2.7)$$

Details of other statistical methods are given in the relevant table legends.

Computer simulation techniques

Details of the mathematical methods used in the computer simulation of tracer experiments are given in Appendix B.

*Elsewhere in this thesis the standard error of the mean and the sample standard deviation are denoted by S.E.M. and s.d., respectively. This notation was not retained above since it makes the equations unnecessarily cumbersome.

3 EXPERIMENTAL RESULTS

3.1 ASSESSMENT OF METHODS

A number of preliminary experiments were carried out in order to establish the details of methods to be adopted in *in vivo* experiments. Some of these experiments are described in this section. In particular, it was necessary to decide which amino acids should be used in the measurement of rates of protein synthesis and which route of administration should be adopted. In addition to this a number of practical problems were encountered during the present study. The major problems arose from the need to count low levels of radioactivity in aqueous solution. Measurement of the specific activity of amino acids was further complicated as a result of the altered ion-exchange properties of isotopically substituted amino acids. A brief assessment of some of the simple procedures that were adopted to overcome these difficulties is also given in this section.

3.1.1 MODE OF PRECURSOR ADMINISTRATION

Precursor administration is mentioned in Section 1.1.3 in relation to the problem of stress-induced changes in amino acid incorporation rates. On the basis of a report (DUNN, 1975) that intracerebral puncture causes an inhibition of the incorporation into brain protein of subcutaneously administered amino acids only peripheral routes of administration were considered. During trial experiments in which radioactive amino acids were administered by intraperitoneal injection, no radioactivity was found in the brain or liver of a few rats. This was assumed to result from badly placed injections. It was therefore decided that labelled amino acids would be given subcutaneously. Further, subcutaneous injection was assumed to be a relatively stress free procedure. When the present work was nearing completion, however, SCHOTMAN *et al.* (1977) reported that the mild stress associated with subcutaneous injection is sufficient to elevate plasma corticosterone, which may, in turn, affect rates of protein synthesis. ENTINGH and DAMSTRA (1976) reported that even the handling of mice results in an elevation of lysine incorporation into brain protein. This clearly represents a problem whatever route of administration is chosen.

3.1.2 ASSESSMENT OF METHODS USED IN THE DETERMINATION OF COMPARATIVE RATES OF AMINO ACID INCORPORATION INTO TCA-PRECIPITABLE MATERIAL

A number of researchers have suggested that TCA-soluble radioactivity may be used as an index of precursor specific activity and that incorporation be expressed in terms of a relative incorporation (I_{rel}) defined by

$$I_{rel} = R_2/R_1 \qquad (1.14)$$

where R_1 is the TCA-soluble radioactivity and R_2 the TCA-insoluble radioactivity, both measured at the end of the incorporation period (REES et al., 1974; TIPLADY, 1972). Some of the problems associated with the use of this parameter are discussed in Sections 1.2 and 4.3. Many of the results presented in Section 3.2 are expressed in terms of I_{rel} and some of its properties are therefore examined in this section.

According to equation (3.2.6), if I_{rel} is to be a meaningful index of absolute incorporation rates, then the time-average precursor specific activity and TCA-soluble radioactivity must be linearly correlated within the group of experimental animals. Further, if the inter-animal variation in the rate of protein synthesis is significantly less than the variation in the final soluble radioactivity, then TCA-insoluble and TCA-soluble radioactivity should be linearly correlated within the group of animals. The relationship between TCA-insoluble and TCA-soluble radioactivity was investigated in one experiment in which these parameters were measured in rats killed 30min after the intraperitoneal administration of L-[4,5-^{3}H]leucine. It can be seen from the data given in Table 3.1.1 that the coefficient of variation of I_{rel} is smaller than that of either R_1 or R_2. Further, R_2 and R_1 were correlated (r_s=0.94, P<0.05, see Table A1). This is consistent with inter-animal variation in the uptake of labelled amino acid into the brain being the major cause of variability in TCA-insoluble radioactivity and indicates that the simple procedure based on equation (1.14) is useful and to some extent corrects for inter-animal variability in precursor specific activity.

The above experiment was carried out as part of a study in which L-[^{3}H]leucine and L-[^{3}H]valine incorporation into TCA-precipitable material of brain and

TABLE 3.1.1 ASSESSMENT OF I_{rel} AS AN INDEX OF COMPARATIVE RATES OF AMINO ACID INCORPORATION INTO BRAIN PROTEIN

TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	*TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	**Relative incorporation ($I_{rel} = R_2/R_1$)
$552 \pm 81(6)$	$199 \pm 30(6)$	$2.78 \pm 0.18(6)$
$C(R_2) = 0.147$	$C(R_1) = 0.151$	$C(I_{rel}) = 0.065$

Six rats (aged between 4 and 6 weeks) were injected (ip) with L-[4,5-^{3}H] leucine (10uCi/animal in 1ml of normal saline) and killed 30min later. Brain tissue was processed as described in Section 2.3.1. The results are given as the mean $\pm$ s.d. together with the coefficient of variation (C(x)) which is defined by $C(x) = s.d.(x)/\bar{x}$.

* TCA-soluble radioactivity was calculated using : soluble radioactivity = total involatile tissue radioactivity - TCA-insoluble radioactivity.

** I_{rel} was calculated using individual figures for R_2 and R_1 and not the group means.

liver was measured at various times following intraperitoneal or subcutaneous administration. The results of these experiments (Tables 3.1.2 and 3.1.3) are included here because they illustrate some of the properties of I_{rel}. During the time interval 15 to 45min after the administration of L-[^{3}H]valine, brain involatile TCA-soluble radioactivity decreased steadily indicating that the specific activity of the brain soluble valine pool had probably reached a maximum within 15min of precursor administration (Table 3.1.2). By 45min 85% of brain soluble radioactivity was in the form of volatile metabolites. In the case of valine, the relative rate of incorporation in brain (R_{rel}) defined by

$$R_{rel} = I_{rel}/a \qquad (3.1.1)$$

where a is the incorporation time, exhibited a time-dependent increase between 15 and 45min (Table 3.1.3). This probably arose mainly because after precursor specific activity has reached a maximum, the rate at which TCA-soluble radioactivity decreases with time is faster than the rate at which time-average precursor specific activity decreases (see the theoretical lysine data given in Table 4.8a). The relative rate of leucine incorporation did not show this time-dependent increase (Table 3.1.3). It can be seen from Table 3.1.2 that the rate at which the involatile TCA-soluble radioactivity decreased following the subcutaneous injection of L-[4,5-^{3}H]leucine was slower than that observed following the administration of L-[G-^{3}H]valine. This may, in part, be explained by the observation that in contrast to L-[G-^{3}H]valine, L-[4,5-^{3}H]leucine gives rise to significant quantities of involatile labelled metabolites (Table 3.1.10). It is presumably the presence of these labelled metabolites that masked the time-dependent increase in the relative rate of incorporation of [^{3}H]leucine in brain. The relationship between I_{rel} and absolute incorporation is further examined in Section 4.3. Because the numerical values of relative incorporation rates have no obvious physiological meaning, and bear no simple relationship to the absolute incorporation rates, the results of the L-dopa experiments described in Section 3.2 have not been converted to relative rates of incorporation but have been expressed simply in terms of I_{rel}.

TABLE 3.1.2 INCORPORATION OF L-[4,5-^{3}H] LEUCINE AND L-[G-^{3}H]VALINE INTO TCA-PRECIPITABLE AND TCA-SOLUBLE FRACTIONS OF RAT BRAIN AND LIVER

	Time (min)	Radioactive content of total homogenate (A) (d.p.m./mg prot.)	Radioactive content of dried homogenate (B) (d.p.m./mg prot.)	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	Involatile TCA-soluble radioactivity (R_1=B-R_2) (d.p.m./mg prot.)	Relative incorporation (I_{rel} = R_2/R_1)	% of soluble radioactivity lost on drying $\left(\frac{100(A-B)}{A-R_2}\right)$
Brain	15	944 ± 123	720 ± 120	437 ± 72	283 ± 47	1.547 ± .007	45 ± 4
leucine	30	1019 ± 31(6)	751 ± 44(6)	552 ± 33(6)	199 ± 12(6)	2.78 ± .07(6)	57 ± 4(6)
i.p.	45	985 ± 32	622 ± 21	488 ± 12	134 ± 20	3.83 ± .68	73 ± 3
	15	643 ± 107	477 ± 76	275 ± 38	202 ± 38	1.40 ± .11	45 ± 2
leucine	30	955 ± 76	696 ± 75	486 ± 67	209 ± 11	2.31 ± .25	55 ± 4
s.c.	45	1041 ± 100	733 ± 111	570 ± 86	163 ± 26	3.50 ± .12	65 ± 5
	15	760 ± 51	476 ± 22	244 ± 11	252 ± 11	0.89 ± .01	53 ± 2
valine	30	763 ± 123	475 ± 99	327 ± 67	148 ± 32	2.21 ± .03	67 ± 3
s.c.	45	929 (785) (1072)	532 (449) (615)	458 (398) (518)	74 (51) (97)	6.6 (7.8) (5.3)	85 (87) (82)
Liver	15	3207 ± 696	3116 ± 731	2475 ± 531	641 ± 208	4.17 ± .66	15 ± 13
leucine	30	3741 ± 351	3596 ± 299	3058 ± 264	538 ± 57	5.74 ± .50	19 ± 9
i.p.	45	3465 ± 127	3422 ± 108	2926 ± 79	495 ± 31	5.94 ± .22	8 ± 3
	15	1478 ± 191	1312 ± 161	1039 ± 139	274 ± 23	3.78 ± .19	37 ± 3
leucine	30	1995 ± 92	1790 ± 92	1485 ± 90	304 ± 6	4.88 ± .27	40.3 ± 0.3
s.c.	45	1913 ± 183	1697 ± 158	1457 ± 159	240 ± 6	6.07 ± .70	47 ± 3
	15	2179 ± 214	1938 ± 190	1518 ± 162	420 ± 49	3.67 ± .47	37 ± 2
valine	30	2630 ± 679	2318 ± 629	2070 ± 565	248 ± 67	8.28 ± .78	56 ± 6
s.c.	45	2602 (2017) (3187)	2343 (1764) (2921)	2081 (1560) (2602)	262 (204) (319)	7.9 (7.6) (8.2)	50 (45) (55)

Rats were injected with [^{3}H] amino acid as indicated (see Table 3.1.1 for details) and tissue processed as described in Section 2.3. Three animals were used in each determination except where indicated. Where only two animals were used the individual results are shown in parenthesis.

TABLE 3.1.3 THE RELATIVE INCORPORATION RATE OF L-[4,5-^{3}H] LEUCINE AND L-[G-^{3}H] VALINE IN BRAIN

Amino acid (and mode of administration)	Incorporation time (a) (min)	Relative rate of incorporation ($R_{rel} = I_{rel}/a$)
leucine (i.p.)	15	0.103
	30	0.093
	45	0.085
leucine (s.c.)	15	0.093
	30	0.077
	45	0.078
valine (s.c.)	15	0.059
	30	0.074
	45	0.147

Relative incorporation rates (R_{rel}) were calculated from the data given in Table 3.1.2 by dividing the mean relative incorporation by the incorporation time.

Effect of controlled feeding on the incorporation of L-[4,5-^{3}H]lysine into TCA-precipitable and TCA-soluble fractions of rat brain and liver

During their study of protein synthesis in rat liver, AIRHART et al. (1974) found that if for seven days previous to each experiment they allowed the experimental animals to have access to food for only a single 4h period each day, the animal variation in all parameters studied was significantly reduced. Having shown that variability in the uptake of [^{3}H]leucine into the brain makes a major contribution to inter-animal variation in the rate at which the label is incorporated into cerebral TCA-insoluble material, one experiment was carried out in order to establish whether the procedure of Airhart and co-workers might be adopted in the present study as a means of reducing the scatter associated with incorporation data. Two groups of 4 rats were housed under a 12h light - 12h dark régime. One group was allowed free access to food at all times, while the other group was given food at the start of each dark cycle. All uneaten food was removed 4h later. This routine was maintained for six days before the start of the experiment which was carried out on the seventh day between 7 and 9h after the end of the dark cycle. Rats were injected with L-[4,5-^{3}H]lysine and killed after a 15min incorporation period. L-[4,5-^{3}H]lysine was used in this experiment rather than L-[4,5-^{3}H]leucine because the former radiochemical gave rise to a lower level of radioactive cerebral metabolites (see Section 3.1.3). Although the incorporation of radioactivity into the brain TCA-insoluble and TCA-soluble fractions was significantly greater in the 4h-fed group of animals, no marked difference between the two groups was observed with respect to the coefficient of variation associated with the various parameters (Table 3.1.4). The procedure of controlled feeding was not therefore adopted in subsequent experiments.

3.1.3 A STUDY OF THE METABOLISM OF RADIOACTIVE AMINO ACIDS USED IN THE MEASUREMENT OF COMPARATIVE RATES OF PROTEIN SYNTHESIS

If experiments are carried out in which TCA-soluble radioactivity is used as an index of time-average specific activity and incorporation is expressed in terms of I_{rel} it is important that a labelled amino acid is chosen which does not produce significant quantities of radioactive metabolites. BANKER and COTMAN (1971) have suggested that

TABLE 3.1.4 EFFECT OF CONTROLLED FEEDING ON BODY WEIGHT AND ON THE INCORPORATION OF L-[4,5-^{3}H] LYSINE INTO TCA-INSOLUBLE AND TCA-SOLUBLE FRACTIONS OF RAT BRAIN AND LIVER

A. Brain incorporation data and body weights

	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)	Body weight (g)
Ad libitum fed	65 ± 15 $C(R_2) = 0.23$	387 ± 79 $C(R_1) = 0.20$	0.170 ± 0.022 $C(I_{rel}) = 0.13$	106 ± 14
4 h fed	107 ± 25 $C(R_2) = 0.23$	549 ± 69 $C(R_1) = 0.13$	0.193 ± 0.021 $C(I_{rel}) = 0.11$	78 ± 11

Percentage changes

TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation	Body weight
+65 $P<0.05$	+42 $P<0.05$	+14 NS	-26 $P\simeq0.02$

Table 3.1.4 Continued

B. Liver incorporation data

	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Ad lib. fed	800 ± 240 $C(R_2) = 0.30$	1006 ± 181 $C(R_1) = 0.18$	0.79 ± 0.15 $C(I_{rel}) = 0.19$
4 h fed	882 ± 218 $C(R_2) = 0.25$	1002 ± 231 $C(R_1) = 0.23$	0.89 ± 0.13 $C(I_{rel}) = 0.15$

Percentages changes

TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
+10 NS	0 NS	+13 NS

Two groups of 4 rats were housed under a 12h light - 12h dark régime. For six days one group was allowed free access to food at all times while the other group was given food at the start of each dark cycle. All uneaten food was removed 4h later. On day seven the rats were injected with L-[4,5-^{3}H]lysine (200uCi/kg,40uCi/ml in normal saline, s.c.) and were killed after a 15 min incorporation period. Tissue was processed as described in Section 2.3. The results are expressed as the mean $\pm$ s.d. (n=4). C(x) is the coefficient of variation defined by $C(x) = s.d.(x)/\bar{x}$.

carboxyl-labelled amino acids be used since the radioactive carbon is lost as $^{14}CO_2$. Alternatively, tritiated amino acids may be employed providing the positions of isotopic substitution are such that the only labelled metabolite formed in a significant quantity is tritiated water (BANKER and COTMAN, 1971; TIPLADY, 1972; REES et al., 1974). This should also ensure that radioactivity is not incorporated into TCA-insoluble material in the form of radioactive metabolites. L-[2,3-^{3}H]valine and L-[4,5-^{3}H]lysine satisfy this requirement (BANKER and COTMAN, 1971; TIPLADY, 1972). In contrast, L-[4,5-^{3}H]leucine produces large quantities of involatile radioactive metabolites including other amino acids (BANKER and COTMAN, 1971; SCHOTMAN et al., 1974).

On the basis of the results shown in Table 3.1.2 it was decided that an incorporation time of 15min represented an acceptable compromise between the stipulation that short incorporation periods should be used (see Section 1.2) and the need to obtain a measurable level of incorporation without having to use prohibitively large quantities of labelled amino acids. Having established that labelled precursors would be given by subcutaneous injection (see Section 3.1.1) and that an incorporation period of not longer than 15min would be used, experiments were carried out in which the distribution of radioactivity between acidic and basic metabolites was measured 15min after the subcutaneous injection of L-[G-^{3}H]valine and L-[4,5-^{3}H]lysine. For comparison the same experiments were performed with L-[4,5-^{3}H]leucine. Estimates of the radiochemical purity (RCP) of heated TCA-soluble extracts of brain and liver were made in order to test the validity of the assumption that all TCA-soluble involatile radioactivity is present as the administered amino acid. TCA-soluble extracts were applied to micro-cation-exchange columns at pH 2, which were eluted first with water and then with NH_4OH (4.5M) (see Section 2.3.2). For convenience, all materials which appeared in the water eluate, which included ^3H_2O, are referred to as acidic metabolites and materials eluted with dil NH_4OH, which included all the amino acids, are referred to as basics. Using this simple ion-exchange procedure a reproducible separation of acidic and basic materials was achieved (Table 3.1.5). The radiochemical purity of vacuum dried basic eluates was measured after the separation of the parent amino acid from other material using a Joel amino acid analyzer.

TABLE 3.1.5 ASSESSMENT OF THE RELIABILITY OF THE CATION-EXCHANGE CHROMATOGRAPHIC PROCEDURE USED IN THE SEPARATION OF RADIOACTIVE AMINO ACIDS FROM THEIR ACIDIC METABOLITES

a) Recovery of amino acid

Fraction	Eluting medium	% of applied radioactivity
1	Water (15ml)	3.7 ± 0.6
2	NH_4OH (10ml)	95.2 ± 0.6
3	NH_4OH (10ml)	2.0 ± 0.3
4	NH_4OH (10ml)	0.23 ± 0.03

Authentic L-[4,5-^{3}H]leucine (95% RCP 3-4 months previously) was applied to Bio-Rad cation-exchange columns (AG 50W-X4, 5ml) which were then eluted as described in Section 2.3.2. The percentage recovery of radioactivity in the fractionated eluate was determined. The results, which are given as the mean ± S.E.M. (n=3) indicate that a quantitative recovery of [^{3}H] lysine was achieved.

b) Separation of acidic metabolites

Fraction	% of recovered acidic metabolites	
	Animal 1	Animal 2
1 (10ml)	97.2	93.3
2 (5ml)	1.2	4.0
3 (5ml)	1.7	2.7

Table 3.1.5 Continued

Two rats were killed 15min after the subcutaneous administration of L-[4,5-^{3}H]lysine (10uCi). A brain TCA-soluble extract was prepared for cation-exchange chromatography as described in Section 2.3.2. The extracts were applied to Bio-Rad cation-exchange columns and eluted with water. The radioactive content of the fractionated eluates was determined. More than 97% of the acidic radioactivity was eluted in the first 15ml. On the basis of this result, cation-exchange columns were routinely washed with 15ml of water prior to the elution of basic materials with NH_4OH.

Fifteen minutes after the subcutaneous administration of L-[4,5-^{3}H]lysine, 28% of brain soluble radioactivity was in the form of acidic metabolites. About 12% of this 28% was involatile (Table 3.1.6). The radiochemical purity of the vacuum dried basic fraction was 95%. On the basis of this data the radiochemical purity of heated TCA-soluble samples was estimated to be 92% (Table 3.1.12). The radiochemical purity of the basic fraction obtained from TCA-soluble extracts of liver was 78% (Table 3.1.7), and the estimated radiochemical purity of heated TCA-soluble extracts was 76% (Table 3.1.12). Fifteen minutes after the subcutaneous injection of L-[G-^{3}H]valine, 45% of the total brain soluble radioactivity was present as acidic metabolites. About 22% of the radioactivity in this acidic fraction was involatile (Table 3.1.8). The radiochemical purity of vacuum dried basic samples was about 97% and the radiochemical purity of heated TCA-soluble samples was estimated to be 85% (Table 3.1.12). A similar distribution of radioactivity was found in the liver (Table 3.1.9). The radiochemical purity of vacuum dried basic samples was 93%, but a large portion of the basic radiochemical impurities were lost on heating at 100°C (note that 100D/E = 92.9). The radiochemical purity of heated TCA-soluble liver samples was estimated to be 84% (Table 3.1.12). L-[4,5-^{3}H]leucine gave rise to large quantities of both acidic and basic involatile radioactive metabolites in the brain and liver (Tables 3.1.10 and 3.1.11).

These results are consistent with the known catabolic pathways of the amino acids in question. Lysine is degraded at a relatively slow rate in the brain (CHANG, 1976) and the majority of the radioactive metabolites found in this tissue at 15min were presumably formed at some peripheral site of degradation and transported via the blood to the brain. Lysine is one of the few amino acids which does not undergo initial deamination, which explains the presence of a significant level of labelled basic materials in the liver. In contrast, valine undergoes initial transamination to produce acidic metabolites. Tritium substituted at C2 and C3, and the majority of that at C4 is expected to rapidly appear as water. Not more than 30% of the tritium substituted at C4 is expected to enter the TCA cycle, but much of this will also be lost as water. L-[4,5-^{3}H] leucine differs from L-[G-^{3}H]valine in that a smaller percentage loss of radioactivity occurs during the initial stages of metabolism and a significant percentage is therefore expected to enter the TCA cycle as acetyl-CoA

TABLE 3.1.6 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF BRAIN FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[4,5-^{3}H]LYSINE

a)

Fraction	% of applied radioactivity
Acidic (A)	27.7 ± 1.5
Basic (B)	71.1 ± 0.6
Recovery	98.7 ± 0.8

b)

Fraction	% of radioactivity remaining after drying
Acidic fraction heated at 100°C(C)	11.8 ± 0.2
Basic fraction heated at 100°C(D)	Exp 1)*93.8 ± 1.3 Exp 2) 92.3 ± 2.1 mean = 93.1
Vacuum dried basic fraction (E)	*94.7 ± 0.6

c)

RCP of vac dried basic fraction (F)	94.8 ± 0.7%
% of vac dried basic fraction remaining after heating at 100°C $\left(\frac{100D}{E}\right)$	98.3

Table 3.1.6 Continued

a) Rats were killed 15min after the subcutaneous administration of L-[4,5-^{3}H] lysine (10uCi/animal). TCA-soluble extracts were prepared and separated into acidic and basic fractions by cation-exchange chromatography as described in Section 2.3.2. The radioactivity recovered in each fraction is expressed as a percentage of the total radioactivity applied to the column. Extracts from three rats were used in each determination.

b) Samples of the acidic and basic fractions were dried either by heating at 100°C or by vacuum drying, and the percentage of radioactivity remaining was determined. The radioactive content of each fraction is expressed as a percentage of that present in each fraction prior to drying. (*This data was obtained from separate experiments in which L-[U-^{14}C]lysine had been added to the extract as an internal standard. The percentage of radioactivity remaining after drying was calculated from the change in the ^{3}H:^{14}C ratio.)

c) The radiochemical purity of samples of the vacuum dried basic fraction was determined after the separation of lysine from other basic materials using a Joel amino acid analyzer as described in Section 2.3.3. L-[U-^{14}C] lysine was added as an internal standard. (100D/E) was calculated from the data given in (b). The radiochemical purity of the internal standard was determined at the time of the experiment using paper chromatography (see Section 2.4.4) and a correction made for the presence of radiochemical impurities.

TABLE 3.1.7 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF LIVER FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[4,5-^{3}H]LYSINE

a)

Fraction	% of total radioactivity
Acidic (A)	18.2 ± 0.4
Basic (B)	82.0 ± 1.5
Recovery	100.2 ± 1.4

b)

Fraction	% of radioactivity remaining after drying
Vacuum dried acidic fraction (C)	37.7 ± 5.6
Basic fraction heated at 100°C (D)	89.2 ± 1.1
Vacuum dried basic fraction (E)	Exp 1)*93.9 ± 0.6 Exp 2) 97.3 ± 1.5 mean = 95.6

Table 3.1.7 Continued

c)

RCP of vac dried basic fraction (F)	77.7 ± 2.8
% of vac dried basic fraction remaining after heating at 100°C $\left(\frac{100D}{E}\right)$	93.3

Experimental and other details are given in Table 3.1.6. NB. Samples of the acidic fraction of liver extracts were dried under vacuum instead of drying at 100°C because when oven dried some of these samples became so intensely coloured that the measurement of radioactivity by scintillation counting was not practicable.

TABLE 3.1.8 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF BRAIN FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[G-^{3}H]VALINE*

a)

Fraction	% of total radioactivity
Acidic (A)	45.0 ± 3.0
Basic (B)	61.4 ± 2.3
Recovery	106.4 ± 3.1

b)

Fraction	% of radioactivity remaining after drying
Acidic fraction heated at 100°C (C)	22.3 ± 2.1
Basic fraction heated at 100°C (D)	*90.7 ± 0.8
Vacuum dried basic fraction (E)	*93.8 ± 1.5

Table 3.1.8 Continued

c)

RCP of vac dried basic fraction (F)	96.7 ± 0.4%
% of vac dried basic fraction remaining after heating at 100°C $\left(\frac{100D}{E}\right)$	96.7

Experimental and other details are given in Table 3.1.6. *This radiochemical was marketed as L-[2,3-^{3}H]valine, but analysis of some batches of this material has indicated the presence of significant levels of substitution at C4 (see main text).

TABLE 3.1.9 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF LIVER FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[G-^{3}H]VALINE*

a)

Fraction	% of total radioactivity
Acidic (A)	36.3 ± 1.4
Basic (B)	64.7 ± 1.1
Recovery	101.1 ± 1.6

b)

Fraction	% of radioactivity remaining after drying
Vacuum dried acidic fraction (C)	32.9 (32.7) (33.0)
Basic fraction heated at 100°C (D)	91.7 ± 2.0
Vacuum dried basic fraction (E)	98.7 ± 0.2

Table 3.1.9 Continued

c)

RCP of vac dried basic fraction (F)	93.2 ± 0.5%
% of vac dried basic fraction remaining after heating at 100°C $\left(\frac{100D}{E}\right)$	92.9

Experimental and other details are given in Tables 3.1.6 and 3.1.7. *This radiochemical was marketed as L-[2,3-^{3}H]valine, but analysis of some batches of this material has indicated the presence of significant levels of substitution at C4.

TABLE 3.1.10 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF BRAIN FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[4,5-^{3}H]LEUCINE

a)

Fraction	% of total radioactivity
Acidic (A)	59.3 ± 2.0 (4)
Basic (B)	44.6 ± 2.9 (4)
Recovery	103.9 ± 1.3 (4)

b)

Fraction	% of radioactivity remaining after drying
Acidic fraction heated at 100°C (C)	16.9 ± 5.7 (3)

c)

RCP of vac dried basic fraction (F)	62.5 ± 3.6%

Experimental and other details are given in Table 3.1.6.

TABLE 3.1.11 THE DISTRIBUTION OF RADIOACTIVITY BETWEEN THE ACIDIC AND BASIC SOLUBLE FRACTIONS OF LIVER FIFTEEN MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[4,5-^{3}H]LEUCINE

a)

Fraction	% of total radioactivity
Acidic (A)	42.1 ± 0.8
Basic (B)	63.0 ± 1.1
Recovery	105.1 ± 1.8

b)

Fraction	% of radioactivity remaining after drying
Vac dried acidic fraction (C)	42.3 ± 5.7

c)

RCP of vac dried basic fraction (F)	68.0 ± 1.6%

Experimental and other details are given in Tables 3.1.6 and 3.1.7.

TABLE 3.1.12 ESTIMATED RADIOCHEMICAL PURITY OF HEATED TCA-SOLUBLE EXTRACTS OF THE BRAIN AND LIVER OF RATS KILLED 15 MINUTES AFTER THE SUBCUTANEOUS ADMINISTRATION OF L-[4,5-^{3}H]LYSINE OR L-[G-^{3}H]VALINE

Amino acid	Tissue	Estimated RCP
L-[4,5-^{3}H]lysine	Brain	92%
L-[4,5-^{3}H]lysine	Liver	76%
L-[G-^{3}H]valine	Brain	85%
L-[G-^{3}H]valine	Liver	84%

Estimates of the radiochemical purity (RCP) of heated TCA-soluble extracts were obtained by substituting the data given in Tables 3.1.6 to 3.1.9 into the equation

$$\%RCP = \frac{B\ E\ F}{(A\ C) + (B\ D)} \qquad (3.1.2.)$$

where $(BEF/10^{4})$ is the percentage of the total radioactivity present as the parent amino acid, $(AC/100)$ is the percentage of the total radioactivity present as involatile acidic metabolites and $(BD/100)$ the percentage of total radioactivity present as involatile basic metabolites.

and become incorporated into TCA cycle derived amino acids and other materials.

To summarise, these results confirm that in experiments of short duration (15min or less), the majority (>85%) of involatile brain soluble radioactivity remains as the parent amino acid following the peripheral administration of L-[G-^{3}H]valine or L-[4,5-^{3}H]lysine. L-[^{3}H]lysine did, however, give rise to a larger (about 25%) quantity of involatile radioactive metabolites in the liver. In contrast, large quantities of involatile radioactive metabolites accumulated in both the brain and liver following the administration of L-[4,5-^{3}H]leucine. These metabolites are expected to include other amino acids.

It is important to emphasise that the metabolic fate of the administered radioactivity depends as much upon the position of substitution as it does on the amino acid itself. The batch of tritiated valine used in the above experiments was marketed as being labelled at C2 and C3, but subsequent tritium n.m.r. spectroscopy showed that there was a significant level of substitution at C4 (hence the designation L-[G-^{3}H]valine). This radiochemical is prepared by the catalytic reduction of an unsaturated intermediate with tritium gas. The presence of tritium at C4 indicates that proton migration occurs during this process. ADRIAENS et al. (1975) reported differences in the distribution of tritium between C2, C3 and C4 in L-[2,3-^{3}H]valine supplied by different manufacturers and between different batches from the same manufacturer. At the Radiochemical Centre it was found that there was a greater percentage of C4 substitution in batches of higher specific activity (personal communication). An examination of one such batch of high specific activity [^{3}H]valine indicated that 64% of the label was substituted at position 4. Non-specificity of substitution clearly represents a problem if TCA-soluble radioactivity is used as an index of precursor specific activity. The radiochemical purities (RCP) of dried TCA-soluble extracts given in Table 3.1.12 were calculated on the basis of results obtained using single batches of [^{3}H] valine and [^{3}H]lysine. Subsequent drug studies were carried out using a number of different batches of these radiochemicals, but no experiments were performed to determine whether the RCP of dried tissue extracts varied significantly between experiments in which different batches of

labelled amino acids were used. A recent n.m.r. spectroscopic examination of a batch of high specific activity L-[4,5-^{3}H]lysine showed, however that 95% of the label was equally distributed between positions 4 and 5 (Radiochemical Centre data sheet) suggesting that proton migration is not a problem in the preparation of this material.

3.1.4 MEASUREMENT OF LOW LEVELS OF RADIOACTIVITY IN AQUEOUS SOLUTION

Financial limitations did not permit the routine use of large amounts of radiochemicals and consequently, on many occasions, the problem of counting very low levels of radioactivity was encountered. Triton X-100 was routinely used for the emulsification of aqueous solutions in the preparation of samples for scintillation counting. The Triton scintillant suffers from the disadvantage that chemiluminescence often causes the background count rate to be high and variable. Large differences were observed between the background count rate obtained using different batches of Triton X-100.

A series of experiments is described in Section 3.1.3 in which TCA-soluble extracts were separated into acidic and basic fractions by cation-exchange chromatography. The results obtained from five such replicate experiments are shown in Table 3.1.13 and it can be seen that in two of these the apparent recovery of radioactivity was significantly greater than 100%. The variability of the apparent recovery of radioactivity in the basic fractions suggested that the problem was associated with the measurement of radioactivity in basic samples. Only in those experiments (3,4 and 5) in which basic samples were acidified prior to the addition of scintillant were sensible recoveries of radioactivity obtained. In subsequent experiments basic samples were therefore acidified with concentrated HCl. Although this greatly reduced the chemiluminescence problem, high recoveries of radioactivity were still obtained in some experiments. This is consistent with the report that chemiluminescence can also present a problem when counting strongly acidic solutions in the presence of emulsifying agents (FOX, 1976).

Two solutions to the problem were considered, firstly, titration of each basic sample to pH7.0 before the addition of scintillant, and secondly,

TABLE 3.1.13 CHEMILUMINESCENCE IN BASIC TRITON X-100 EMULSIONS

Exp	Total radioactivity applied (A) (d.p.m.)	Radioactivity recovered in acidic fraction (B) (d.p.m.)	Radioactivity recovered in basic fraction (C) (d.p.m.)	$\frac{100\ B}{A}$	$\frac{100\ C}{A}$	Total percentage recovery
1	22218	9990	23219	45	105	150
2	19620	9230	29054	47	148	195
3	15954	7879	*8794	49	55	104
4	16561	6509	*10177	39	61	100
5	23387	10798	*15139	46	65	111

This data was obtained from experiments carried out during a study of amino acid metabolism. Brain TCA-soluble extracts (pH2.0) prepared from rats injected with L-[G-^{3}H]valine were applied to Bio-Rad cation-exchange columns (AG50W-X4) and the acidic metabolites eluted with water. Basic metabolites were eluted with 4.5M NH_4OH (see Section 2.3.2). The radioactive content of the original extract and of the acidic and basic fractions was determined after emulsification using Triton X-100 (see Section 2.4.1.1). The total radioactivity applied to the column is shown together with the radioactivity recovered in the acidic and basic fractions. Recovered radioactivity is also expressed as a percentage of that applied to the column. *These samples were acidified with concentrated HCl prior to the addition of the scintillant.

the addition of a quantity of some weak acid sufficient to bring the final pH of the solution within acceptable limits. The second procedure was adopted because it was less time consuming and acetic acid was chosen as a suitable weak acid. However, while aqueous NH_4Cl/toluene/Triton X-100 emulsions were stable over a relatively wide range of temperature and NH_4Cl concentration, the stability of aqueous NH_4OAc/toluene/Triton X-100 emulsions was more sensitive to concentration and temperature changes. It was estimated that the concentration of NH_4OH in basic eluates from cation-exchange columns would be in the range 2.3 to 4.5 M and consequently the stability of ammonium acetate containing emulsions was determined at these two concentrations. As shown in Table 3.1.14 it was not possible to prepare samples of differing composition that were stable at both 4°C and room temperature and so the composition of the emulsion had to be selected on the basis of the temperature of the counting chamber. Radioactive measurements were routinely made at 4°C and samples were therefore prepared by adding acetic acid (100ul) plus Triton scintillant to the column eluates (1ml) with no additional water. Although the resulting mixtures did not form a single phase emulsion at room temperature, stable emulsions formed on shaking mixtures precooled to 4°C.

Measurement of counting efficiency

The external standard channels ratio (ESCR) method for the measurement of counting efficiency was extensively used in the present study. When using this method, if samples of different composition are to be counted, it may be necessary to construct a separate quench correction curve for each type of sample. The use of chemical quench calibration curves to correct for colour quenching can lead to considerable error, particularly when counting tritium (NOUJAIM et al., 1971). In a situation in which counting efficiency is reduced through both colour and chemical quenching the measurement of counting efficiency by the ESCR method is complicated (see for example, LANG, 1971) and it may be easier to use the internal standard method of calibration. Alternatively, samples may be decolourized but peroxide treatment can lead to chemiluminescence (see for example WINKELMAN and SLATER, 1967) and loss of radioactivity (BENEVENGA et al., 1968). The procedure outlined in Fig. 2.1 involved heating aliquots of tissue

TABLE 3.1.14 THE EFFECT OF TEMPERATURE AND WATER CONTENT ON THE STABILITY OF AQUEOUS AMMONIUM ACETATE/TOLUENE/TRITON X-100 EMULSIONS

Amount of water added (ml)	Stability			
	Room temp		4°C	
	A	B	A	B
0.0	U	S	S	S
0.1	S	S	S	S
0.2	U	S	S	S
0.3	S	S	S	S
0.4	U	S	S	S
0.5	S	S	S	S
0.6	S	S	U	U
0.7	S	S	U	U
0.8	S	U	U	U
0.9	S	U	U	U
1.0	S	S	U	U

The stability of aqueous ammonium acetate/toluene/Triton X-100 emulsions was determined at room temperature and at 4°C. The compositions of the emulsions were A. NH_4OH (4.5M, 1ml), acetic acid (100ul), toluene/Triton X-100 (2:1, $^{v}/_{v}$) containing PPO (3.33g/l) (10ml). B. NH_4OH (2.3M, 1ml), all other components as in A. Water was added as indicated. S = Stable, U = Unstable.

homogenate at 100°C in order to remove volatile radioactive amino acid metabolites. Some samples, particularly those prepared from liver, became very coloured on heating and a significant level of colour quenching was therefore expected in the scintillation mixtures in which these samples were subsequently dissolved. During one early experiment five of the most highly coloured samples were selected and counting efficiencies determined using both the ESCR and internal standard methods. The results showed that acceptable estimates of counting efficiencies were obtained using the ESCR method (Table 3.1.15) and this was therefore adopted in subsequent experiments.

The spectroscopic properties of heterocyclic aromatics are usually highly pH dependent and consequently it was not possible to predict whether a chemical quench curve obtained using neutral samples would adequately correct for quenching in strongly acidic samples. As described in Section 2.4.4.1 some samples were acidified with excess concentrated HCl prior to the addition of scintillation fluid and consequently the final pH was low. The results in Table 3.1.16 indicate, however, that this was not a problem since quenching in samples of different pH was adequately corrected for using the same quench correction curve.

Counting low levels of tritium in a narrow energy channel

With some batches of Triton X-100 the background count rate of neutral emulsions in a pre-set tritium channel was too high and variable to be acceptable. PATTERSON and GREENE (1965) reported that Triton X-100 contains phosphorescent impurities which can be removed by treatment with silica gel. In contrast, SCALES (1972) found that luminescence in Triton X-100 emulsions was not reduced by a silica gel pretreatment but that it could be eliminated by counting in a narrow energy channel. The latter approach was adopted in the present study because, as shown in Fig. 3.1.1A, the chemiluminescent background could be completely eliminated with only a moderate loss of tritium counting efficiency by counting in a channel in which the lower energy discriminator was raised. The upper discriminator was set at a level equivalent to the E_{max} for tritium at the highest counting efficiency that would be attained in the samples to be counted (see Fig. 3.1.1B).

TABLE 3.1.15 COMPARISON OF THE EXTERNAL STANDARD CHANNELS RATIO (ESCR) AND INTERNAL STANDARD (IS) METHODS FOR THE DETERMINATION OF COUNTING EFFICIENCY IN COLOURED SAMPLES

A. Colourless samples			B. Coloured samples		
Counting efficiency (%)		Difference	Counting efficiency (%)		Difference
IS method	ESCR method	(IS-ESCR)	IS method	ESCR method	(IS-ESCR)
33.7	34.4	-0.7	25.5	23.9	+1.6
33.8	34.4	-0.6	23.3	22.0	+1.3
33.3	33.5	-0.2	24.7	23.5	+1.2
36.1	34.2	+1.9	23.2	21.7	+1.5
35.4	33.7	+1.7	21.9	20.4	+1.5

Five of the most highly coloured samples obtained from one of the experiments described in Section 3.1.2 were selected and a comparison was made of the counting efficiencies determined using the ESCR and IS methods. The same comparison was made using colourless samples. n-[1,2(n) - ^{3}H] hexadecane was used as an internal standard. ESCR counting efficiencies were calculated using the equation E(%) = 69.22 (ESCR) - 18.6, which was a best fit (correlation coefficient 0.998) to data obtained using toluene samples containing PPO (5g/l), standardized n-[1,2(n) - ^{3}H] hexadecane and chloroform (0.0-0.5ml). Measurements were made using a Packard Tri-Carb Spectrometer (Model 3390).

TABLE 3.1.16 COMPARISON OF THE EXTERNAL STANDARD CHANNELS RATIO (ESCR) AND INTERNAL STANDARD (IS) METHODS FOR THE MEASUREMENT OF COUNTING EFFICIENCY IN ACIDIC EMULSIONS

Sample	Experiment I			Experiment II		
	Counting efficiency (%)		Difference	Counting efficiency (%)		Difference
	IS method	ESCR method	(IS-ESCR)	IS method	ESCR method	(IS-ESCR)
1ml water	29.2	31.4	-2.2	29.3	31.2	-1.9
0.2ml cHCl +1ml4.5M NH_4OH	27.7	29.7	-2.0	26.5	27.9	-1.4
0.2ml cHCl +1ml2.3M NH_4OH	24.8	26.1	-1.3	24.4	25.2	-0.8
1ml 8% TCA adjusted to pH 2.0	26.6	28.5	-1.9	26.9	27.6	-0.7

Table 3.1.16 Continued

The samples listed above were added to 10ml of toluene/Triton X-100 (2:1, $^{v}/_{v}$) containing PPO (3.33g/l) and standardized [^{3}H] toluene. The mixtures were chosen as simulating the composition of samples applied to cation-exchange columns as well as the composition of acidified column eluates (see Sections 2.3.2 and 2.4.1.1). ESCR counting efficiencies were determined as outlined in Table 3.1.15 using standardized [^{3}H] toluene. Measurements were made using a Packard Tri-Carb Spectrometer (Model 3390).

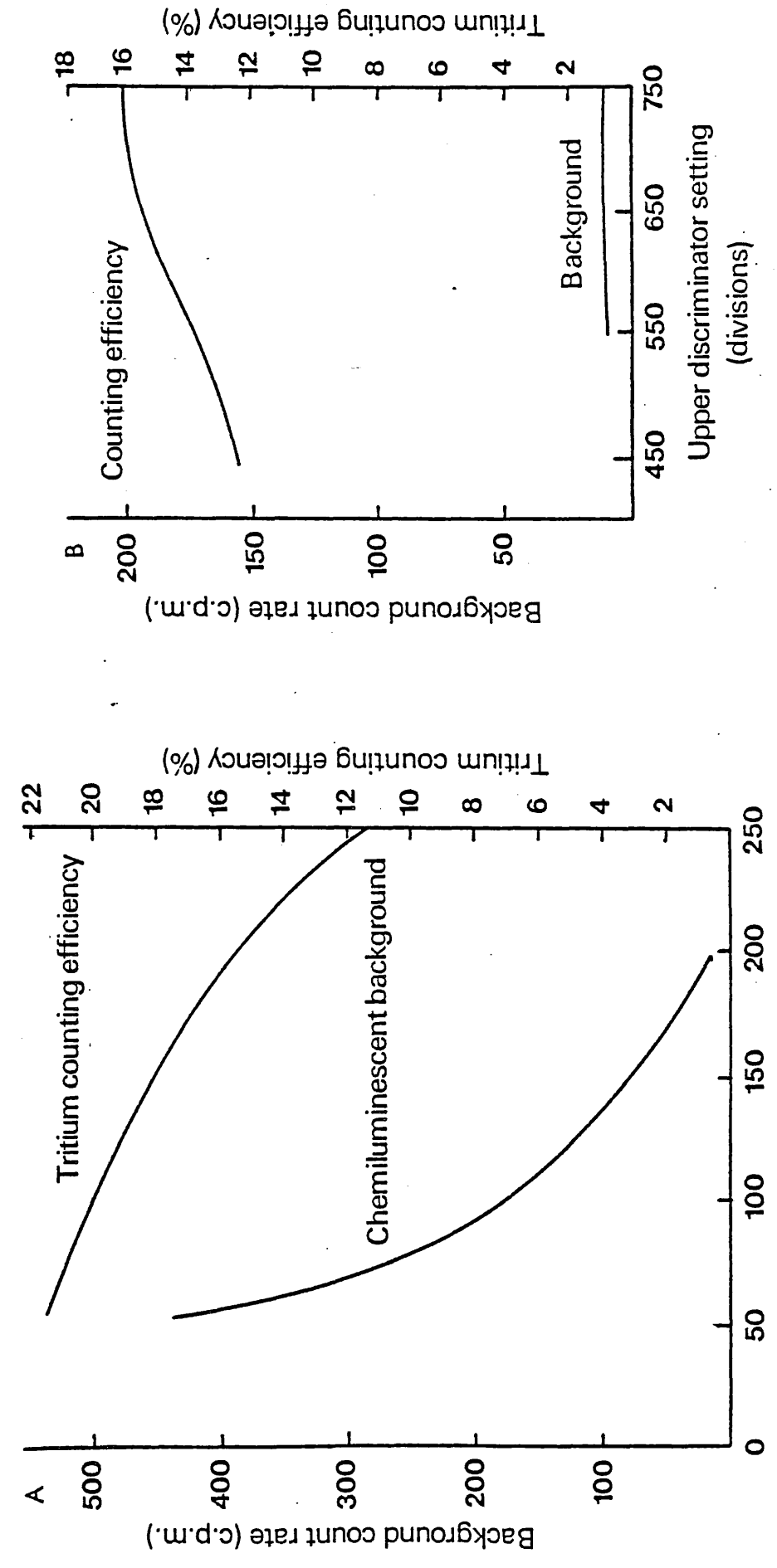


FIG. 3.1.1 THE EFFECTS OF CHANNEL ENERGY WIDTH ON TRITIUM COUNTING EFFICIENCY AND BACKGROUND COUNT RATE

FIG. 3.1.1 Continued.....(2)

The background vial contained a mixture of toluene/Triton X-100 (2:1, $^{v}/_{v}$) (10ml), PPO(3.33g/l) and water (1ml). The emulsion had been prepared several weeks previously and left at room temperature in darkness. Counting efficiencies were determined using an emulsion consisting of toluene/Triton X-100 (2:1, $^{v}/_{v}$) (10ml), PPO (3.33g/l), standardized n-[1,2(n)-^{3}H] hexadecane and sufficient water to cause a level of quenching equivalent to the lowest quenching (ie. highest counting efficiency) expected in samples to be counted. A. Upper discriminator, 650 divisions; gain, 100%. B. Lower discriminator, 200 divisions; gain, 100%. Measurements were made using a Packard Tri-Carb Spectrometer (Model 3390).

Using figures taken from a typical experiment and assuming a reproducible background count rate calculation showed that no significant difference was expected between the error associated with the net count rate obtained in the pre-set tritium and narrow energy channels (Table 3.1.17). In practice, however, when counting Triton X-100 emulsions in which chemiluminescence resulted in a variable background count rate in the pre-set tritium channel, the error associated with the net count rate was significantly reduced by counting in the narrow energy channel since the background count rate became low and reproducible. This procedure was therefore routinely adopted for the measurement of radioactivity in aqueous samples when Triton X-100 was used as an emulsifier.

3.1.5 ALTERED ION-EXCHANGE PROPERTIES OF ISOTOPICALLY SUBSTITUTED AMINO ACIDS

A series of experiments is described in Section 3.1.3 in which the radiochemical purity of TCA-soluble extracts was determined after the separation of administered tritiated amino acids from their metabolites using an automatic amino acid analyzer. [U-^{14}C]amino acids were added to the extracts as internal standards and the radiochemical purity calculated from the difference in the ^{3}H : ^{14}C ratio before and after purification. During the course of this work it was noted that L-[4,5-^{3}H]leucine, L-[4,5-^{3}H]lysine and [L- G-^{3}H]valine do not co-elute with the corresponding L-[U-^{14}C] amino acids (Table 3.1.18) and consequently the ^{3}H :^{14}C ratio could not be determined from the ratio in individual fractions of the peak. Instead, the ratios were calculated from the total area under the ^{3}H and ^{14}C peaks. A similar problem was encountered in experiments in which the specific activities of tritiated amino acids in TCA-soluble extracts were determined (Section 3.2). Again it was found that isotopic substitution altered the ion-exchange properties of amino acids and that [^{1}H]amino acids did not co-elute with their tritiated analogues. Consequently, it was not possible to estimate the true specific activity of tritiated amino acids from the specific activity of any single fraction of the amino acid analyzer eluate. In this case, however, it was not feasible to determine accurately the total radioactivity and total amino acid eluted. Both the radioactive and amino acid contents of the samples were low and their accurate measurement at the tails of the peaks was not therefore possible. To overcome this problem the amino acid specific activity (S) was calculated using the equation

TABLE 3.1.17 COMPARISON OF THE ERRORS ASSOCIATED WITH COUNTING LOW LEVELS OF TRITIUM IN CHANNELS OF DIFFERENT ENERGY WIDTH

Channel	Radioactive content of sample (d.p.m.)	Background count rate (c.p.m.)	Sample count rate (c.p.m.)	Net count rate (c.p.m.)	Var(net count rate) (c.p.m.)	%C(net count rate)
Preset tritium channel	500	40	160	120	20.0 for 10 min count	3.7 for 10 min count
Narrow energy channel	500	10	95	85	10.5 for 10 min count	3.8 for 10 min count

Aqueous samples containing tritium at a level of about 500 d.p.m. were routinely counted using Triton X-100 as an emulsifier. Counting efficiencies in a preset tritium channel were typically 24% and the corresponding efficiency in a narrow energy channel (see Fig. 3.1.1) 17%. As a result of chemiluminescence the background count rate in the preset channel was often high (40 c.p.m. or more) and variable, but was reduced to 10 c.p.m. or less in the narrow channel. Calculation shows that if the background count rate in the preset channel had been constant at about 40 c.p.m. there would be no significant difference in the accuracy of the net count rate measured in the two channels despite the lower counting efficiency in the narrow energy channel. Because the background in the preset channel was variable, however, the error associated with the net count rate was in practice significantly greater than that given above and was reduced through the use of the narrow energy channel. The narrow channel settings were: lower discriminator, 200 divisions; upper discriminator, 650 divisions; gain, 100%. B, background count rate; net count rate = sample count rate - B; var(net count rate)= var(sample count rate)+var(B); var(sample count rate)= sample count rate/counting time; %C(net count rate) = percentage coefficient of variation of the net count rate = $100\sqrt{[\text{var(net count rate)}]}/\text{net count rate}$. Measurements were made using a Packard Tri-Carb Spectrometer (Model 3390).

TABLE 3.1.18 THE PARTIAL SEPARATION OF TRITIATED AMINO ACIDS FROM THEIR ^{14}C-ANALOGUES ON CATION-EXCHANGE COLUMNS

a) Separation of L-[4,5-^{3}H]leucine from L-[U-^{14}C]leucine

Fraction	^{3}H c.p.m.	^{14}C c.p.m.	$^{3}H/^{14}C$
1	200	5	40.0
2	1289	111	11.6
3	2487	358	6.9
4	4139	760	5.4
5	6158	1668	3.7
6	7244	2633	2.8
7	7239	3555	2.0
8	6261	4030	1.6
9	4416	5814	0.8
10	2745	2739	1.0
11	1548	2300	0.7
12	808	1237	0.7
13	169	227	0.7

Table 3.1.18 Continued

b) Separation of L-[G-^{3}H]valine from L-[U-^{14}C] valine

Fraction	^{3}H d.p.m.	^{14}C d.p.m.	^{3}H/^{14}C
1	90	7	12.9
2	2420	337	7.2
3	11503	4248	2.7
4	4658	4597	1.0
5	122	163	0.7
6	42	12	3.5

c) Separation of L-[4,5-^{3}H]lysine from L-[U-^{14}C]lysine

Fraction	^{3}H d.p.m.	^{14}C d.p.m.	^{3}H/^{14}C
1	80	6	13.3
2	260	44	5.9
3	704	122	5.8
4	1104	281	3.9
5	1351	433	3.1
6	755	276	2.7
7	574	246	2.3
8	282	149	1.9
9	95	48	2.0

Table 3.1.18 Continued

The data were obtained from experiments in which the radiochemical purities of tissue extracts were determined after the separation of parent [^{3}H]amino acids from their radioactive metabolites using a Joel amino acid analyzer (see Sections 2.3.2 and 2.3.3 for experimental details). [^{14}C]amino acids were added as internal standards and the tritium and carbon-14 content of the fractionated eluates determined as described in Section 2.4.1. a) 0.5min fractions, flow rate = 0.8ml/min. b) 1.0min fractions, flow rate = 0.8 ml/min. c) 0.5min fractions, flow rate = 1.2ml/min.

$$S = 0.5 \left\{ \sum_{x=-1}^{x=+1} \left\{ \frac{a_x}{\sum_{x=-1}^{x=+1} a_x} S_x^R \right\} + \sum_{x=-1}^{x=+1} \left\{ \frac{b_x}{\sum_{x=-1}^{x=+1} b_x} S_x^A \right\} \right\} \qquad (3.1.3)^*$$

where a_{-1} is the radioactive content of the fraction prior to the radioactive peak, a_o the radioactive content of the peak radioactive fraction and a_{+1} the radioactive content of the fraction following the radioactive peak. S_x^R is the specific activity of the amino acid in each fraction. Similarly, b_{-1} is the amino acid content of the fraction prior to the amino acid peak, b_o the amino content of the peak amino acid fraction, b_{+1} the amino acid content of the fraction following the amino acid peak and S_x^A the corresponding specific activities. The data given in Table 3.1.19 shows that in a situation in which the area under the radioactive and amino acid peaks could be accurately determined there is a good agreement between the specific activity calculated using equation (3.1.3) and that calculated from the total peak areas. In Table 3.1.20 it is shown that, as expected, equation (3.1.3) also yields a good estimate of $^3H:^{14}C$ ratios.

Although it is not shown here, the separation between unsubstituted amino acids and their tritiated analogues was less pronounced than the separation between tritiated amino acids and their ^{14}C-substituted analogues. BELLOBONO (1968) has reported the partial chromatographic separation of [^{12}C] amino acids from their ^{14}C- analogues and has discussed some of the factors that may be involved.

*It is assumed that an estimate of the true amino acid specific activity (S) may be obtained using the equation

$$S = 0.5 \left(S_{max}^R + S_{max}^A \right) \qquad (3.1.4)$$

where S_{max}^R is the amino acid specific activity at the radioactive peak and S_{max}^A is the specific activity at the [^1H]amino acid peak. Because fractions of the column eluate were not symmetrically distributed about the radioactive peak (ie., $a_{+1} \neq a_{-1}$) it was not possible to determine S_{max}^R directly. Instead it was calculated from specific activity measurements made on the three fractions distributed about the radioactive peak (see above) each weighted according to the radioactive content of the fraction. The total radioactive content of the three fractions is $\sum_{x=-1}^{x=+1} a_x$ and hence the weighting coefficient is $a_x / \sum_{x=-1}^{x=+1} a_x$ and $S_{max}^R \simeq \sum_{x=-1}^{x=+1} \left[\left(a_x / \sum_{x=-1}^{x=+1} a_x \right) S_x^R \right]$. Similarly the second term in (3.1.3) is an estimate of S_{max}^A.

TABLE 3.1.19 A COMPARISON OF METHODS FOR CALCULATING AMINO ACID SPECIFIC ACTIVITIES

Fraction	Radioactivity (d.p.m./aliquot)	Amino acid content (nmol/aliquot)	Specific activity (d.p.m./nmol)
1	5155	-	-
2	28088	3.7	7591
3	215588	50.2	4295
4	314969	119.8	2629
5	106484	56.6	1881
6	14454	9.7	1490
7	-	1.5	-
Total peak	684738 d.p.m.	241.5 nmol	2835 d.p.m./nmol

A mixture of L-[4,5-^{3}H]lysine and [^{12}C]lysine was applied to the short column of a Joel amino acid analyzer and eluted with sodium citrate buffer (pH5.25) (see Section 2.3.3 for details). The lysine peak was fractionated (45sec fractions, flow rate = 1.2 ml/min) and the radioactive and amino acid content of each fraction determined as described in Sections 2.4.1.1 and 2.4.2. The specific activity calculated using equation (3.1.3) is 2939 d.p.m./nmol compared with 2835 d.p.m./nmol, the figure calculated from the total radioactive and total lysine content of the peak.

TABLE 3.1.20 A COMPARISON OF METHODS FOR CALCULATING $^{3}H:^{14}C$ RATIOS

Fraction	^{3}H d.p.m.	^{14}C d.p.m.	$^{3}H/^{14}C$
1	80	6	13.33
2	260	44	5.91
3	704	122	5.77
4	1104	281	3.93
5	1351	433	3.12
6	755	276	2.74
7	574	246	2.33
8	282	149	1.89
9	95	48	1.98
10	35	22	1.59
Total peak	5240	1627	3.22

This data is taken from an experiment in which L-[4,5-^{3}H]lysine was separated from its metabolites using a Joel amino acid analyzer as described in Sections 2.3.2 and 2.3.3. [U-^{14}C]lysine had been added as an internal standard. Radioactivity determinations were carried out as outlined in Section 2.4.1. The $^{3}H:^{14}C$ ratio calculated using equation (3.1.3) is 3.28:1.00 compared with 3.22:1.00, the figure calculated from the total radioactivity under the tritium and carbon-14 peaks.

3.2 THE EFFECT OF L-DOPA ON ANIMAL BEHAVIOUR AND IN VIVO PROTEIN SYNTHESIS

Preliminary experiments were carried out to substantiate the results of ROEL et al. (1974) who reported that L-dopa inhibits rat brain protein synthesis. Because large numbers of animals are required for experiments in which time-average soluble amino acid specific activities are determined it was decided not to attempt to determine absolute incorporation rates but to make measurements only at the end of the incorporation period and to express amino acid incorporation in terms of either I_{rel} or I_{app} (see Section 1.2). Male rats were injected with L-dopa (usually 500mg/kg,ip) followed 45min later by the administration of either L-[3,4(n)-^{3}H]valine or L-[4,5-^{3}H]lysine (sc.). They were killed after a $7\frac{1}{2}$ or 15min incorporation period and brain and liver tissue processed as described in Section 2.3.

3.2.1 BEHAVIOURAL RESPONSES TO L-DOPA

The majority (say 5 out of a group of 6) of rats given L-dopa (500mg/kg,ip.) showed characteristic behavioural changes. Within a few minutes of drug administration the animals exhibited pilo-erection and exophthalmus. This was usually followed by a period of hyperactivity or stereotypy. Hyperactive animals moved rapidly around the cage, occasionally showing signs of aggression towards other animals. A few animals vocalized, even in the absence of any obvious stimulus. In response to tactile or acoustic stimuli many rats became intensely hyperactive. Stereotype behaviour was characterized by head-movements (up and down or side to side), bizarre 'dancing' movements of the front legs and prolonged periods of sniffing, particularly while the animal stood on its hind legs in the cage corner. The majority of rats were predominantly hyperactive. In these animals hyperactivity was interspersed by only brief periods of stereotypy. In contrast, a few rats were predominantly stereotypic and showed no signs of hyperactivity. Hyperactivity was often followed by a period of catatonia, particularly of the hind legs. Catatonic rats lay flat on the cage floor. Their breathing was exagerated and when these rats attempted to move they did so by hauling themselves along with their front legs. Occasionally apparently catatonic rats unexpectedly became hyperactive for a very brief period and then lapsed back into a catatonic state.

A few dopa-treated rats showed none of the behavioural responses outlined in the previous paragraph. Tissue from these animals was processed and a figure for I_{rel} obtained but the results were not included in the group means. Although no measurements of tissue dopa levels were made it was assumed that in these non-responding animals the drug had failed to reach a sufficient concentration in the brain to elicit a response. This may have resulted from faulty administration. A similar problem had been encountered during a previous series of experiments in which radioactive amino acids were administered by intraperitoneal injection. No radioactivity was found in the brain and liver of a few of these animals. Levels of TCA-insoluble radioactivity extracted from the brain and liver of non-responding, dopa-treated rats injected with L-[^{3}H]lysine lay in the control range, confirming that these animals should be excluded from the experiment. In those experiments in which [^{3}H]valine was used as the precursor, the difference between the incorporation level in dopa-treated and control rats was not always statistically significant (Tables 3.2.2-3.2.4) and so it was not possible to judge whether the level of incorporation observed in non-responding dopa-treated rats was clearly different from that seen in responding animals. However, any dopa-treated rat that failed to show at least one of the behavioural responses outlined above was disregarded.

3.2.2 THE EFFECT OF L-DOPA ON THE UPTAKE OF RADIOACTIVE AMINO ACIDS INTO BRAIN AND LIVER AND THEIR SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL

3.2.2.1 Effect of L-dopa on the relative incorporation of L-[3,4(n)-^{3}H] valine

Four separate experiments were carried out in which the effect of L-dopa on the incorporation of L-[3,4(n)-^{3}H]valine into TCA-insoluble and soluble material of rat brain and liver was studied. In the first instance the incorporation for each animal was expressed in terms of a relative incorporation (I_{rel}) which is a dimensionless parameter defined by

$$I_{rel}(t=a) = R_2(t=a)/R_1(t=a) \qquad (1.14)$$

where $R_1(t=a)$ is the TCA-soluble radioactivity and $R_2(t=a)$ is the radioactive content of protein, both measured at the end of the incorporation period (i.e., when t=a). A fifteen minute incorporation period was chosen for initial experiments (see Section 3.1.3) and so equation (1.14) becomes

$$I_{rel}(15min) = R_2(15min)/R_1(15min). \qquad (3.2.1)$$

In one experiment 200mg L-dopa/kg had no significant effect on L-[3,4(n)-^{3}H]valine incorporation in brain (Table 3.2.1) and the dose was therefore increased to 500mg/kg for subsequent experiments. At this increased dose level the results obtained in three replicate experiments using [^{3}H]valine were variable (Tables 3.2.2-3.2.4). In one experiment, no statistically significant change was observed in any of the parameters measured (Table 3.2.2), while in the other two experiments the incorporation of [^{3}H]valine into cerebral TCA-precipitable material was reduced by 50% and 45%, respectively, in L-dopa-treated rats (Tables 3.2.3 and 3.2.4). In the first of these two experiments there was an accompanying 37% decrease in brain TCA-soluble radioactivity and consequently the L-dopa-induced decrease in the mean relative incorporation was only 22%, which was not statistically significant (Table 3.2.3). In the second of these two experiments there was no change in TCA-soluble radioactivity and the mean relative incorporation was therefore reduced by 47% in L-dopa-treated rats although, again, the effect was not statistically significant (Table 3.2.4).

Measurements were also made in liver. While it was recognized that inter-animal variation in hepatic amino acid levels would render the simple procedure adopted in this study somewhat inadequate for the study of protein synthesis in liver (see Section 4.3), these measurements were made simply to gain some indication as to whether the L-dopa-induced changes observed in brain also occurred in liver. In general, the same effects were seen in liver as were observed in brain. A significant reduction in TCA-insoluble radioactivity and I_{rel} was observed in only one of the three experiments (Tables 3.2.2-3.2.4).

TABLE 3.2.1 THE EFFECT OF L-DOPA (200mg/kg) ON THE UPTAKE OF L-[3,4(n)-^{3}H] VALINE INTO THE BRAIN AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 1)

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	*TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	**Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	6	202 ± 21	337 ± 18	0.599 ± 0.054
L-Dopa-treated group	6	228 ± 9	341 ± 25	0.683 ± 0.040

Percentage L-dopa-induced changes

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	+13 NS	+1 NS	+14 NS

Rats were injected intraperitoneally with L-dopa (200mg/kg in 0.05M HCl, 20mg/ml) or with 0.05M HCl (10ml/kg), followed 45min later by the subcutaneous administration of L-[3,4(n)-^{3}H] valine (20CuCi/kg, 50uCi/ml in normal saline) (see Section 2.2.1 for details) and were killed after a 15 min incorporation period. Tissue was processed as described in Section 2.3. The animals were 33 days old. Body weights: Control group, 87±1(6)g; Dopa group, 90±1(6)g.

*TCA-soluble radioactivity was calculated using: soluble radioactivity = (total involatile tissue radioactivity) - (TCA-insoluble radioactivity).

**I_{rel} was calculated using individual figures for R_2 and R_1 and not the group means.

The results are given as the mean ± S.E.M. n is the number of animals used. P values were determined by Student's t test (two tailed). NS, not significant at the 5% level.

TABLE 3.2.2 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[3,4(n)-^{3}H] VALINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 2)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	4	242 ± 29	385 ± 48	0.643 ± 0.076
L-Dopa-treated group	3	201 ± 59	322 ± 105	0.641 ± 0.070

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-17 NS	-16 NS	0

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	3	1221 ± 147	316 ± 57	4.00 ± 0.43
L-dopa-treated group	3	884 ± 293	229 ± 43	3.77 ± 0.72

Table 3.2.2 Continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100(\frac{\text{dopa}}{\text{control}}-1)$	-28 NS	-28 NS	-6 NS

Rats were injected intraperitoneally with L-dopa (500mg/kg in 0.05MHCl, 50mg/ml) or with 0.05M HCl (10ml/kg) followed 45 min later by the subcutaneous administration of L-[3,4(n)-^{3}H] valine (200uCi/kg, 40uCi/ml in normal saline) and were killed after a 15min incorporation period.

The animals were between 28 and 35 days old. Body weights: Control group, $73\pm1(4)$g; Dopa-group $68\pm1(3)$g. See Table 3.2.1 for further details.

TABLE 3.2.3 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[3,4(n)-^{3}H] VALINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 3)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	135 ± 8	254 ± 32	0.55 ± 0.04
L-dopa-treated group	5	67 ± 14	161 ± 9	0.43 ± 0.11

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-50 P<0.01	-37 P<0.05	-22 NS

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	881 ± 62	400 ± 29	2.21 ± 0.09
L-dopa-treated group	6	407 ± 93	328 ± 13	1.26 ± 0.29

Table 3.2.3. continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-54 P<0.01	-18 P<0.05	-43 P<0.02

Rats were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg), followed 45min later by the subcutaneous administration of L-[3,4(n)-^{3}H] valine (200uCi/kg, 40 uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were 35 days old. Body weights: Control group, $148\pm5(5)$g; Dopa group, $148\pm6(6)$g. See Table 3.2.1 for further details.

TABLE 3.2.4 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[3,4(n)-^{3}H] VALINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 4)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	4	172 ± 13	186 ± 31	1.01 ± 0.19
L-Dopa-treated group	4	94 ± 14	186 ± 23	0.54 ± 0.11

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-45 P<0.01	0	-47 NS

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	4	1055 ± 74	422 ± 24	2.50 ± 0.07
L-dopa-treated group	4	751 ± 126	379 ± 48	2.16 ± 0.53

Table 3.2.4. continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-29 NS	-10 NS	-14 NS

Rats were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg), followed 45min later by the subcutaneous administration of L-[3,4(n)-^{3}H] valine (200uCi/kg, 40uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were 36 or 37 days old. Body weights: Control group, $128\pm1(4)$g; Dopa group, $126\pm4(4)$g. See Table 3.2.1 for further details.

3.2.2.2 The effect of L-dopa on the relative incorporation of L-[4,5-^{3}H] lysine

The results of two experiments in which L-[4,5-^{3}H]lysine was used as the labelled precursor differed markedly from those in which L-[3,4(n)-^{3}H] valine was employed. In both lysine experiments the radioactive content of the TCA-insoluble material extracted from the brains of rats given 500mg/kg L-dopa was reduced by 73-75% of the control level (Tables 3.2.5 and 3.2.6). L-dopa had no significant effect on TCA-soluble radioactivity and consequently the mean relative incorporation of [^{3}H]lysine in brain was decreased by 76% and 70% in the two experiments, respectively. The results obtained for liver were more variable. In one experiment TCA-insoluble radioactivity was reduced by 40% and the relative incorporation by 44% (Table 3.2.5). In the second experiment L-dopa caused an 80-83% decrease in both the TCA-insoluble radioactivity and the relative incorporation of [^{3}H]lysine (Table 3.2.6).

3.2.2.3 Comparison of the effect of L-dopa on the relative incorporation of L-[^{3}H]valine and L-[^{3}H]lysine

Two questions needed to be answered regarding the above observations.

(1) In no experiment was the mean inhibitory effect observed with [^{3}H] valine as large as that seen with [^{3}H]lysine, but the results of the three separate valine experiments were very variable. This raised the question as to whether the apparent difference between the effect of L-dopa on [^{3}H]valine and [^{3}H]lysine incorporation was statistically significant.

(2) Did L-dopa cause a significant inhibition of [^{3}H]valine incorporation according to the combined results of the three valine experiments?

As a first step to providing answers to these questions an analysis of variance was performed on the lysine relative incorporation data obtained from experiments 5 and 6 in order to determine whether the two sets of data could be combined. The result (Table A2) indicated that although L-dopa produced a significant reduction in I_{rel} in both experiments, there was a significant difference between the duplicate experiments. The results of these two experiments could not therefore legitimately be combined and used in the t-test in the same way as the data obtained from single experiments. For this reason,

TABLE 3.2.5 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 5)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	92 ± 10	510 ± 40	0.180 ± 0.013
L-dopa-treated group	5	25 ± 11	584 ± 45	0.043 ± 0.018

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-73 P<0.01	+15 NS	-76 P<0.001

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	573 ± 23	885 ± 63	0.66 ± 0.04
L-dopa-treated group	5	343 ± 132	925 ± 66	0.37 ± 0.14

Table 3.2.5 continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-40 NS	+5 NS	-44 NS

Rats were injected intraperitoneally with L-dopa (500 mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg), followed 45min later by the subcutaneous administration of L-[4,5-^{3}H] lysine (200uCi/kg, 50uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were 30 days old. Body weights: Control group, $105\pm3(5)$g; Dopa group $108\pm2(5)$g. See Table 3.2.1. for further details.

TABLE 3.2.6 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL (EXPERIMENT 6)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	65.3 ± 6.3	470 ± 41	0.138 ± 0.003
L-dopa-treated group	5	16.3 ± 2.8	398 ± 29	0.042 ± 0.008

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-75 P<0.001	-15 NS	-70 P<0.001

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	401 ± 19	789 ± 59	0.513 ± 0.025
L-dopa-treated group	5	70 ± 18	677 ± 108	0.105 ± 0.026

Table 3.2.6 continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-83 P<0.001	-14 NS	-80 P<0.001

Rats were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg) followed 45min later by the subcutaneous administration of L-[4,5-^{3}H]lysine (200uCi/kg, 40uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were between 33 and 35 days old. Body weights: Control group, $100\pm3(5)$g; Dopa group, $101\pm4(5)$g. See Table 3.2.1 for further details.

in no instance was the relative incorporation data obtained in separate experiments combined and treated as a single set of results. Instead, a different approach was adopted, the theoretical basis of which is outlined below. Before describing this modified approach, it is first necessary to examine the conditions that must be satisfied if I_{rel} is to be an accurate index of protein synthetic activity. Providing the incorporation time is sufficiently short that the specific activity of the protein-bound amino acid pool is much less than that of the precursor, the radioactive content of protein at the end of the incorporation period ($R_2(t=a)$) is given by

$$R_2(a) = S_2(a)Q_2 \qquad (3.2.2)^*$$

$$= \int_o^a F\, S_p(t)dt \qquad (3.2.3)$$

$$= F\, \overline{S_p(a)}a \qquad (3.2.4)$$

where $S_2(a)$ is the specific activity of protein-bound amino acid at the end of the incorporation period (i.e. when t=a), Q_2 the quantity of that amino acid present in protein, F the rate of amino acid incorporation, $S_p(t)$ the function defining the precursor specific activity-time curve and $\overline{S_p(a)}$ the time-average precursor specific activity. All of these parameters are expected to differ for different amino acids. Rearrangement of (3.2.4) yields

$$F = R_2(a) \,/\overline{S_p(a)}\, a. \qquad (3.2.5)$$

It is easily shown that I_{rel} can only be a reliable index of F if within the group of m experimental animals

$$\overline{S_{pk}(a)} = C(a)\, R_{1k}(a),\ k = 1,2,\ldots,m \qquad (3.2.6)$$

where $\overline{S_{pk}(a)}$ and $R_{1k}(a)$ are the time-average precursor specific activity and the final radioactive content of the soluble amino acid pool for the kth animal of the group, respectively, and C is constant for a given amino acid (and incorporation time). Thus from equations (3.2.5)

* For brevity, R(t=a) and S(t=a) are written R(a) and S(a).

and (3.2.6)

$$F = R_{2k}(a)/C(a)R_{1k}(a)a \qquad (3.2.7)$$

$$= I_{rel_k}/C(a)a \qquad (3.2.8)$$

and hence I_{rel} will be a reliable index of rates of amino acid incorporation. (NB. Equation (3.2.6) requires that C is unaffected by L-dopa.)

The modified treatment of the data takes advantage of the fact that experiments were carried out using matched-paired dopa-treated and control rats (see Section 2.2.1). Thus, while it is still necessary to assume that L-dopa can only affect F and R_1 (and hence S_1), the proportionality factor C need only be constant within each matched pair. That is,

$$C_i^D(a) = C_i^C(a) = C_i(a) \qquad (3.2.9)$$

where the postscripts D and C indicate dopa and control parameters, respectively, and the subscript i indicates the ith matched pair of animals. Thus

$$\frac{F_i^D}{F_i^C} = \frac{R_{2i}^D(a)}{C_i(a)R_{1i}^D(a)a} \cdot \frac{C_i(a)R_{1i}^C(a)a}{R_{2i}^C(a)} \qquad (3.2.10a)$$

$$= \frac{R_{2i}^D(a)}{R_{1i}^D(a)} \cdot \frac{R_{1i}^C(a)}{R_{2i}^C(a)} \cdot \qquad (3.2.10b)$$

By definition the relative incorporation is

$$I_{rel} = R_2(a)/R_1(a) \qquad (1.14)$$

and therefore

$$\frac{F_i^D}{F_i^C} = \frac{I_{rel_i}^D}{I_{rel_i}^C} \qquad (3.2.11a)$$

$$= q_{rel_i}, \qquad (3.2.11b)$$

which is referred to as the relative incorporation quotient. Thus while I_{rel} is a meaningful index of rates of amino acid incorporation only if C(a) is constant for all animals, when the data is expressed in terms of the relative incorporation quotient, it is only necessary to assume that C(a) is constant within each matched pair, that being the assumption made in the derivation of (3.2.11). Although analysis of variance indicated that the relative incorporation data obtained in duplicate lysine experiments was significantly different and should not therefore be combined and treated as a single set of results, there was no significant difference between the relative incorporation quotients obtained in the two experiments ($\overline{q_{rel}}$ = 0.257 and 0.301 in experiment 5 and 6, respectively, see Table A5) as is to be expected if the pairing of animals is a meaningful procedure. Thus the q_{rel} data obtained from the two experiments may be combined. Further, if it is assumed that L-dopa does not specifically affect the turnover of a protein fraction very rich or deficient in a particular amino acid, then the relative incorporation quotient is expected to be independent of the labelled amino acid used in its determination. Thus a comparison of the results obtained with [^{3}H]valine and [^{3}H]lysine is possible.

The quotient of two normally distributed variables is not normally distributed and consequently any analysis that assumes this distribution cannot be applied to the analysis of quotients without modification. Although methods have been devised so that parametric statistics may be applied to this type of problem (see, for example, Section 13.5 of COLQUHOUN, 1971), non-parametric methods were adopted because of their relative simplicity. The Wilcoxon signed-ranks test was used to determine whether, on the basis of the combined data, L-dopa resulted in a significant reduction in the relative incorporation of [^{3}H]valine (that is, whether the mean quotient ($\overline{q_{rel}}$) is significantly less than $\overline{q_{rel}}$ = 1). The result (Tables A3 and A4) shows that the L-dopa-induced decrease in the relative incorporation of [^{3}H]valine in both brain ($\overline{q_{rel}}$ = 0.76) and liver ($\overline{q_{rel}}$ = 0.74) was significant. The Wilcoxon two-sample test was employed to determine whether the L-dopa-

induced inhibition observed with [^{3}H]lysine was significantly greater than that seen with [^{3}H]valine. The results (Tables A5 and A6) indicate that, on the basis of combined experimental data, the decrease in the relative incorporation of [^{3}H]lysine was significantly greater than that of [^{3}H]valine in both brain ($\overline{q_{rel}}$ for lysine = 0.28) and liver ($\overline{q_{rel}}$ for lysine = 0.39).

The difference in the results obtained with the two different amino acids suggests that one or more of the assumptions made in the derivation of (3.2.11) is invalid with respect to one or both amino acids. If the assumption given by (3.2.6) is valid, providing the inter-animal variation in the rate of amino acid incorporation into protein is significantly less than the variability of the uptake of the radioactive amino acid into the tissue in question, TCA-insoluble radioactivity and TCA-soluble radioactivity will be linearly correlated. This was found to be the case in one experiment in which L-[4,5-^{3}H]leucine was used as the labelled precursor (see Section 3.1.2).

Spearman rank correlation analysis was performed on the data obtained from the control groups of rats of two valine experiments (experiments 3 and 4). The analysis was carried out on the basis of both separate and combined data. In no case was a significant correlation observed (Table A7), indicating that the assumption given by (3.2.6) may not be valid with respect to these valine experiments. If equation (3.2.6) is invalid then I_{rel} and q_{rel} cannot be reliable indices of rates of protein synthesis. In contrast, in the case of the two [^{3}H]lysine experiments (experiments 5 and 6) a correlation was observed between the TCA-insoluble and acid-soluble radioactivity within the control group of rats (Table A8), indicating that I_{rel} and q_{rel} may, in this case, be reliable indices of protein synthetic activity, assuming of course that the equality in (3.2.9) is valid. Finally, an analysis of the combined lysine q_{rel} data demonstrated that the rank of q_{rel}^{brain} and that of q_{rel}^{liver} in the same matched pair of rats were correlated (Table A9). The same result was obtained from an analysis of the results obtained with [^{3}H]valine (Table A10). This suggests that some common factor determined the level of inhibition observed in the two tissues.

To summarize, L-dopa caused a statistically significant 24% reduction in the relative incorporation of [^{3}H]valine in brain but an examination of the experimental data indicated that in these experiments I_{rel} was not a reliable index of the absolute rate of brain protein synthesis. In contrast, L-dopa resulted in a significant 72% decrease in the relative incorporation of [^{3}H]lysine in brain and the result of an analysis of the data was consistent with I_{rel} being a reliable index of the absolute rate of lysine incorporation into brain protein in the control group of rats. It was not possible to test the validity of equation (3.2.9). Consequently the question as to whether the observed L-dopa-induced reduction in the relative incorporation of [^{3}H]lysine was caused by an inhibition of brain protein synthesis remained unanswered.

3.2.2.4 The effect of L-dopa on the apparent incorporation of L-[3,4(n)-^{3}H]valine in brain

In order to obtain a more reliable estimate of comparative rates of brain protein synthesis in control and L-dopa-treated rats, valine specific activity measurements were made in two of the four experiments described previously (experiments 3 and 4) and the incorporation of radioactivity into TCA-insoluble material expressed in terms of the *apparent incorporation (I_{app}) which is defined by

$$I_{app}(t=a) = R_2(t=a)/S_1(t=a). \qquad (1.13)$$

Specific activity measurements were made using the same tissue homogenates that were used in the determination of I_{rel}, thus a direct comparison of the effect of L-dopa on I_{rel} and I_{app} can be made. The results are shown in Tables 3.2.7 and 3.2.8. It can be seen that while there was a considerable discrepancy between the two control groups with respect to the mean value of I_{rel}, the difference in I_{app} was not so marked. This is consistent with I_{app} being the more reliable index of protein synthetic activity. L-dopa had no significant effect on this parameter in either experiment.

*The term apparent incorporation is appropriate because I_{app} has the same dimensions as an absolute incorporation.

TABLE 3.2.7 THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[3,4(n)-^{3}H]VALINE IN BRAIN (EXPERIMENT 3 CONTINUED)

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	5	135 ± 8	254 ± 32	206 ± 30	0.55 ± 0.04	0.70 ± 0.10
L-Dopa-treated group	5	67 ± 14	161 ± 9	103 ± 8	0.43 ± 0.11	0.63 ± 0.10

Percentage L-dopa-induced changes

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble valine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-50 P<0.01	-37 P<0.05	-50 P<0.02	-22 NS	-10 NS

Details of experiment 3 are given in Table 3.2.3. Valine specific activity measurements were made after partial purification of brain TCA-soluble extracts by cation-exchange chromatography followed by a final separation of valine from other amino acids using a Joel amino acid analyzer (see Sections 2.3.2 and 2.3.3). (NB. I_{app} was calculated using individual figures for R_2 and S_1 and not the group means.) Relative incorporation data are included for comparison.

TABLE 3.2.8 THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[3,4(n)-^{3}H]VALINE IN BRAIN (EXPERIMENT 4 CONTINUED)

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	4	172 ± 13	186 ± 31	336 ± 63	1.01 ± 0.19	0.55 ± 0.06
L-Dopa-treated group	3	96 ± 19	205 ± 18	165 ± 10	0.49 ± 0.14	0.60 ± 0.16

Percentage L-dopa-induced changes

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble valine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-44 P<0.02	+10 NS	-51 NS	-51 NS	+9 NS

See Table 3.2.7 for explanation. Details of experiment 4 are given in Table 3.2.4. It should be noted that because one sample was lost on the amino acid analyser the number of replicates in the dopa group is one less than shown in Table 3.2.4. For the purpose of comparison a new mean I_{rel} has been calculated on the basis of the remaining three replicates.

Using a procedure similar to that adopted in Section 3.2.2.3 it is possible to determine whether the apparent incorporation of an amino acid is indicative of its absolute incorporation rate. It follows from (3.2.5) that I_{app} can only be an accurate index of the absolute rate of amino acid incorporation into protein if

$$\overline{S_{pk}(a)} = b(a)S_{1k}(a),\ k=1,2,\ldots,m \qquad (3.2.12)$$

within the group of m experimental animals, where $S_{pk}(a)$ is the final specific activity of the soluble amino acid pool for the kth animal of the group and b is constant for a given amino acid (and incorporation time). Thus when this condition is fulfilled

$$F = R_{2k}(a)/b(a)S_{1k}(a)a \qquad (3.2.13)$$

$$= I_{app_k}/b(a)a. \qquad (3.2.14)$$

If variability in precursor specific activity is the major cause of inter-animal variation in the rate of incorporation of radioactivity into protein then, according to (3.2.13), TCA-insoluble radioactivity and the final specific activity of the soluble amino acid pool should be linearly correlated within the group of experimental animals. Statistical analysis of the combined data obtained from the two experiments in which valine specific activity measurements were made indicated that such a correlation existed within the control animals (Table A11). Further, this correlation was still observed when data obtained from L-dopa-treated rats were combined with the control data (Table A12). While this is consistent with the hypothesis that L-dopa had no effect on rates of protein synthesis and no effect on the proportionality factor b of (3.2.12), the possibility that both parameters were affected by L-dopa, but in such a way that

$$F^C b^C(a) = F^D b^D(a) \qquad (3.2.15a)$$

but

$$F^C \neq F^D \qquad (3.2.15b)$$

and

$$b^C(a) \neq b^D(a) \qquad (3.2.15c)$$

cannot be ruled out.

3.2.2.5 The effect of L-dopa on the apparent incorporation of L-[4,5-^{3}H]lysine in brain

The specific activity of the brain soluble lysine pool was measured in experiments 5 and 6 and the data used in the calculation of I_{app}. The results are shown in Tables 3.2.9 and 3.2.10, together with the relative incorporation data which is included for comparison. The L-dopa-treatment caused a 55 - 59% decrease in the apparent incorporation of [^{3}H]lysine, compared with the 70 - 76% decrease in I_{rel}. The observed 42 - 44% L-dopa-induced decrease in the specific activity of the soluble lysine pool was not reflected by a parallel decrease in TCA-soluble radioactivity. This probably contributed to the discrepancy between the effect of L-dopa on I_{rel} and I_{app}. This matter is discussed further in Section 3.2.2.7.

Statistical analysis showed that within the control group of rats, TCA-insoluble radioactivity and the specific activity of free lysine were correlated (Table A13), which is in agreement with equation (3.2.13) and indicates that the control I_{app} data obtained in experiments 5 and 6 were reliable indices of brain protein synthetic activity. It was not possible, however, to determine whether the proportionality factor b of (3.2.12) was affected by the L-dopa-treatment. Consequently, the question as to whether the drug-induced reduction in I_{app} reflected a reduction in rates of brain protein synthesis could not be answered.

3.2.2.6 Comparison of the effect of L-dopa on the apparent incorporation of L-[3,4(n)-^{3}H]valine and L-[4,5-^{3}H]lysine in brain

As mentioned in Section 3.2.2.3, experiments were carried out using matched-paired animals and therefore an apparent incorporation quotient may be defined

$$q_{app_i} = I^D_{app_i} / I^C_{app_i} \qquad (3.2.16)$$

where $I^D_{app_i}$ and $I^C_{app_i}$ are the apparent incorporation for the dopa-treated and control rats of the ith pair, respectively. Providing the proportionality factor of (3.2.12) is constant within each matched pair of rats, that is,

TABLE 3.2.9 THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[4,5-^{3}H]LYSINE IN BRAIN (EXPERIMENT 5 CONTINUED)

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	5	92 ± 10	510 ± 40	122 ± 10	0.180 ± 0.013	0.75 ± 0.05
L-Dopa-treated group	5	25 ± 11	584 ± 45	71 ± 7	0.043 ± 0.018	0.31 ± 0.12

Percentage L-dopa-induced changes

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble lysine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-73 P<0.01	+15 NS	-42 P<0.01	-76 P<0.001	-59 P<0.01

Details of experiment 5 are given in Table 3.2.5. Lysine specific activity measurements were made after partial purification of brain TCA-soluble extracts by cation-exchange chromatography followed by a final separation of lysine from other amino acids using a Joel amino acid analyzer (see Sections 2.3.2 and 2.3.3). (NB. I_{app} was calculated using individual figures for R_2 and S_1 and not the group means.) Relative incorporation data are included for comparison.

TABLE 3.2.10 THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[4,5-^{3}H]LYSINE IN BRAIN (EXPERIMENT 6 CONTINUED)

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	5	65.3 ± 6.3	470 ± 41	98.3 ± 5.8	0.138 ± 0.003	0.662 ± 0.037
L-Dopa-treated group	5	16.3 ± 2.8	398 ± 29	54.6 ± 4.7	0.042 ± 0.008	0.301 ± 0.048

Percentage L-dopa-induced changes

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble lysine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-75 P<0.001	-15 NS	-44 P<0.001	-70 P<0.001	-55 P<0.001

See Table 3.2.9 for explanation. Details of experiment 6 are given in Table 3.2.6.

$$b_i^C(a) = b_i^D(a) = b_i(a) \qquad (3.2.17)$$

then the apparent incorporation quotient should be an accurate index of the effect of L-dopa on rates of amino acid incorporation. Thus from (3.2.13) and (3.2.17)

$$\frac{F_i^D}{F_i^C} = \frac{R_{2i}^D(a)}{b_i(a)S_{1i}^D(a)a} \cdot \frac{b_i(a)S_{1i}^C(a)a}{R_{2i}^C(a)}. \qquad (3.2.18)$$

Since

$$I_{app} = R_2(a)/S_1(a), \qquad (1.13)$$

$$\frac{F_i^D}{F_i^C} = \frac{I_{app_i}^D}{I_{app_i}^C} \qquad (3.2.19a)$$

$$= q_{app_i}. \qquad (3.2.19b)$$

If, as before, the additional assumption is made that L-dopa does not specifically affect the turnover rate of a protein fraction particularly rich or deficient in a given amino acid, then q_{app} should be independent of the radioactive amino acid used in its determination.

The mean apparent incorporation quotient for valine ($\overline{q_{app}^{val}}$), based on the combined results of experiments 3 and 4 was 1.07, which is not significantly different from $\overline{q_{app}} = 1$, that is, no drug effect (Table A14) (compare with Section 3.2.2.4). The mean quotient for lysine ($\overline{q_{app}^{lys}}$) based on the combined results of experiments 5 and 6 was 0.45, which is significantly less than $\overline{q_{app}^{val}} = 1.07$ (Table A15). This discrepancy between $\overline{q_{app}^{val}}$ and $\overline{q_{app}^{lys}}$ means that one or more of the assumptions made in the derivation of (3.2.19) is invalid with respect to lysine and/or valine.

Some indication as to which of the above assumptions is incorrect may be obtained through an examination of the time dependence of the effect of L-dopa on I_{app} and I_{rel}. As mentioned in Section 3.2.2.4 the observed correlation between TCA-insoluble radioactivity and the specific activity of the soluble valine pool within a group of animals consisting of both control and dopa-treated rats is compatible with the hypothesis that the proportionality factor of (3.2.12) was unaffected by L-dopa in those experiments in which [^{3}H]valine was used as the radioactive precursor. No evidence has been presented, however, to show this to be true with respect to the [^{3}H]lysine experiments. The proportionality factor b is time dependent, but if the assumption that it is unaffected by L-dopa is correct (see equation (3.2.17)) then

$$\frac{b^D(t_1)}{b^C(t_1)} = \frac{b^D(t_2)}{b^C(t_2)} = 1. \qquad (3.2.20)$$

If, however, (3.2.17) is incorrect, then it is likely that $b^D(t)$ and $b^C(t)$ will be different functions of time and that

$$\frac{b^D(t_1)}{b^C(t_1)} \neq \frac{b^D(t_2)}{b^C(t_2)} \neq 1. \qquad (3.2.21)$$

The effect of L-dopa on I_{app} will then depend upon the length of the incorporation period. If b is affected by L-dopa, then C(t) of equations (3.2.6)-(3.2.8) will also be affected and the magnitude of the L-dopa-induced decrease in I_{rel} will also depend upon the length of the labelling period. For this reason, additional experiments were carried out using [^{3}H]lysine but with a 7½min incorporation period. The results of two such experiments are shown in Tables 3.2.11 and 3.2.12. In the first experiment, L-dopa resulted in a 15% increase in the relative incorporation of lysine in brain, whilst in the second a 22% decrease was observed. Neither of these changes was statistically significant. The apparent lysine incorporation in brain was reduced by 1% and 15% in the two experiments, respectively. Again, neither of these changes was statistically significant. These observations differ markedly from those of the 15min lysine experiments (experiments

TABLE 3.2.11 THE EFFECT OF L-DOPA ON THE UPTAKE OF L-[4,5-^{3}H]LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL DURING A $7\frac{1}{2}$ MIN PERIOD (EXPERIMENT 7)

A.1. Effect of L-Dopa on (a) the relative incorporation and (b) the apparent incorporation of [^{3}H]lysine in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	5	32.4 ± 2.5	512 ± 43	99 ± 7	0.0634 ± 0.0012	0.327 ± 0.005
L-Dopa-treated group	4	22.0 ± 6.2	285 ± 49	65 ± 13	0.0731 ± 0.0095	0.324 ± 0.033

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble lysine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-32 NS	-44 P<0.02	-34 P<0.05	+15 NS	-1 NS

TABLE 3.2.11 continued

B.1. Effect of L-Dopa on the relative incorporation of [^{3}H]lysine in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.$^{-1}$)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.$^{-1}$)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	231 ± 19	1229 ± 65	0.188 ± 0.008
L-Dopa-treated group	4	206 ± 50	647 ± 120	0.313 ± 0.020

B.2. Percentage L-Dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-11 NS	-47 P<0.01	+66 P<0.001

Rats were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg) followed 45min later by the subcutaneous administration of [L- 4,5-^{3}H]lysine (400uCi/kg, 80uCi/ml in normal saline) and were killed after a 7.5min incorporation period. Tissue was processed as described in Section 2.3. The animals were between 33 and 35 days old. Body weights: Control group, 142±4(5)g; Dopa group, 148±3(4)g. See Table 3.2.1 for further details.

TABLE 3.2.12 THE EFFECT OF L-DOPA ON THE UPTAKE OF L-[4,5-^{3}H]LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL DURING A $7\frac{1}{2}$ MIN PERIOD (EXPERIMENT 8)

A.1. Effect of L-Dopa on (a) the relative incorporation and (b) the apparent incorporation of [^{3}H]lysine in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Specific activity of soluble valine (S_1) (d.p.m./nmole)	Relative incorporation ($I_{rel} = R_2/R_1$)	Apparent incorporation ($I_{app} = R_2/S_1$) (nmole/mg prot.)
Control group	6	40.5 ± 3.2	575 ± 38	114.6 ± 8.4	0.0727 ± 0.0093	0.364 ± 0.043
L-Dopa-treated group	6	18.6 ± 2.0	333 ± 35	61.0 ± 6.6	0.0564 ± 0.0051	0.311 ± 0.034

A.2. Percentage L-Dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Specific activity of soluble lysine	Relative incorporation	Apparent incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-54 P<0.001	-42 P<0.001	-47 P<0.001	-22 NS	-15 NS

TABLE 3.2.12 continued

B.1. Effect of L-Dopa on the relative incorporation of [^{3}H]lysine in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	6	244 ± 20	966 ± 41	0.254 ± 0.024
L-Dopa-treated group	6	123 ± 29	710 ± 96	0.179 ± 0.042

B.2. Percentage L-Dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-50 P<0.01	-27 P<0.05	-30 NS

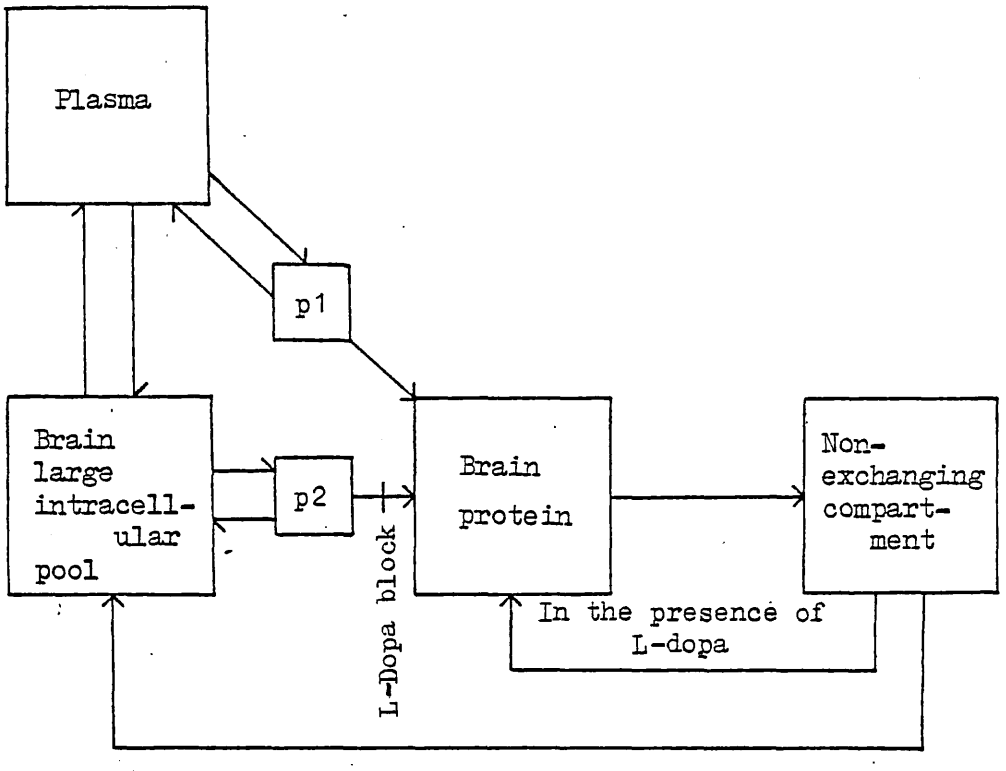
Rats were injected intraperitoneally with L-Dopa (500mg/kg in 0.05 M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg), followed 45min later by the subcutaneous administration of L-[4,5-^{3}H]lysine (400uCi/kg, 80uCi/ml in normal saline) and were killed after a 7.5min incorporation period. Tissue was processed as described in Section 2.3. The animals were 38 days old. Body weights: Control group, 149±4(6)g; Dopa group, 151±3(6)g. See Table 3.2.1 for further details.

5 and 6, see Tables 3.2.9 and 3.2.10) in which a significant decrease in both the relative and apparent incorporation of lysine was observed. The effect of L-dopa on the relative incorporation of lysine in liver, as measured after a 7½min incorporation period, was variable. In the first experiment a 66% increase was observed, which contrasts with the 30% decrease seen in the second experiment. It is shown in Section 4.3, however, that I_{rel} is sensitive to changes in amino acid pool sizes. Hepatic amino acid levels fluctuate over a considerable range (see for example VIDRICH et al., 1977) and consequently I_{rel} is not expected to be an accurate index of rates of hepatic protein synthesis. This may account for the discrepancy between the two results.

The difference between the effect of L-dopa on the incorporation of [^{3}H]lysine and [^{3}H]valine into brain protein may be explained by a variety of hypotheses. It could be postulated, for example, that L-dopa specifically inhibits the turnover of a protein fraction particularly rich in lysine or deficient in valine. A more plausible explanation is that I_{app} and I_{rel} are not reliable indices of rates of lysine and/or valine incorporation into brain protein. The observation that the apparent incorporation of [^{3}H]lysine in brain was unaffected by L-dopa when measured after a 7½min incorporation period but was grossly inhibited after 15min suggests that the relationship between the time-average precursor lysine specific activity and the final specific activity of the soluble lysine pool was affected in some way by the drug (i.e., the proportionality factor b of equations 3.2.12 - 3.2.14 and therefore 'C' of equations 3.2.6 - 3.2.8 are L-dopa dependent).

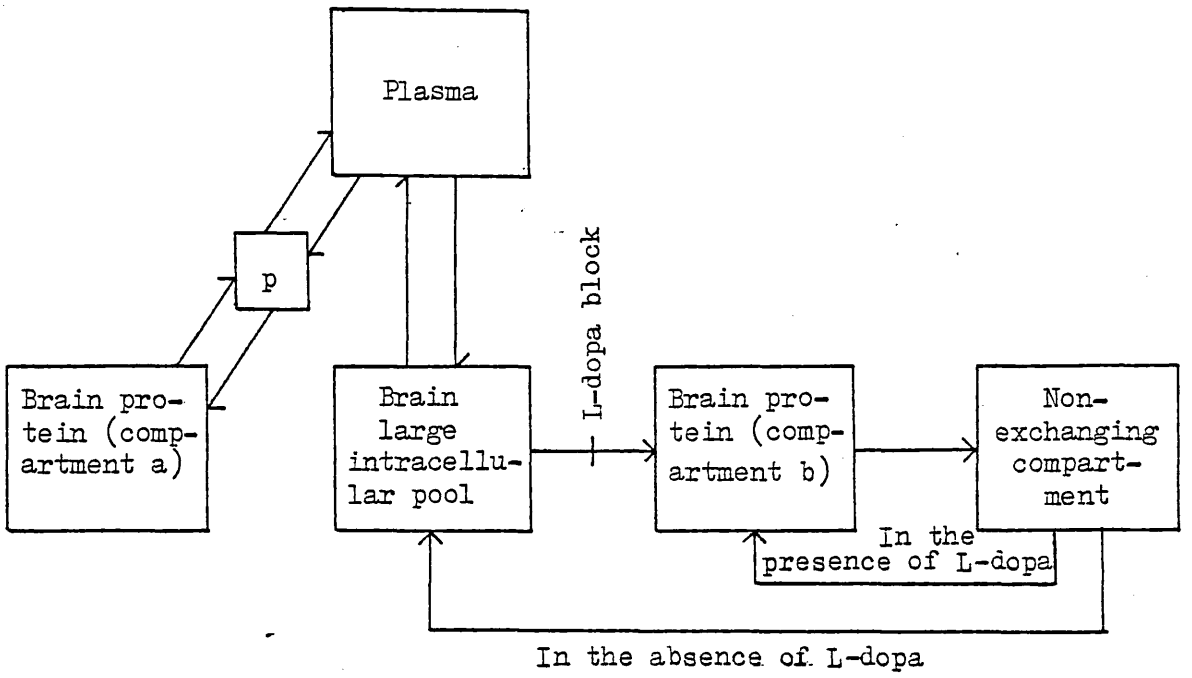
Computer simulation was used to test a number of models of lysine compartmentation that might account for the above experimental results. A detailed description of two models that yield incorporation data that is in agreement with the experimental observations is given in Section 4.1. A summary of these models is outlined in Figs. 3.2.1 and 3.2.2. The two schemes yield virtually identical theoretical incorporation curves and these are shown in Fig. 4.6 together with the incorporation data obtained from the 7½min and 15min lysine experiments.

FIG. 3.2.1 MODEL 1. SUBCELLULAR MODEL OF BRAIN PRECURSOR LYSINE COMPARTMENTATION



Outlined above are the important features of the model shown in Fig. 4.4. p1 and p2 represent two precursor lysine compartments. p1 equilibrates rapidly with plasma lysine while p2 exchanges rapidly with the large brain intracellular lysine pool. Lysine released from degraded protein enters the large intracellular pool via a nonexchanging compartment. In the presence of L-dopa p2 ceases to be a source of lysine for protein synthesis, the deficit being derived from the nonexchanging compartment.

FIG. 3.2.2 MODEL 2. CELLULAR MODEL OF BRAIN PRECURSOR LYSINE COMPARTMENTATION



Outlined above are the important features of the model shown in Fig. 4.5. The model assumes the presence of two protein compartments, possibly associated with different cell types. One protein compartment (a) derives its lysine from a small precursor compartment (p) that exchanges rapidly with the plasma pool. In the absence of L-dopa the other protein compartment (b) derives its lysine from the large intracellular pool, while in the presence of L-dopa this pathway is blocked and, in order that protein synthesis may continue, lysine released into the nonexchanging compartment is recycled directly back into protein.

In both models precursor lysine is compartmented. One model assumes the presence of two distinct protein compartments and associated precursor compartments (Fig. 3.2.2), while in the other model a single protein compartment derives its lysine from more than one precursor pool (Fig. 3.2.1, compare with the models of AIRHART et al., 1974; KHAIRALLAH and MORTIMORE, 1976; VIDRICH et al., 1977). Both models assume that (1) rates of protein synthesis are unaffected by L-dopa, which is consistent with the valine data given in Tables 3.2.7 and 3.2.8, and (2) that L-dopa specifically inhibits the incorporation into protein of lysine present in one of the precursor compartments. According to these models apparent and relative lysine incorporation data cannot be used as indices of absolute protein synthetic rates since changes in precursor specific activity are not necessarily reflected by a proportional change in the specific activity of the total soluble lysine pool. This matter is examined in detail in Chapter 4.

An important feature of the above models is that they account for the 7½min and 15min lysine incorporation data (Fig. 4.6 and Table 4.6). It should be emphasised, however, that extreme caution is required when comparing the 7½min data with the 15min results since these were obtained from separate experiments. Clearly, it would have been preferable to carry out experiments in which incorporation was measured as a function of time. This was not done because it was not considered feasible to maintain the pace demanded by the injection schedule employed in these experiments (see Table 2.1) for the time required to process the large numbers of animals that such an experiment demands. Further, brain protein synthetic activity is reported to undergo diurnal changes (RICHARDSON and ROSE, 1971) and it was therefore considered desirable to keep the duration of experiments to a minimum.

3.2.2.7 The effect of L-dopa on lysine metabolism

The data given in Tables 3.2.9 and 3.2.10 indicate that although L-dopa caused a decrease in both the relative and apparent incorporation of lysine in brain, the decrease in I_{rel} was larger than the corresponding decrease in I_{app}. It is shown in this section that an L-dopa-induced change in the metabolism of L-[4,5-^{3}H]lysine probably contributed to this discrepancy.

The relationship between the time-average precursor specific activity ($\overline{S_p(a)}$) and the final radioactive content of the TCA-soluble pool ($R_1(a)$) (see equation (3.2.6)) will, in part, depend upon whether or not the administered amino acid gives rise to radioactive metabolites. In order to examine the consequence of an accumulation of radioactive metabolites it is convenient to define a new variable $I_{rel_{corr}}$ as

$$I_{rel_{corr}} = R_2(t=a) \; / R_1(t=a)_{corr} \qquad (3.2.22)$$

where R_2 is the TCA-insoluble radioactivity and $R_{1_{corr}}$ is the TCA-soluble radioactivity present in the form of the administered amino acid, both measured at the end of the incorporation period. Since

$$R_1(a)_{corr} = R_1(a)P \qquad (3.2.23)$$

where P is the radiochemical purity of the TCA-soluble fraction, it follows that

$$\frac{I^D_{rel_{corr}}}{I^C_{rel_{corr}}} = \frac{R^D_2(a)}{R^D_1(a)P^D} \; \frac{R^C_1(a)\,P^C}{R^C_2(a)} \qquad (3.2.24)$$

$$= \frac{I^D_{rel}}{I^C_{rel}} \; \frac{P^C}{P^D} \cdot \qquad (3.2.25)$$

Since

$$R_1(a)\,P = Q_1 S_1(a) \qquad (3.2.26)$$

(3.2.24) can be rewritten

$$\frac{I^D_{rel_{corr}}}{I^C_{rel_{corr}}} = \frac{R^D_2(a)}{Q^D_1 S^D_1(a)} \quad \frac{Q^C_1 S^C_1(a)}{R^C_2(a)} \qquad (3.2.27)$$

$$= \frac{I^D_{app}}{I^C_{app}} \quad \frac{Q^C_1}{Q^D_1} \qquad (3.2.28)$$

Equating the R.H.S. of (3.2.25) and (3.2.28) yields

$$\frac{I^D_{rel}}{I^C_{rel}} \quad \frac{P^C}{P^D} = \frac{I^D_{app}}{I^C_{app}} \quad \frac{Q^C_1}{Q^D_1},$$

that is

$$\frac{I^D_{rel}}{I^C_{rel}} = \frac{I^D_{app}}{I^C_{app}} \quad \frac{Q^C_1}{Q^D_1} \quad \frac{P^D}{P^C}. \qquad (3.2.29)$$

Thus the observation that after a 15min incorporation period the L-dopa-induced reduction in I_{rel} was larger than the corresponding decrease in I_{app} may be explained in terms of an increased accumulation of involatile radioactive metabolites in the brains of L-dopa-treated rats (i.e., $P^D < P^C$) and/or an increase in the size of the brain soluble lysine pool (i.e., $Q^D_1 > Q^C_1$). Although not all of the parameters of equation (3.2.29) were measured directly, some indication of the cause of the disparity between the relative and apparent incorporation data may be obtained from the available data and an examination of the outcome of assuming that L-dopa has no effect on lysine metabolism. To this end amino acid pool sizes were estimated using (3.2.26). The radiochemical purity of the brain TCA-soluble lysine pool 15min after the administration of L-[4,5-^{3}H]lysine was taken to be 92% (Table 3.1.12). Substitution into (3.2.26) of *data obtained from the 15min lysine experiments

*R_1 was equated to the TCA-soluble radioactivity remaining after extracts had been heated at 100°C to remove tritiated water, the major radioactive metabolite (see Section 3.1.3).

(experiments 5 and 6) yielded results indicating that L-dopa caused an apparent 54 - 105% increase in the size of the brain soluble lysine pool 60min after the administration of the drug, while according to the results of the 7½min experiments (experiments 7 and 8), L-dopa had no significant effect on lysine pool sizes 52½min after its administration (Tables 3.2.13 and 3.2.14). In contrast, the magnitude of the L-dopa-induced decrease in the specific activity of the soluble lysine pool was similar in all four experiments and lay in the range 34-47%. It is considered unlikely that endogenous brain lysine levels could have risen abruptly between the 52nd and 60th minute after L-dopa administration while the specific activity of the soluble lysine pool remained unchanged. A more plausible explanation is that the assumption that L-dopa had no effect on [^{3}H]lysine metabolism is incorrect and that in the presence of L-dopa L-[4,5-^{3}H]lysine produced a larger percentage of involatile radioactive metabolites in peripheral tissue, and that these metabolites were subsequently transported to the brain. As shown by equation (3.2.29), such an increase in the concentration of labelled metabolites would explain the discrepancy between the relative and apparent incorporation data obtained from the 15min experiments. In order to account for the fact that no significant L-dopa-induced change is observed in the apparent lysine pool sizes calculated on the basis of the 7½min experimental data (Table 3.2.14) it is necessary to postulate that in dopa-treated rats the concentration of radioactive cerebral metabolites increased with time in such a way that these metabolites accounted for only a small percentage of brain soluble radioactivity 7½min after the administration of the label, but that by 15min the concentration of radioactive impurities had risen to a significant level. It is recognised that these arguments are based on speculation and that the discrepancy between the 15min relative and apparent incorporation data may have arisen fortuitously through random error in the experimental data. This matter can only be resolved by further experimentation. The present interpretation of the data is, however, consistent with reports that L-dopa has no significant effect on brain lysine levels (ROEL *et al.*, 1974; WEISS *et al.*, 1971; LIU *et al.*, 1972).

TABLE 3.2.13 APPARENT SOLUBLE LYSINE POOL SIZES IN THE BRAIN OF CONTROL AND L-DOPA-TREATED RATS

	Apparent soluble lysine pool sizes in brain			
	15min incorporation period		$7\frac{1}{2}$min incorporation period	
	Experiment 5	Experiment 6	Experiment 7	Experiment 8
Control group	3.9 ± 0.2(5)	4.39 ± 0.24(5)	5.16 ± 0.09(5)	5.05 ± 0.24(6)
L-Dopa-treated group	8.0 ± 1.2(5)	6.75 ± 0.24(5)	4.51 ± 0.37(4)	5.50 ± 0.22(6)

Apparent brain soluble lysine pool sizes were calculated using equation (3.2.26) assuming that L-dopa had no effect on the metabolism of L-[4,5-^{3}H] lysine and that the radiochemical purity (P) of brain TCA-soluble extracts of control and L-dopa-treated rats was therefore not significantly different. The radiochemical purity of TCA-soluble extracts 15min after precursor administration was taken to be 92% (Table 3.1.12). Radiochemical purity measurements were not made $7\frac{1}{2}$min after precursor administration and calculations were therefore performed using $P(t = 7\frac{1}{2}) = 100\%$. It should be noted that the resulting error in the calculated $7\frac{1}{2}$min apparent pool sizes is expected to be less than 8% (ie., $P(t = 7\frac{1}{2})$ is expected to be greater than $P(t = 15)$). Further, according to the assumption that the radiochemical purity of extracts of control and dopa-treated animals were not significantly different, the percentage changes in lysine pool sizes given in Table 3.2.14 are independent of P.

TABLE 3.2.14

Parameter	15min incorporation period		$7\frac{1}{2}$min incorporation period	
	Experiment 5	Experiment 6	Experiment 7	Experiment 8
Specific activity of soluble lysine	-42 P<0.01	-44 P<0.001	-34 P<0.05	-47 P<0.001
Soluble radioactivity	+15 NS	-15 NS	-44 P<0.02	-42 P<0.001
Apparent lysine pool size	+105 P<0.01	+54 P<0.001	-13 NS	+9 NS
I_{rel}	-76 P<0.001	-70 P<0.001	+15 NS	-22 NS
I_{app}	-59 P<0.01	-55 P<0.001	-1 NS	-15 NS

The effect of L-dopa on the specific activity of the brain soluble lysine pool (S_1) and the radioactive content of this pool (R_1), both measured $7\frac{1}{2}$min and 15min after precursor administration are given together with the magnitude of the L-dopa-induced changes in parameters calculated on the basis of R_1 and S_1. Data for experiment 5 are taken from Table 3.2.9, experiment 6 from Table 3.2.10, experiment 7 from Table 3.2.11 and experiment 8 from Table 3.2.12. Percentage changes in apparent pool sizes were calculated from the data given in Table 3.2.13.

In their study, ROEL et al. (1974) found no evidence for an L-dopa-induced change in lysine metabolism, but their chromatographic procedure was probably not adequate for the separation of lysine from some of its basic metabolites. Also, since only 32-40% of radioactivity applied to cation-exchange columns was recovered (see Table 2 of ROEL et al., 1974) it is not possible to comment on the chemical form of the remaining 60-68% of radioactivity. Further, the experimental procedure adopted by Roel and co-workers differed from the procedure used in the present study with respect to the mode of precursor administration. Roel et al. administered their radioactive amino acids by intracisternal injection, and since brain has relatively little capacity for lysine metabolism (CHANG, 1976), not until the radioactive lysine had been transported to some peripheral site of metabolism (e.g. the liver), and metabolites transported back into the brain could appreciable levels of radioactive impurities accumulate in the brain. This would, in part, account for their failure to detect radioactive lysine metabolites in the brain of L-dopa-treated rats 30min after the intracisternal administration of the label, assuming, of course, that L-dopa does bring about an increased rate of lysine degradation in the periphery, as suggested above.

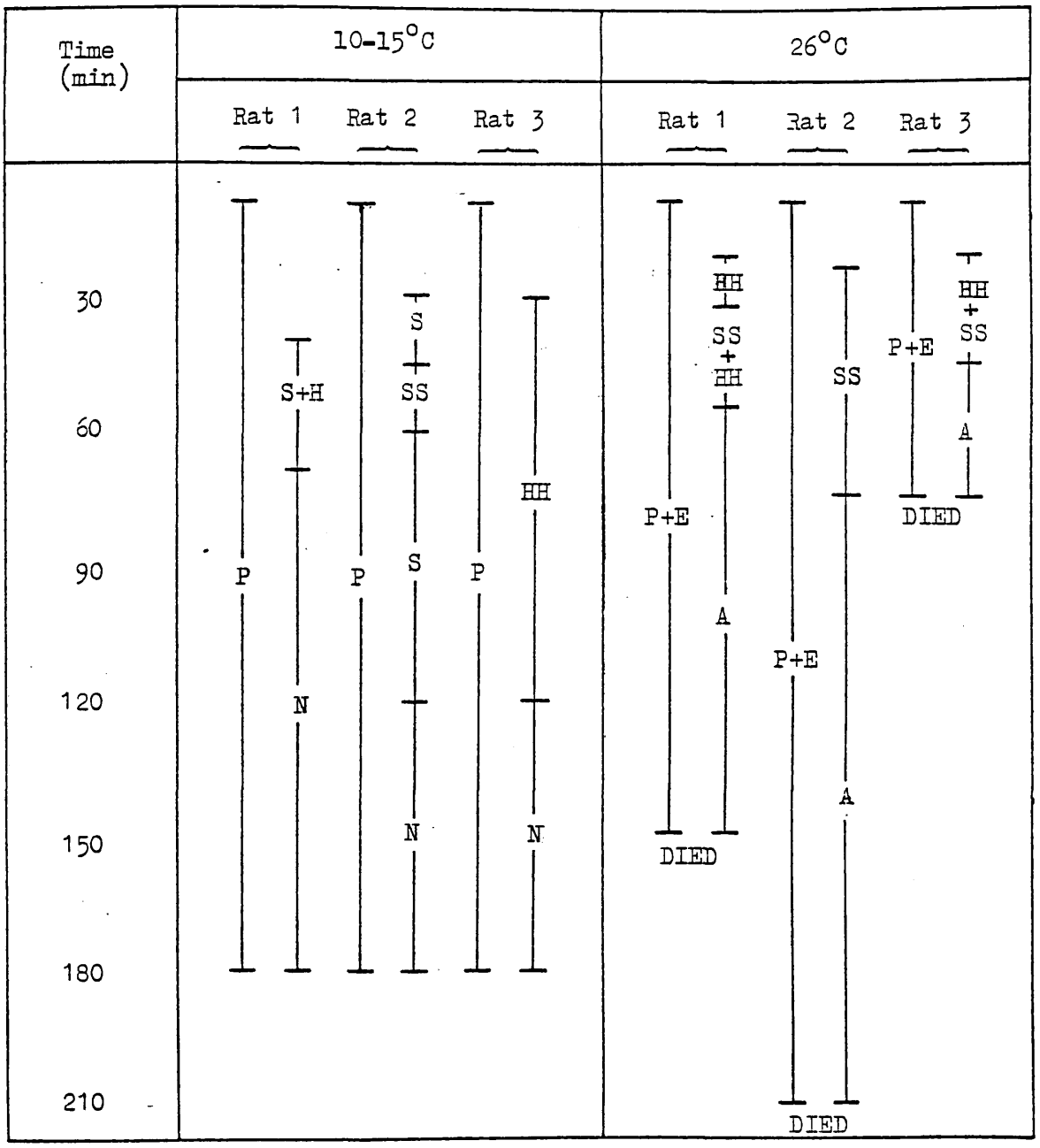
3.2.2.8 The effect of ambient temperature on L-dopa-induced behavioural changes

When the experiments described in the previous sections were nearing completion MOSKOWITZ et al. (1977) published their work on the role of hyperthermia in the L-dopa and D-amphetamine-induced disaggregation of cerebral polysomes. This study was prompted by the previous observation that on some occasions these drugs failed to have a significant effect on the integrity of brain polysomes. Moskowitz and co-workers found that both L-dopa and D-amphetamine disaggregated brain polysomes in animals maintained at 26°C, while polysomes isolated from drug-treated animals maintained at 18°C or 10°C were not significantly affected. It was also reported that L-dopa and D-amphetamine produced hyperthermia when administered to rats kept at 26°C, had little effect on the body temperature of rats kept at 18°C and produced a hypothermic response in rats maintained at 10°C. Hyperthermia itself was not as effective as drug-induced hyperthermia in disaggregating brain polysomes. On the basis of these results it was suggested that hyperthermia plays a 'permissive role' in drug-induced polysome disaggregation. It was also suggested

that differences in the temperature at which animals were maintained after drug administration may have been the cause of the previously observed variability in drug responses.

Experiments 1-8 of the present study were carried out under conditions in which ambient temperature was not controlled. As mentioned previously, in these experiments a few rats failed to show a behavioural response to L-dopa. Although these animals were excluded from the experiment, tissue taken from them was processed, and it was found that in those experiments in which lysine had been used as the labelled precursor, the level of incorporation in non-responding dopa-treated rats lay in the control range (see Section 3.2.1). This observation, together with those of Moskowitz and co-workers led to the notion that there exists a critical temperature range, above 18°C but below 26°C, within which some rats become hyperthermic, exhibit behavioural changes and a decreased apparent rate of lysine incorporation, while other rats fail to become hyperthermic, fail to show behavioural responses and incorporate lysine at a normal apparent rate. A preliminary experiment was therefore carried out to test the hypothesis that L-dopa-induced behavioural changes are dependent upon the temperature at which the animals are maintained after drug administration. Six rats were injected with L-dopa (500mg/kg), three of which were kept at 10-15°C and three at 26°C. While it is important to emphasise that no specialised equipment (e.g. activity recording cages) was used in this experiment, there were definite behavioural differences between the two groups of rats. The major behavioural responses are summarised in Fig. 3.2.3. Within 10 min of receiving L-dopa, rats maintained at 26°C exhibited pilo-erection and exophthalmus. By 25 min two of the three rats showed very intense hyperactivity which was later interrupted by periods of stereotyped behaviour. Stereotypy consisted mainly of head movements and sniffing usually while the animals stood on their hind legs in the cage corner. This was followed by a period of ataxia during which the rats lay flat on the floor of the cage. Their hind quarters appeared to be particularly affected and when they moved they did so by hauling themselves along with their front legs. Very occasionally, an apparently ataxic rat exhibited hyperactivity for a few seconds and then lapsed back into an ataxic state. All three rats died within $3\frac{1}{2}$h. Control rats kept at 26°C exhibited no behavioural changes.

FIG. 3.2.3 THE BEHAVIOURAL EFFECTS OF L-DOPA (500mg/kg) IN RATS MAINTAINED AT 10-15°C AND 26°C



Three rats were maintained at 10-15° and three at 26°C for 1h before and throughout the experiment. They were injected with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) and their behaviour observed. HH, intense hyperactivity; H, hyperactivity; SS, intense stereotypy; S, slight stereotypy; P, pilo-erection; E, exophthalmus; A, ataxia; N, normal. The animals were between 32 and 34 days old.

Body weights: 10-15°C group, 111 ± 8 (3)g; 26°C group, 117 ± 8 (3)g.

Rats kept at 10-15°C showed pilo-erection. Slight to moderate stereotypy was the predominant behavioural response. This was interrupted by brief periods of hyperactivity in two of the three rats, but this was much less intense than that seen at 26°C. Stereotypy was similar to that seen at 26°C except that it included bizarre dancing movements of the front legs in addition to head movements etc. Within three hours of dopa-administration the 10-15°C rats appeared normal. They were kept at room temperature for a number of weeks after the experiment and during this time they showed no ill effects. Control rats maintained at 10-15°C showed pilo-erection but no behavioural responses. They lay curled in a tight ball with their eyes closed.

To summarise, L-dopa-treated rats maintained at 10-15°C showed behavioural changes that were generally less intense than that exhibited by rats kept at 26°C. Rats maintained at 26°C died within 3½h of drug administration while those kept at 10-15°C showed no permanent ill effects.

During the course of previous experiments carried out at room temperature it had been noted that L-dopa-treated rats sometimes became quiet for a period of time, but that any stimulus (acoustic or tactile) would precipitate an episode of intense hyperactivity, often accompanied by vocalisation. During one experiment carried out at 10°C (experiment 9) dopa-treated rats showed no behavioural responses until, some time after the start of the experiment, they were unintentionally subjected to high intensity machine noise. Coincident with the onset of this acoustic stimulus, L-dopa-treated rats became intensely hyperactive and exhibited little stereotype behaviour. This was in contrast to the L-dopa response seen at 10°C in the experiment described above. In a third experiment carried out at 10°C (experiment 10) rats showed no hyperactivity and little or no stereotype behaviour. In general they stood still in the cage corner showing slight signs of exophthalmus and pilo-erection. On the basis of these observations it is tentatively suggested that some stimulus is required to initiate an episode of hyperactivity. It is also suggested that at room temperature and above a mild stimulus is sufficient, while at lower temperatures a stronger stimulus is required to elicit a response. However, no systematic study of the effect of ambient temperature on the behavioural response to L-dopa was carried out and consequently this suggestion remains highly speculative.

3.2.2.9 The effect of L-dopa on the incorporation of radioactive amino acids into protein in rats maintained at different ambient temperatures

Having demonstrated that the behavioural response to L-dopa is temperature dependent, experiments were carried out to investigate the effect of ambient temperature on the L-dopa-induced decrease in the rate of [^{3}H] lysine incorporation into protein. Although the work described in Sections 3.2.2.1 - 3.2.2.3 had shown that I_{rel} is not a reliable index of absolute rates of protein synthesis, specific activity measurements were not made in these experiments and the results are expressed only in terms of the relative incorporation of [^{3}H]lysine, recognising that this gives no measure of the true rate of lysine incorporation. In two experiments in which L-dopa was administered to rats maintained at 10°C, the drug caused a 24-32% decrease in brain TCA-insoluble radioactivity measured after a 15min incorporation period (Tables 3.2.15 and 3.2.16), although in neither experiment was the effect statistically significant. A parallel 21-36% decrease in brain TCA-soluble radioactivity was observed and consequently there was no difference between the dopa and control groups with respect to the relative incorporation of [^{3}H]lysine in brain. These results contrast with the 70-76% decrease in the relative incorporation of brain lysine seen at room temperature (Tables 3.2.5 and 3.2.6). In one 10°C experiment there was a statistically significant 33% decrease in the relative incorporation of [^{3}H]lysine in the liver of L-dopa-treated rats while in the other experiment a non-significant 23% decrease was observed.

The finding that L-dopa caused a gross reduction in the relative incorporation of [^{3}H]lysine in the brains of rats kept at room temperature but not in rats maintained at 10°C appears to be in agreement with the observation made by MOSKOWITZ et al. (1977) that L-dopa disaggregates cerebral polysomes at 26°C but not at 10°C and may indicate that both phenomena result from a dopa-induced inhibition of cerebral protein synthesis. This interpretation is not consistent, however, with the observation that, at room temperature, L-dopa had no significant effect on the apparent incorporation of [^{3}H]valine in brain (Tables 3.2.7-3.2.8) and no significant effect on the apparent incorporation of [^{3}H]lysine after a 7½ min labelling period (Tables 3.2.11 and 3.2.12). Although these experimental observations have been explained in terms of lysine compartmentation (see Section 4.1), it was recognised that the apparent

TABLE 3.2.15 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL IN RATS MAINTAINED AT 10°C (EXPERIMENT 9)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	6	82.7 ± 8.8	600 ± 11	0.137 ± 0.013
L-Dopa-treated group	5	63.2 ± 8.9	474 ± 61	0.136 ± 0.012

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-24 NS	-21 NS	-1 NS

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	6	1278 ± 106	2538 ± 116	0.511 ± 0.052
L-Dopa-treated group	5	769 ± 101	2262 ± 280	0.341 ± 0.030

Table 3.2.15 continued

B.2. Percentage L-Dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-40 P<0.01	-11 NS	-33 P<0.05

Rats were maintained at 10°C for 1-2h before and throughout the experiment. They were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05 M HCl (10ml/kg) followed 45min later by the subcutaneous administration of L-[4,5-^{3}H]lysine (200uCi/kg, 40uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were 28 days old. Body weights: Control group, $95\pm1(6)$g; Dopa-group, $93\pm2(5)$g. See Table 3.2.1 for further details.

TABLE 3.2.16 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL IN RATS MAINTAINED AT 10°C (EXPERIMENT 10)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	47.4 ± 2.8	490 ± 15	0.0966 ± 0.0037
L-Dopa-treated group	6	32.1 ± 6.5	316 ± 40	0.0967 ± 0.0085

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-32 NS	-36 P<0.01	0 NS

B.2. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	531 ± 48	1769 ± 169	0.313 ± 0.042
L-Dopa-treated group	6	246 ± 37	1046 ± 101	0.240 ± 0.031

Table 3.2.16 continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-54 P<0.001	-41 P<0.01	-23 NS

See Table 3.2.15 for experimental details. Rats were between 34 and 38 days old. Body weights: Control group, $99\pm4(5)$g; Dopa group, $100\pm4(6)$g.

discrepancy between the data obtained using different labelled amino acids and different incorporation times could have arisen from the failure to control ambient temperature. Thus, although it was considered an unlikely occurrence, additional experiments were carried out at 23-26°C to rule out the possibility that a low ambient temperature was the cause of the failure to observe a dopa-induced decrease in the apparent incorporation of amino acids in some experiments.

Both of the models of lysine compartmentation outlined in Section 4.1 assume that L-dopa has no effect on rates of cerebral protein synthesis. This assumption is based on the observed insensitivity of valine incorporation to inhibition by L-dopa and it was therefore imperative that this be demonstrated unequivocally. Measurement of absolute rates of protein synthesis is greatly simplified if precursor specific activity is maintained at a constant level throughout the incorporation period (see Section 1.2). DUNLOP et al. (1975a) have demonstrated that this may be achieved by injecting [^{14}C]amino acids together with a large quantity of carrier amino acid. Although it had been decided that, because of the relatively high cost of [^{14}C]amino acids, this flooding technique could not be used routinely, this method was adopted in two experiments in order to obtain additional evidence for the insensitivity of valine incorporation to inhibition by L-dopa.

Rats were maintained at *23-26°C for one to two hours before the start of each experiment. L-dopa (500mg/kg,ip) or carrier medium was administered, followed 45min later by L-[U-^{14}C]valine (9.6 mmole/kg, ip). (It should be noted that in their study, DUNLOP et al. (1975a) used uniformly labelled [^{14}C]amino acids with the exception of L-[1-^{14}C]valine. L-[U-^{14}C] valine was used in the present study because the specifically substituted radiochemical was prohibitively expensive.) The labelling period was increased from 15 to 35min in order to obtain a higher and more measureable incorporation of radioactivity into protein.

*For convenience, experiments carried out at a controlled temperature in the range 23-26°C are referred to as 26°C experiments. The actual temperature recorded during each experiment is given with each table of results.

In one "flooding" experiment (experiment 11) L-dopa had no effect* on the rate at which valine was incorporated into cerebral protein (Table 3.2.17), which is in agreement with the apparent incorporation data obtained in the two experiments carried out at room temperature and using trace quantities of [^{3}H]valine (Tables 3.2.7 and 3.2.8). In the second flooding experiment (experiment 12) L-dopa caused a 30% decrease in the valine incorporation rate in brain, but this was not significant at the 5% level. Although the possibility that L-dopa causes a small inhibition of cerebral protein synthesis cannot be ruled out, the results indicate that the large L-dopa-induced reduction in I_{rel} and I_{app} observed in brain fifteen minutes after the administration of [^{3}H]lysine cannot be attributed to a gross inhibition of protein synthesis. This interpretation assumes that L-dopa does not specifically reduce the turnover rate of a protein fraction particularly rich in lysine and devoid of valine.

* During a trial experiment using the flooding technique, virtually no radioactivity was found in the brain of one animal injected with 10 mmole of [^{14}C]valine/kg body weight. Similar observations were made when trace quantities of radioactivity were given intraperitoneally. This was attributed to badly placed injections. In experiment 11 this problem was observed in one dopa-treated and one control rat. The levels of radioactivity recovered from the two animals were 4 to 13 times less than that recovered from other animals. These two results were not included in the calculation of the means given in Table 3.2.17. When the data obtained from these animals are included, the mean $\pm$ S.E.M. for the rate of valine incorporation into cerebral protein in the L-dopa-treated and control groups is 1.75 $\pm$ 0.57 (4) and 1.96 $\pm$ 0.29 (6) nmole/mg prot./h, respectively, so the conclusion remains unchanged.

TABLE 3.2.17 THE EFFECT OF L-DOPA (500mg/kg) ON THE RATE OF INCORPORATION OF L-[U-14C]VALINE INTO BRAIN AND LIVER TCA-PRECIPITABLE MATERIAL IN RATS MAINTAINED AT 24-26°C

A. Effect of L-dopa in brain

	Rate of valine incorporation (nmole/mg prot./h)	
	Experiment 11	Experiment 12
Control group	2.23 ± 0.08(5)	2.30 ± 0.08(4)
L-Dopa-treated group	2.26 ± 0.35(3)	1.61 ± 0.30(4)

B. Effect of L-dopa in liver

	Rate of incorporation of L-[U-^{14}C] valine (d.p.m./mg prot./h)	
	Experiment 11	Experiment 12
Control group	161 ± 7(5)	293 ± 10(4)
L-Dopa-treated group	130 ± 35(3)	99 ± 17(4)

Table 3.2.17 continued

C. Percentage L-dopa-induced changes

Brain		Liver	
Experiment 11	Experiment 12	Experiment 11	Experiment 12
+1 NS	-30 NS	-19 NS	-66 P<0.001

Rats were maintained at 24-26°C before and throughout the experiment. They were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl (10ml/kg) followed 45min later by the intraperitoneal injection of L-[U-^{14}C]valine (9.6 mmole/kg, 0.48umole/ml, 6.7 uCi/mmole in exp. 11 and 12.7 uCi/mmole in exp. 12) and were killed after a 35min incorporation period. Rates of cerebral valine incorporation were calculated assuming the precursor specific activity to be the same as that of the injected amino acid. The liver incorporation data has not been converted to a rate of valine incorporation but is given in terms of the incorporation of radioactivity. Rats were 37-42 days old in experiment 11 and 27 days old in experiment 12. Body weights: Control group of exp. 11, $121\pm6(5)$g; Dopa group of exp. 11, $128\pm11(3)$g; Control group of exp. 12, $89\pm3(4)$g; Dopa group of exp. 12, $87\pm3(4)$g.

A discrepancy was observed between the results of the two flooding experiments with respect to the L-dopa effect in liver. The rate of [^{14}C]valine incorporation into hepatic TCA-insoluble material was reduced by 66% in one experiment, while a non-significant 19% decrease was observed in the other. No experiments were carried out to establish whether the administration of 10 mmole of valine /kg body weight is sufficient to swamp the endogenous soluble valine pool in liver, and whether or not the rate at which radioactivity is incorporated into TCA-insoluble material is a reliable index of rates of protein synthesis in that tissue. It is not possible, therefore, to assess whether L-dopa had an effect on hepatic protein synthesis. In order to answer this question it may be necessary to adopt a different experimental approach. Liver perfusion experiments would probably yield more reproducible data, but were not carried out in the present study.

To confirm that L-dopa causes a gross inhibition of [^{3}H]lysine incorporation under the same temperature controlled conditions that valine incorporation in brain was comparatively unaffected, two experiments were carried out at 23-26^{o}C using trace quantities of L-[4,5-^{3}H]lysine. L-Dopa resulted in an 80% decrease in TCA-insoluble radioactivity in one experiment (Table 3.2.18) and a 67% decrease in the other (Table 3.2.19). Although these results are similar to those obtained at room temperature (Tables 3.2.5 and 3.2.6), at 23-26^{o}C the final TCA-soluble radioactivity in the brain of dopa-treated rats was reduced by 47% and 35% in the two experiments, respectively, and consequently the relative incorporation of brain lysine was decreased by 50-59%. These results contrast with those previously obtained at room temperature. At that temperature L-dopa had no significant effect on brain TCA-soluble radioactivity and caused a 70-76% decrease in the relative incorporation of cerebral lysine. The difference between the effect of L-dopa on the relative incorporation of brain lysine at room temperature ($\overline{q_{rel}}$ = 0.279) and 23-26^{o}C ($\overline{q_{rel}}$ = 0.450) was not statistically significant (Table A16). It should be noted, however, that even if this difference had been significant, according to the models of lysine compartmentation outlined in Section 4.1, this would not necessarily indicate that the effect of L-dopa on the incorporation of lysine into protein is temperature dependent, since the effect could equally well have arisen from

TABLE 3.2.18 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL IN RATS MAINTAINED AT 23 - 25°C (EXPERIMENT 13)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	98.2 ± 5.2	695 ± 20	0.141 ± 0.005
L-Dopa-treated group	5	19.2 ± 2.5	367 ± 56	0.058 ± 0.010

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-80 P<0.001	-47 P<0.001	-59 P<0.001

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	417 ± 32	914 ± 42	0.455 ± 0.016
L-Dopa-treated group	5	41 ± 11	742 ± 95	0.061 ± 0.017

Table 3.2.18 continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-90 P<0.001	-19 NS	-87 P<0.001

Rats were maintained at 23 - 25°C for 1-2h before and throughout the experiment. They were injected intraperitoneally with L-dopa (500mg/kg in 0.05M HCl, 50mg/ml) or with 0.05M HCl(10ml/kg) followed 45min later by the subcutaneous administration of L-[4,5-^{3}H]lysine (200uCi/kg, 100uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were 31 days old. Body weights: Control group, 111±1(5)g; Dopa-group, 110±1(5)g. See Table 3.2.1 for further details.

TABLE 3.2.19 THE EFFECT OF L-DOPA (500mg/kg) ON THE UPTAKE OF L-[4,5-^{3}H] LYSINE INTO BRAIN AND LIVER AND ITS SUBSEQUENT INCORPORATION INTO TCA-PRECIPITABLE MATERIAL IN RATS MAINTAINED AT 24 - 25°C (EXPERIMENT 14)

A.1. Effect of L-dopa in brain

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	150 ± 10	1339 ± 62	0.112 ± 0.004
L-dopa-treated group	5	49 ± 11	872 ± 84	0.056 ± 0.013

A.2. Percentage L-dopa-induced changes in brain

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-67 P<0.001	-35 P<0.01	-50 P<0.01

B.1. Effect of L-dopa in liver

	n	TCA-insoluble radioactivity (R_2) (d.p.m./mg prot.)	TCA-soluble radioactivity (R_1) (d.p.m./mg prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
Control group	5	861 ± 67	1980 ± 142	0.436 ± 0.018
L-dopa-treated group	5	318 ± 108	1515 ± 157	0.232 ± 0.082

Table 3.2.19 continued

B.2. Percentage L-dopa-induced changes in liver

	TCA-insoluble radioactivity	TCA-soluble radioactivity	Relative incorporation
$100\left(\frac{\text{dopa}}{\text{control}}-1\right)$	-63 P<0.01	-23 NS	-47 P<0.05

Rats were maintained at 24-25°C for 1-2h before and throughout the experiment. They were injected intraperitoneally with L-dopa (500 mg/kg in 0.05M HCl, 50 mg/ml) or with 0.05M HCl (10ml/kg) followed 45min later by the subcutaneous administration of L-[4,5-^{3}H]lysine (400uCi/kg, 80uCi/ml in normal saline) and were killed after a 15min incorporation period. The animals were between 29 and 37 days old. Body weights: Control group, 130±5(5)g; Dopa group, 127±5(5)g. See Table 3.2.1 for further details.

a difference in the rate at which radioactive lysine accumulates in the brain following its subcutaneous administration to animals maintained at different temperatures. There was a discrepancy between the two 26°C lysine experiments with respect to the effect seen in liver. L-dopa resulted in an 87% reduction in the relative incorporation of lysine in one experiment (Table 3.2.18) while a reduction of only 47% was observed in the other (Table 3.2.19).

To conclude, the series of 26°C experiments for the most part confirmed the room temperature experimental data and supports the proposition that the difference between the effect of L-dopa on lysine and valine incorporation did not arise artifactually as a result of a failure to control ambient temperature.

3.3 THE EFFECT OF L-DOPA ON IN VITRO PROTEIN SYNTHESIS

WEISS et al. (1975) proposed that L-dopa inhibits protein synthesis via a cellular mechanism involving a soluble dopamine receptor. In support of this hypothesis they pointed out that the magnitude of the dopa-induced disruption of cerebral polysomes indicates that the total population of brain cells is affected by L-dopa, i.e., polysomes of both neuronal and glial origin are disaggregated. An alternative hypothesis is outlined in Section 1.1.1.2 according to which the effect is mediated indirectly via a synaptic dopaminergic system. If a cytoplasmic receptor were involved in the L-dopa-induced inhibition of lysine incorporation into protein, then one might expect to observe the effect in brain slices. On the other hand, if the drug acts via an indirect synaptic mechanism, it would be expected to be without effect in vitro. Experiments were therefore performed in order to determine whether L-dopa has any effect on amino acid incorporation into protein in chopped brain tissue. Chopped brain was used in these experiments in preference to brain slices to overcome the problem of structural heterogeneity and variability in the sensitivity of different brain regions to L-dopa.

3.3.1 THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-PRECIPITABLE MATERIAL IN CHOPPED RAT BRAIN

Effect of oxygenation

FRANCK(1972) has presented a detailed account of the changes that occur

in brain slices incubated under different conditions and he recommends that in order to prevent swelling, the tissue be incubated in a permanently oxygenated medium. For oxygenation to be effective the gas should be passed through the incubation mixture at a rate sufficient to attain a continuous agitation of the slices. Initial incubations were carried out in accordance with these recommendations, but during preliminary experiments two problems were encountered. Firstly, when oxygen was passed through L-dopa-containing solutions the rate at which the material underwent oxidative polymerization was greatly accelerated. Subsequent experiments showed, however, that this did not cause the concentration of L-dopa to fall significantly. For example, after a 165min incubation (at 35°C with continuous oxygenation) the final L-dopa concentration was greater than 95% of the starting concentration, indicating that in this respect oxidation did not represent a serious problem. Oxidation products of L-dopa could, however, themselves affect protein synthesis. In this connection it is interesting to note that SOURKES (1971) has suggested that some of the pharmacological effects of L-dopa may be mediated via hydroxylated derivatives of noraporphine formed by the phenolic oxidation of the condensation product of dopamine and its metabolite 3,4-dihydroxyphenylacetaldehyde. One isomer of tetrahydroxynoraporphine is structurally very similar to the dopaminergic agonist apomorphine.

The second problem encountered was that of protein denaturation caused by the rapid agitation of incubation mixtures. To overcome this, it was decided to abandon the idea of passing oxygen through the medium, although this presumably meant accepting a submaximal rate of protein synthesis. Two alternative procedures were considered. Either incubations could be carried out using pre-oxygenated buffer without further oxygenation, or oxygenation could be continued by passing the gas over the surface of the medium. One experiment was carried out to establish whether incubations could be carried out in the absence of a continuous supply of oxygen without causing a further impairment of protein synthesis. The results showed that although the rate of [^{3}H]lysine incorporation into protein may have been slightly increased by continuous oxygenation, the effect was not marked (Table 3.3.1). Consequently, subsequent dopa experiments were carried out in the absence of a continuous oxygen supply since under these conditions the rate at which L-dopa underwent

TABLE 3.3.1 THE EFFECT OF CONTINUOUS OXYGENATION ON THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-PRECIPITABLE MATERIAL IN CHOPPED RAT BRAIN

Incorporation time (min)	Cycloheximide	Air			Oxygen		
		TCA-insoluble radioactivity (R_2) (d.p.m./ug prot.)	TCA-soluble radioactivity (R_1) (d.p.m./ug prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)	TCA-insoluble radioactivity (R_2) (d.p.m./ug prot.)	TCA-soluble radioactivity (R_1) (d.p.m./ug prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
30	+	0.96 (1.15,0.76)	95.9 (99.2,92.5)	0.010 (0.012,0.008)	0.91 (0.70,1.11)	89.6 (70.0,109.2)	0.010 (0.010,0.010)
	-	5.00 (5.70,4.29)	92.0 (94.0,90.0)	0.055 (0.061,0.048)	7.92 (6.57,9.26)	103.8 (97.8,109.7)	0.076 (0.067,0.084)
60	+	1.17 (1.19,1.15)	87.6 (88.5,86.7)	0.013 (0.013,0.013)	1.18 (1.22,1.14)	88.4 (84.1,92.6)	0.013 (0.014,0.012)
	-	8.46 (9.05,7.87)	73.2 (84.3,62.1)	0.117 (0.107,0.127)	9.36 (9.09,9.63)	87.9 (89.1,86.7)	0.107 (0.102,0.111)

Rat brain prisms were prepared as described in Section 2.2.2. Tissue (equivalent to about 2.5mg protein) was incubated in a pre-oxygenated medium (2ml) containing NaCl (108mM), KCl (4.4mM), $MgSO_4$ (1.3mM), $CaCl_2$ (2.6mM), glucose (12mM), HEPES (25mM), K_3PO_4 (1.2mM), NaOH (added to bring the final pH to 7.4, final conc about 12mM) and L-[4,5-^{3}H]lysine (2.5uCi). Cycloheximide (final conc 1mM) was added where indicated. One set of incubations was carried out in stoppered, air filled conical flasks, while continual oxygenation of the other set of incubation mixtures was performed by passing the gas over the surface of the medium. Both sets of incubation mixtures were agitated on a shaking water bath at 35^{o}C. Incorporation of radioactivity was stopped after 30min or 60min by freezing the tissue in liquid nitrogen. The tissue was then processed as described in Section 2.3.1. The results are expressed as the average of duplicates, the individual results being given in parenthesis.

oxidation was reduced. It is important to stress that the rate of replacement of protein-bound amino acid under these conditions is expected to be somewhat less than 0.08-0.09% per hour, the rate observed in brain slices of optimum thickness incubated under oxygen (DUNLOP et al., 1975b).

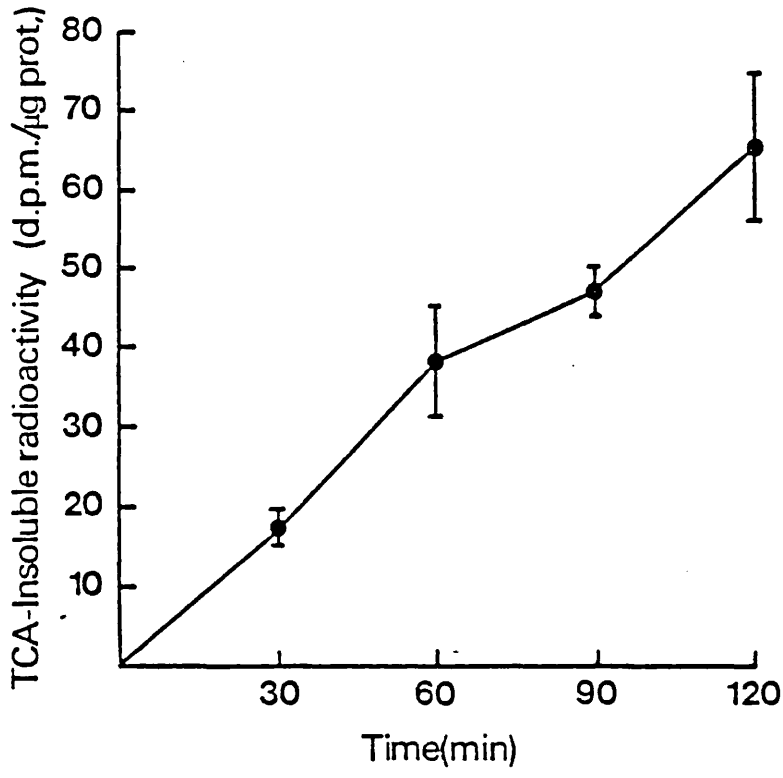
The incorporation of L-[4,5-^{3}H]lysine into TCA-insoluble and TCA-soluble fractions of chopped rat brain

One experiment was performed in which chopped rat brain was prepared and incubated as described in Section 2.2.2 and TCA-insoluble radioactivity (R_2) measured as a function of time. The incorporation of radioactivity into TCA-precipitable material continued at a near linear rate for 2h (Fig.3.3.1). TCA-soluble radioactivity (R_1) was also determined and lysine relative incorporation (I_{rel}) data calculated together with the coefficients of variation of R_2, R_1 and I_{rel} (Table 3.3.2). It can be seen that the coefficient of variation of I_{rel} was not, in general, less than that of R_2 and R_1, which indicated that variability in [^{3}H]lysine uptake did not make a major contribution to the variability in its rate of incorporation into TCA-precipitable material. This is in contrast to the observation made in vivo (see Section 3.1.2). The results of the following experiments are therefore expressed only in terms of TCA-precipitable radioactivity.

3.3.2 THE EFFECT OF L-DOPA ON THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-PRECIPITABLE MATERIAL IN CHOPPED RAT BRAIN

The incorporation of L-[4,5-^{3}H]lysine into TCA-precipitable material was measured in chopped rat brain tissue preincubated for 30min in the presence of various concentrations of L-dopa. The drug had no significant effect in the concentration range 20-640ug/ml (Tables 3.3.3 and 3.3.4). In two experiments L-[^{3}H]lysine incorporation was measured in cortical slices taken from L-dopa-pretreated and control rats. The animals were maintained at 26°C for about one hour before the start of each experiment (see Sections 3.2.2.8 and 3.2.2.9) and were then injected with L-dopa (500 mg/kg body weight) or with carrier medium. Forty-one minutes after drug administration the animals were killed and cortical slices cut and incubated for 20min in the presence of L-[4,5-^{3}H]lysine. The two experiments were carried out one immediately after the other and the data combined and treated as a single set of results. The L-dopa

FIG. 3.3.1 THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-INSOLUBLE MATERIAL IN CHOPPED RAT BRAIN



Rat brain prisms were prepared as described in Section 2.2.2 and incubated as described in Table 3.3.1 (except that each 2ml incubation mixture contained 5uCi [^{3}H] lysine). Each circle and bar represents the mean of the results obtained from three incubations and the standard error, respectively.

TABLE 3.3.2 THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-PRECIPITABLE AND TCA-SOLUBLE FRACTIONS OF CHOPPED RAT BRAIN

Incorporation time (min)	TCA-insoluble radioactivity (R_2) (d.p.m./ug prot.)	TCA-soluble radioactivity (R_1) (d.p.m./ug prot.)	Relative incorporation ($I_{rel} = R_2/R_1$)
30	17.4 ± 4.0 $C(R_2) = 0.230$	300 ± 44 $C(R_1) = 0.147$	0.0577 ± 0.0085 $C(I_{rel}) = 0.147$
60	38 ± 12 $C(R_2) = 0.316$	250 ± 21 $C(R_1) = 0.084$	0.156 ± 0.059 $C(I_{rel}) = 0.378$
90	47.2 ± 5.4 $C(R_2) = 114$	220 ± 53 $C(R_1) = 0.241$	0.220 ± 0.043 $C(I_{rel}) = 0.195$
120	66 ± 17 $C(R_2) = 0.258$	300 ± 27 $C(R_1) = 0.090$	0.217 ± 0.035 $C(I_{rel}) = 0.161$

This data was obtained from the same experiment as that given in Fig. 3.3.1. The data is expressed as the mean of the results obtained from three incubations $\pm$ s.d. Also given is the coefficient of variation (C(x)) which is defined by $C(x) = s.d.(x)/x$.

TABLE 3.3.3 THE EFFECT OF L-DOPA ON THE INCORPORATION OF L-[4,5-^{3}H] LYSINE INTO TCA-PRECIPITABLE MATERIAL IN CHOPPED RAT BRAIN (EXPERIMENT 1)

Final conc of L-dopa (ug/ml)	TCA-insoluble radioactivity (d.p.m./ug prot.)
0 + CH	2.9 ± 0.2
0	29.7 ± 4.2
20	36.9 ± 4.5
40	31.3 ± 3.5
80	38.2 ± 3.7
160	36.6 ± 2.5
320	28.1 ± 1.3

Rat brain prisms (equivalent to about 2.5mg protein) were incubated for 30min in buffer (1ml) containing various concentrations of L-dopa or cycloheximide (CH) (1mM). L-[4,5-^{3}H]lysine (5uCi) was then added in buffer (1ml) and incubation continued for a further 30min (NB. the dopa concentration is given as the final concentration which is 50% of the preincubation concentration). Further experimental details are given in Table 3.3.1 and Section 2.2.2. The data is given as the mean of the results obtained from three incubations ± S.E.M. The difference between the level of incorporation at different concentrations of L-dopa was not significant. (The result of an analysis of variance is given in Table A17.)

TABLE 3.3.4 THE EFFECT OF L-DOPA ON THE INCORPORATION OF L-[4,5-^{3}H] LYSINE INTO TCA-PRECIPITABLE MATERIAL IN CHOPPED RAT BRAIN (EXPERIMENT 2)

Final conc of L-dopa (ug/ml)	TCA-insoluble radioactivity (d.p.m./ug prot.)
0 + CH	0.8 ± 0.2
0	23.6 ± 1.8
40	21.9 ± 3.1
80	20.5(18.5,22.4)
160	23.2 ± 1.8
320	27.2(23.4,31.0)
640	18.5 ± 0.7

See Table 3.3.3 for details. The data is expressed as the mean of the results obtained from three incubations ± S.E.M., except where n=2, in which case the two individual results are given in parenthesis. The difference between the level of incorporation at 640 ug/ml L-dopa and the control level is not significant (student's t test).

TABLE 3.3.5 THE INCORPORATION OF L-[4,5-^{3}H]LYSINE INTO TCA-PRECIPITABLE MATERIAL IN RAT BRAIN CORTICAL SLICES TAKEN FROM L-DOPA-TREATED AND CONTROL ANIMALS

	n	TCA-insoluble radioactivity (d.p.m./ug prot.)	L-Dopa-induced change (%)
Control group	4	11.3 ± 2.5	
L-dopa-treated group	4	10.3 ± 2.3	-8.8 NS

The above data were calculated from the results of two separate experiments which were carried out one immediately after the other. The animals were maintained at 26°C for one hour before the start of each experiment and were then injected with L-dopa (500mg/kg in 0.05M HCl, 50mg/kg) or with 0.05M HCl (10ml/kg) and returned to the 26°C environment. (The injection schedule is given in Table 2.2.) After 41min the rats were stunned, killed by cervical fracture and one cortical slice cut from each cerebral hemisphere. Each slice was incubated separately in the presence of L-[4,5-^{3}H]lysine (5uCi/2ml incubation, see Table 3.3.1 for details). Incorporation was terminated after 20min and the tissue processed as described in Section 2.3.1. The animals were 35 days old. Body weights: Control group, 92±3(4)g; Dopa-group, 98±5(4)g.

pretreatment had no significant effect on the incorporation of L-[4,5-^{3}H] lysine into TCA-insoluble material (Table 3.3.5). This was in contrast to the gross inhibition of lysine incorporation previously observed *in vivo* (see Section 3.2). In order to eliminate the possibility that the difference between the *in vitro* and *in vivo* results arose artifactually through, for example, a seasonal variability in the sensitivity of rats to L-dopa, a repeat *in vivo* experiment was carried out within a few days of the *in vitro* experiment. A significant L-dopa-induced inhibition of L-[^{3}H]lysine incorporation was observed (Table 3.2.19).

On the basis of these results it was decided to discontinue the planned programme of *in vitro* experiments. Although a meaningful interpretation of the above results is not possible without further experimentation, they are consistent with the hypothesis that neither L-dopa nor its *in vitro* metabolites has a direct effect on the protein synthesizing machinery of the general population of brain cells.

4 A COMPUTER SIMULATION STUDY OF LYSINE COMPARTMENTATION IN THE BRAIN

A computer simulation study of lysine compartmentation in the brain was carried out as an aid to the interpretation of the data obtained from tracer experiments. An account of the mathematical methods applicable to the compartmental analysis of kinetic data is not included in this thesis, but it is appropriate to mention that two distinct approaches may be adopted (see, for example, BERMAN et al., 1962a). It is shown in Appendix B that an n-compartment system is defined by n linear differential equations, the general solution of which are linear combinations of n exponential functions. Consequently, one of the analytical procedures that may be adopted involves fitting the experimental data to sums of exponentials. Once a fit of the experimental data has been achieved, the rate constants for the exchange processes may be evaluated as outlined in Appendix B (see Section B.3).
An alternative approach to the mathematical modelling of compartmental systems involves an initial compilation of feasible models of compartmentation together with the rate equations defining these models. These equations are solved and the solutions used to generate theoretical data. The models are then assessed on the basis of the compatibility of the theoretical and experimental results.

The first of the two procedures outlined above was not adopted in the present study of lysine compartmentation partly because, in many instances, insufficient data were available to permit such an approach. Instead, models of lysine compartmentation were compiled and tested for their capacity to account for the experimental observations. As a starting point models were constructed in which each compartment corresponded to a well-defined morphological or biochemical structure. If a model failed to yield theoretical data that were compatible with the experimental results, the model was either discarded or was modified, usually by the introduction of a fine compartmental structure. Where possible rate constants were calculated using literature data. The remainder were determined empirically. Rather than derive a set of rate equations for each model to be tested, it was more convenient to use a set of equations which describes a general multi-compartmental

system to which any particular model may be fitted (Fig. 4.1). This approach has been adopted by a number of researchers (see for example COBELLI et al., 1977; BERMAN et al., 1962a) and routines are available for fitting mathematical models to kinetic data. BERMAN et al. (1962b) for example, have made available a program which, given a set of experimental data, the equations describing the model, the initial conditions and initial estimates of the variable parameters, uses a least-squares method to obtain by iterative adjustment of the variable parameters a best fit of the experimental results. A lack of suitable input data precluded the use of this type of routine in many of the applications described below. The experiments examined in this chapter were not designed with computer simulation in mind, and consequently in most cases the results were not of sufficient detail or accuracy to warrent or permit a sophisticated analysis. Instead, a preliminary study was carried out using the available data and the simple computer program (Program COMP) given in Appendix B. While it is recognised that in some instances the treatment adopted in this study is extremely unrefined, simple simulation was nevertheless found to be a useful aid to the interpretation of experimental data and for that reason the results are included in this thesis.

In this section extensive use is made of literature data. In particular, some of the work of Lajtha and co-workers has been re-examined and in places a new interpretation of their results is suggested. It must be emphasised that this re-examination of their data is not intended to be in any way detrimental to the original work. On the contrary, it must be acknowledged that only because of the painstaking nature of the original experiments is a detailed examination of the results feasible and that it is not difficult to find fault with early interpretations in the light of knowledge that has been gained in the meantime.

4.1 SIMULATION OF IN VIVO EXPERIMENTS

The examination of the effect of L-dopa on the incorporation of radioactive amino acids into cerebral protein yielded the following results (see Section 3.2). The drug caused a 73-75% inhibition of [^{3}H]lysine

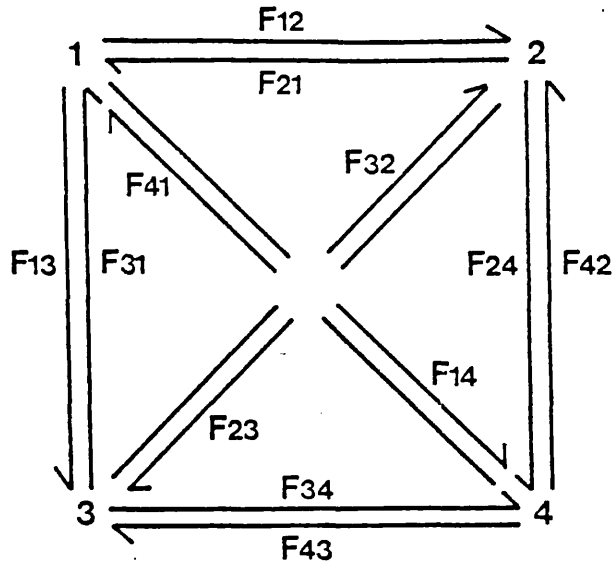


FIG. 4.1 A general n-compartment system consists of n mutually exchanging compartments as shown above for a four-component system. Any particular model may be fitted by setting the appropriate flow rates equal to zero. The system of rate equations defining the general multi-compartment model is given in Appendix B together with a method by which a solution of the system may be obtained.

incorporation into TCA-precipitable material during a 15min incorporation period (Tables 3.2.5 and 3.2.6) and a 32-54% inhibition during a 7½min incorporation period (Tables 3.2.11 and 3.2.12). The incorporation of [^{14}C]valine was inhibited by 30% in one flooding experiment (although the effect was not statistically significant) but was not affected in another (Table 3.2.17). The effect of L-dopa on the incorporation of [^{3}H]valine into TCA-insoluble material was variable, but the drug had no significant effect on the apparent incorporation of this amino acid in brain (Tables 3.2.7 and 3.2.8). A computer simulation study was carried out to establish whether these experimental observations could be accounted for by a single model. All of the models tested assumed that L-dopa had no effect on the rate of cerebral protein synthesis.

It is important to re-emphasise that the simulation procedure outlined below is unrefined. In spite of this, these studies were found to be useful in two respects. Firstly, the very simple procedure adopted here was adequate for the purpose of eliminating models that could not account for the experimental observations. Secondly, it was possible to assess the reliability of the procedures used in the measurement of various parameters (see, for example, Tables 4.2 and 4.3).

4.1.1 A BASIC MODEL FOR THE SIMULATION OF IN VIVO TRACER EXPERIMENTS

Computer simulation of *in vivo* tracer experiments was performed by first setting up a simple model for generating a set of specific activity-time curves for the plasma, brain soluble and brain protein-bound lysine pools. This basic model was then expanded by the introduction of additional compartments so as to incorporate various models of brain lysine compartmentation. Finally, the expanded models were tested for their capacity to generate theoretical data that were in agreement with that obtained experimentally.

The basic in vivo model is shown in Fig. 4.2. Pool sizes and flow rates F_{45} and F_{54} were *calculated using the data given in Table 4.1. In order to obtain physiologically meaningful estimates of the unknown parameters they were adjusted empirically until a fit of the plasma and brain soluble lysine specific activity data given in Table 3 of LAJTHA et al. (1957) was achieved. These specific activity data, which were obtained as part of a study of amino acid and protein metabolism in the mouse, were used because no similar data could be found for the rat.** An optimization routine was not employed. Instead, the flow rates given in Fig. 4.2 were selected on the basis of a visual comparison of the theoretical and experimental data. The simulated plasma and brain soluble lysine specific activity curves are shown in Fig. 4.3 together with the experimental data.

4.1.2 LIMITATIONS OF THE IN VIVO MODEL

1) As stated above a direct simulation of lysine compartmentation in the rat was precluded because insufficient data were available for obtaining reliable estimates of the rates of lysine exchange between the compartments. The acquisition of these data would have been time consuming and was not attempted. Instead the figures given in the scheme outlined in Fig. 4.2 were derived from measurements made in the mouse. In order to account for the effects of L-dopa on the incorporation of radioactive lysine into rat brain protein, this model is expanded in the following pages by the introduction of a "fine compartmental structure". Clearly, an extrapolation to the rat of a model based on measurements made in the mouse is a questionable procedure.

* The computer program requires that all input data are expressed relative to some common factor. Simulation was therefore performed using input data calculated on the basis of a body weight of 100g. All the data given in Section 4.1 are expressed in this manner unless otherwise indicated.

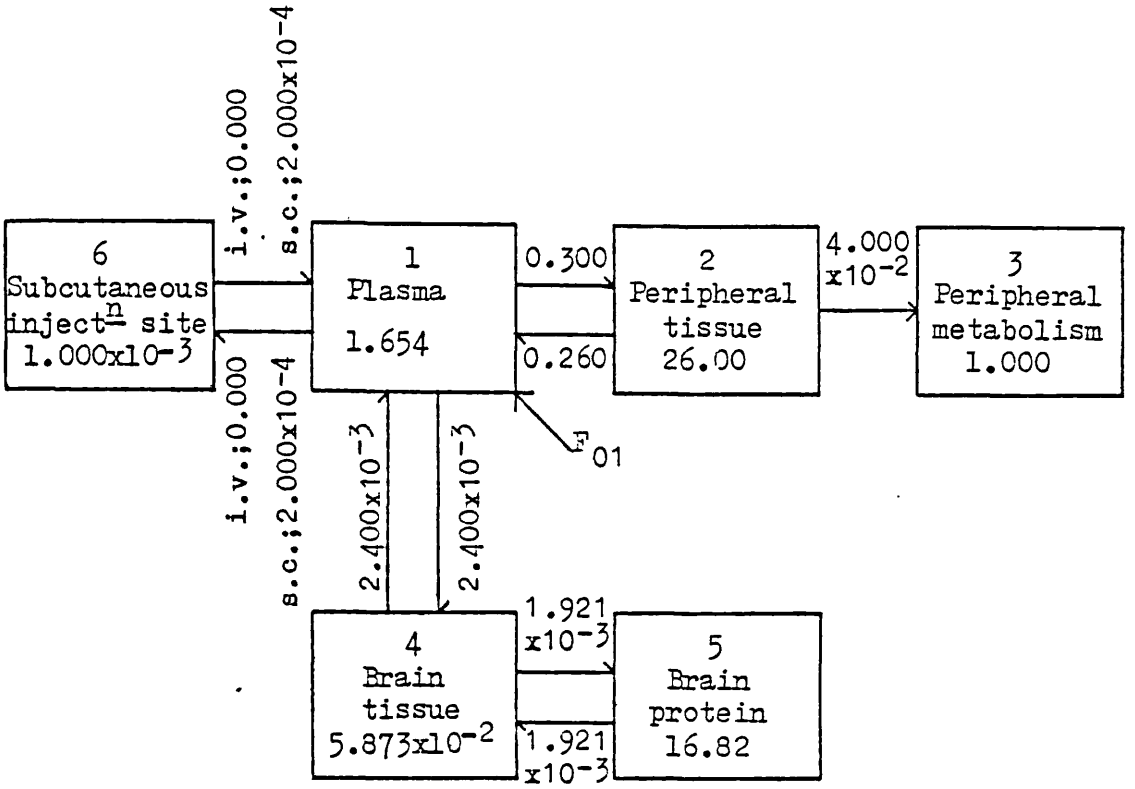
** After this work had been completed rat tissue lysine specific activity-time curves were published by FORD et al. (1977). No data were given, however, for the time interval 0-15min. In order to obtain reliable estimates of the unknown flow rates, specific activity data are required for the time interval during which the radioactive content of the compartments changes rapidly with time, i.e., the first few minutes after the administration of the radioactive amino acid.

TABLE 4.1 DATA USED IN THE SIMULATION OF *IN VIVO* ISOTOPIC LYSINE TRACER EXPERIMENTS IN THE MOUSE

Parameter	Data	Reference
Brain weight	0.29g/100g body wt.	LONG (1961) p. 639
Plasma volume	48.8ml/kg body wt.	ALTMAN & DITTMER (1964) p.264
Lysine content of plasma	63ug/g	LAJTHA *et al.* (1957) Table 3.
	0.42 umole/g (used a figure of 0.339 umole/ml)	TOTH & LAJTHA (1977) Table 1.
Soluble lysine content of brain tissue	0.193umole/g tissue	LEVI *et al.* (1967) Table 1.
	37ug/g tissue (used intermediate figure of 0.2025 umole/g)	LAJTHA *et al.* (1957) Table 3.
Lysine content of brain protein	58umole/g tissue	NEIDLE *et al.* (1975) Table III.
Rate of lysine incorporation into brain protein	0.685%/h	DUNLOP *et al.* (1975a) Table 4.

A direct simulation of *in vivo* isotopic lysine tracer experiments in the rat was precluded since insufficient data were available for optimizing the unknown flow rates. The necessary information was, however, available for the mouse (Table 3 of LAJTHA *et al.*, 1957) and this was therefore used in the present study. For the sake of self-consistency all other parameters were based on measurements made in the mouse. The computer program requires that all parameters are expressed relative to some common factor. All data given in Section 4.1 are therefore expressed in terms of a body weight of 100g, unless otherwise specified.

FIG. 4.2 A SCHEME* FOR THE SIMULATION OF IN VIVO ISOTOPIC LYSINE TRACER EXPERIMENTS IN THE MOUSE



Simulation was carried out using flow rates and pool sizes calculated (per 100g body weight) from the data given in Table 4.1. F_{12}, F_{21}, F_{23}, F_{14}, F_{41} ($F_{16} = F_{61} = 0$) and Q_2 were adjusted to obtain an empirical fit of the experimental specific activity data given in Table 3 of LAJTHA et al. (1957). (NB. F_{ij} is the lysine flow rate from the ith to the jth compartment and Q_i is the size of the ith compartment.) The steady-state approximation fixes $F_{21} = F_{12} - F_{23}$ and $F_{01} = F_{12} - F_{21}$. Compartments 2,3 and 6 are included in the model merely as a means of simulating the experimentally determined plasma lysine specific activity-time curve. Providing this is achieved it is of no consequence if the plasma lysine pool size and the numerical values of the empirically determined parameters are unphysiological. Similarly, because the brain soluble lysine specific activity-time curve was fitted empirically, the precise figure used for the size of the brain soluble lysine compartment is also relatively unimportant. Intravenous injection was simulated by setting the zero-time radioactivity vector $\underline{R}(0) = (1,0,0,0,0,0)$ (see equation B.14). To simulate subcutaneous administration $\underline{R}(0)$ was set equal to $(0,0,0,0,0,1)$ and $F_{16} = F_{61}$ was adjusted empirically to give a plasma specific activity maximum at about 5 min. (ENTINGH and DAMSTRA (1976) found that the level of radioactivity in the plasma reached a maximum within 5 min of the s.c. injection of [^{3}H] lysine.)

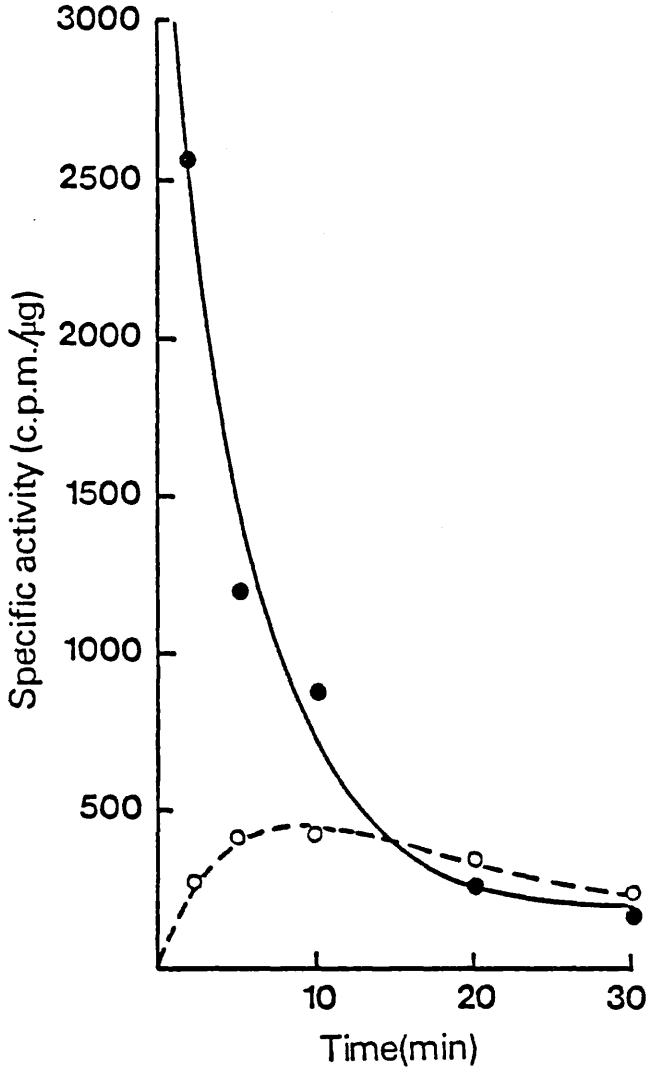
*In the main text this scheme is referred to as the homogeneous precursor lysine model.

Fig. 4.2 Continued.....

An optimization routine was not used to obtain values for the unknown parameters. Instead, flow rates yielding an acceptable agreement between the theoretical and experimental data were selected by visual inspection. These are given (expressed in terms of umole/min/100g body weight**) together with the remaining flow rates and pool sizes. (Pool sizes are expressed as umole/100g body weight.) The simulated lysine specific activity-time curves are shown in Fig. 4.3 together with the experimental data.

**The programs given in Appendix B read the input data as arrays of four digit numbers. For this reason where flow rates and pool sizes were calculated from literature data, the calculation was performed to four figures, recognising that all four digits cannot be significant since this exceeds the accuracy of the original data. In the above scheme (and subsequent figures and tables) all parameters are given as the four figure number supplied to the computer since no attempt was made to determine the variances of the parameters.

FIG. 4.3 THEORETICAL AND EXPERIMENTAL SPECIFIC ACTIVITIES OF THE PLASMA AND BRAIN SOLUBLE LYSINE POOLS



● , Specific activity of plasma lysine (from Table 3 of LAJTHA et al., 1957); o , Specific activity of the brain soluble lysine pool (ibid.); ——, simulated specific activity of plasma lysine; – – – –, simulated specific activity of the brain soluble lysine pool. With the zero-time radioactivity vector $\underline{R}(t=o)$ equal to (1,0,0,0,0,0) (see equation B.14) the sum of the components of the radioactivity vector is unity for all time. In order to compare the theoretical and experimental data, this vector must be multiplied by a constant. This scaler multiplyer was set at a value which, on the basis of a visual inspection of the theoretical and experimental data, yielded an acceptable fit of the experimental plasma lysine specific activity data. This multiplyer then fixes the specific activity curves of all the other compartments. (The simulated specific activity data (in radioactive units/umole) were multiplied by 6101.6 to obtain the above specific activity data (in c.p.m./ug).)

2) Owing to the simplicity of the model in which both peripheral and cerebral soluble lysine are represented as single compartments it was not possible to obtain an agreement between the experimental and theoretical data over a 60min incorporation period. In particular, the cerebrospinal fluid and extracellular space are not represented and the model does not take into account the structural heterogeneity of the brain. It is acknowledged, however, that a better correlation between the experimental and theoretical data might be achieved through the use of an optimization routine. In spite of these limitations the agreement between the theoretical and experimental data over the first 30min was adequate for the present purpose.

4.1.3 MODELS OF LYSINE COMPARTMENTATION IN THE BRAIN

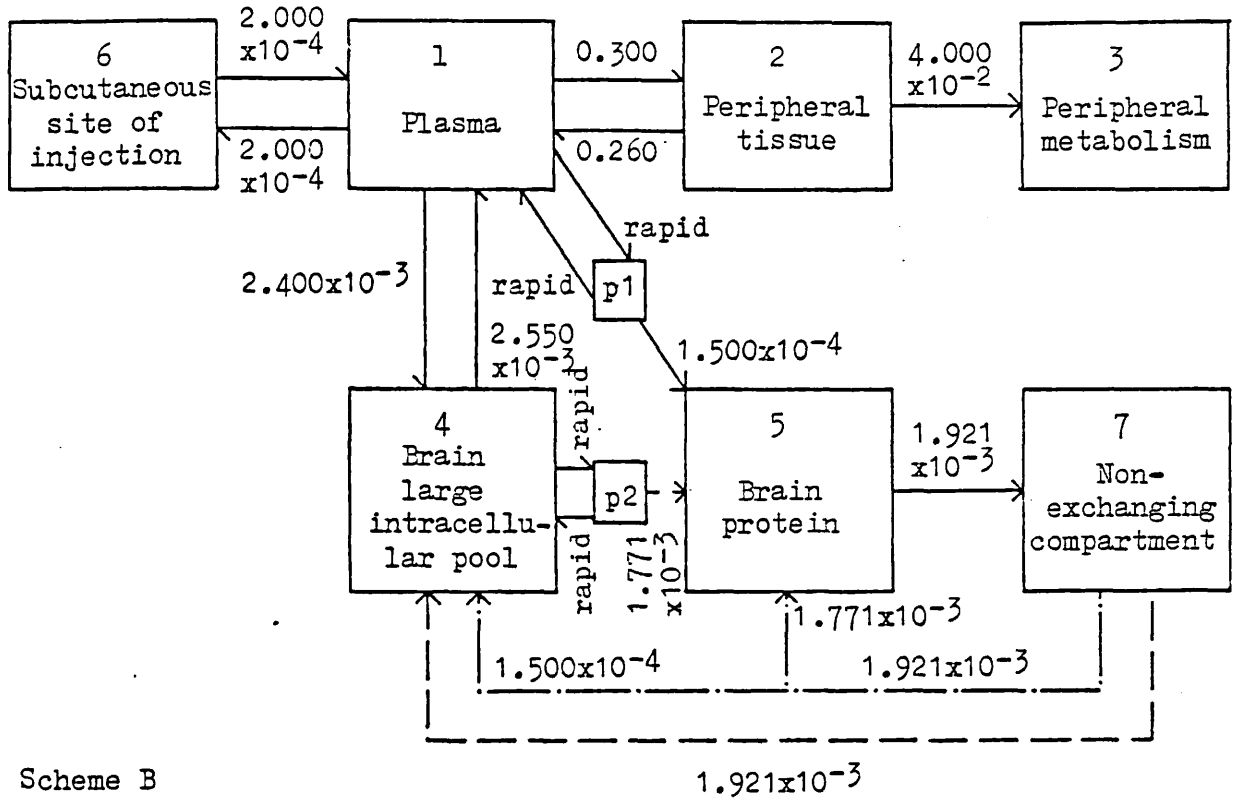
The basic scheme outlined in Fig. 4.2 was expanded in order to accommodate various models of lysine compartmentation. Theoretical TCA-insoluble radioactivity curves were plotted for each model and these compared with the experimental incorporation data (Tables 3.2.5, 3.2.6, 3.2.11 and 3.2.12). Two models yielded results that were consistent with the experimental observations, both models assuming that the precursor lysine pool for brain protein synthesis is heterogeneous.

MODEL 1. Subcellular model of brain precursor lysine compartmentation (Fig. 4.4)

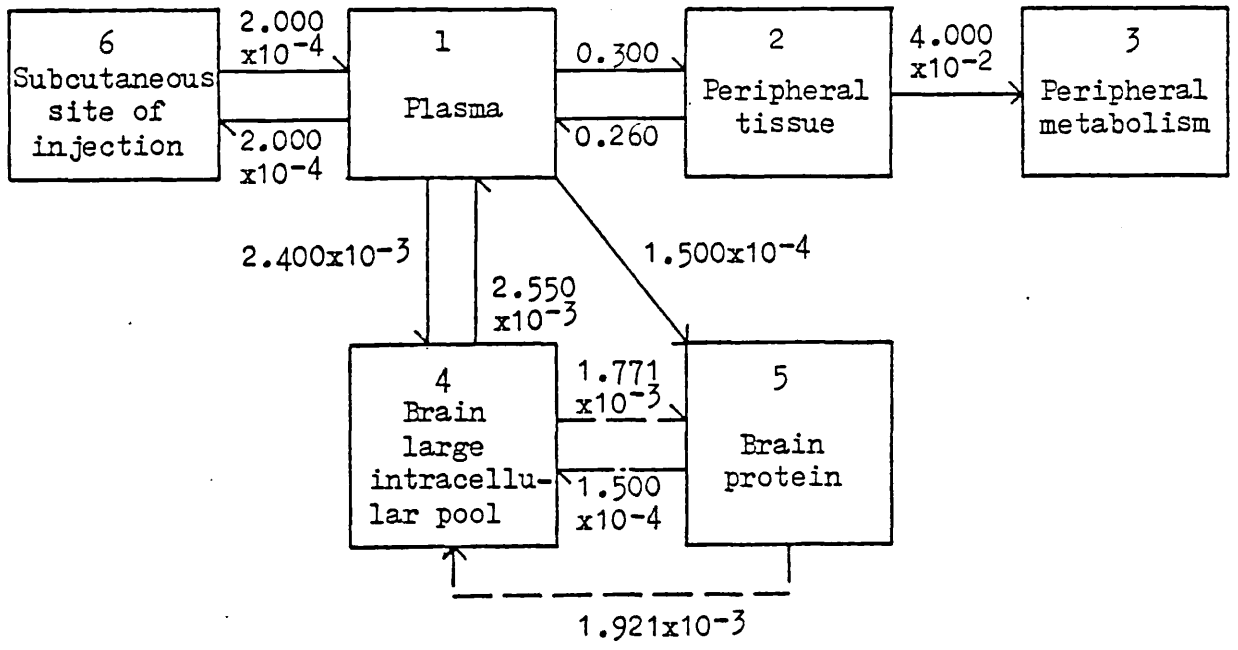
This model assumes that there are normally two brain precursor lysine pools, one of which equilibrates rapidly with the main intracellular lysine compartment, the other undergoing rapid exchange with plasma lysine. Protein-bound lysine is derived from the two precursor pools at the rates shown in Fig. 4.4, the combined rates being equal to the rate of protein synthesis given in Table 4 of DUNLOP *et al.* (1975a). It is also assumed that in the absence of L-dopa there is no direct recycling of lysine released from degraded protein. Instead, newly released lysine flows through a nonexchanging compartment and into the main intracellular pool. In order to account for the observation that L-dopa had little effect on valine incorporation, but caused a gross inhibition of lysine incorporation after a 15min labelling period, it is assumed that protein synthesis is unaffected by L-dopa, but that the flow of radioactive lysine from the main intracellular compartment

FIG. 4.4 MODEL 1. SUBCELLULAR MODEL OF BRAIN PRECURSOR LYSINE COMPARTMENTATION

Scheme A



Scheme B



flow of lysine in control animals

flow of lysine in L-dopa-treated animals

flow common to control and L-dopa-treated animals

Fig. 4.4 Continued.....

The subcellular model of brain precursor lysine compartmentation (Scheme A) assumes that in the absence of L-dopa lysine enters brain protein via two precursor pools, one of which (p1) undergoes rapid exchange with plasma lysine, the other (p2) equilibrating rapidly with the large intracellular pool. Lysine released from degraded protein is fed into the large intracellular soluble pool via a nonexchanging compartment (7). L-Dopa is assumed to inhibit the flow of lysine from the large intracellular pool into protein. In order that protein synthesis may continue in the presence of L-dopa, lysine released into the non-exchanging compartment is recycled directly back into protein.

The proportion of lysine entering protein via each route was determined empirically keeping the total flow rate at 1.921×10^{-3} umole/min. (All data are expressed relative to a body weight of 100g.) Pool sizes are as in Fig. 4.2.

For the purpose of performing the computer simulation, the simplified scheme outlined in B was used. This scheme was derived from Scheme A by making the following approximations. It is assumed that p1 equilibrates so rapidly with plasma lysine that this precursor compartment may be neglected. Thus lysine entering protein via this route is considered to be derived directly from the plasma pool. Similarly, it is assumed that the rate at which p2 equilibrates with the large intracellular lysine pool is sufficiently rapid that p2 can be neglected. For incorporation periods of up to several hours the specific activity of protein-bound lysine remains low compared with that of the plasma and brain soluble pools. For example, by 3h the specific activity of the protein pool has increased to only 2.1% that of the plasma and 3.6% that of the large intracellular compartment. Consequently, the specific activity of compartment 7 remains low irrespective of its size and may also be neglected during this time period. See Fig. 4.2 for further details.

into protein is inhibited and that the deficit is made up from the nonexchanging pool. Theoretical incorporation data generated using this model is shown in Fig. 4.6.

MODEL 2. Cellular model of brain precursor lysine compartmentation (Fig. 4.5).

This model is similar to Model 1 and yields essentially the same specific activity-time curves for short incorporation times (see below). It differs from Model 1 in that the two different precursor compartments are associated with different protein compartments. These compartments could be associated with different cell types.

Assessment of the models

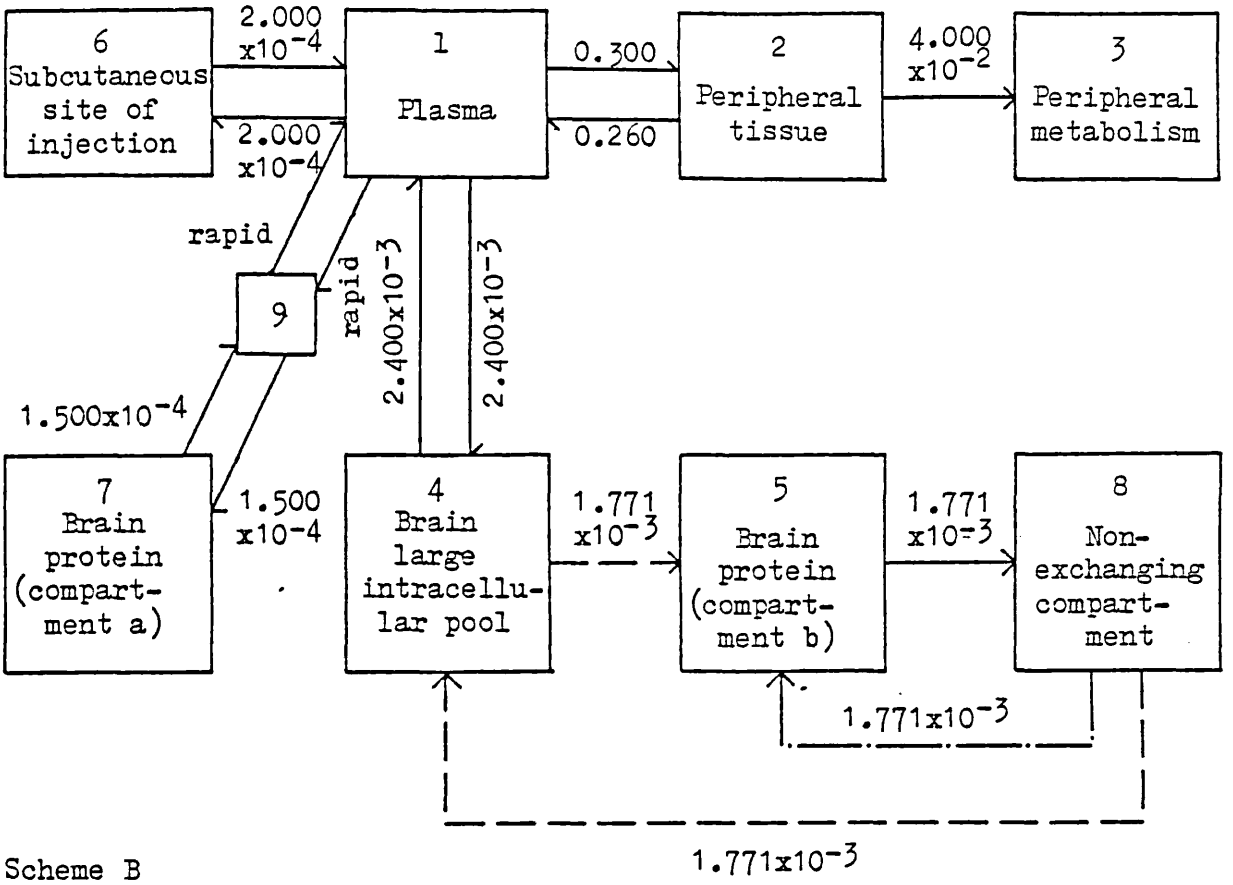
In order to assess the plausibility of the above models a number of their salient features are examined below. In particular a comparison is made of some of the empirically determined parameters with literature data.

1) On the question of lysine reutilization

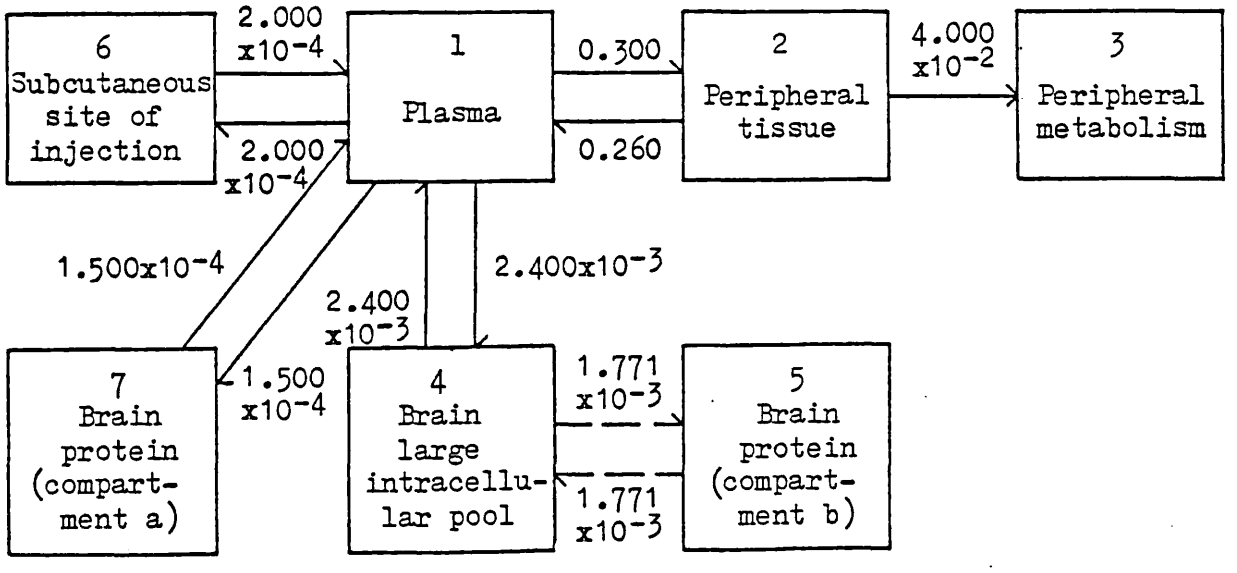
Both of the models outlined above assume that in the absence of L-dopa, no mechanism exists for the direct reutilization of lysine released from degraded protein. In a study of hepatic protein metabolism, SWICK (1958) obtained results contrary to this assumption. He found that the liver protein turnover rate calculated using lysine incorporation data was slow compared with the rate calculated on the basis of the incorporation of other amino acids. He suggested that the discrepancy would be accounted for if, in this tissue, about 50% of lysine released from degraded protein is recycled directly back into the protein-bound lysine pool. However, inspection of the data given in Tables 3 and 4 of DUNLOP et al. (1975a) indicates that rates of brain protein synthesis calculated using lysine incorporation data obtained from trace experiments agree reasonably well with rates calculated on the basis of results obtained with other amino acids in both trace and flooding experiments. (The rates derived from the

FIG. 4.5 MODEL 2. CELLULAR MODEL OF BRAIN PRECURSOR LYSINE COMPARTMENTATION

Scheme A



Scheme B



— — — — — → flow of lysine in control animals

—·——·——·——·→ flow of lysine in L-dopa-treated animals

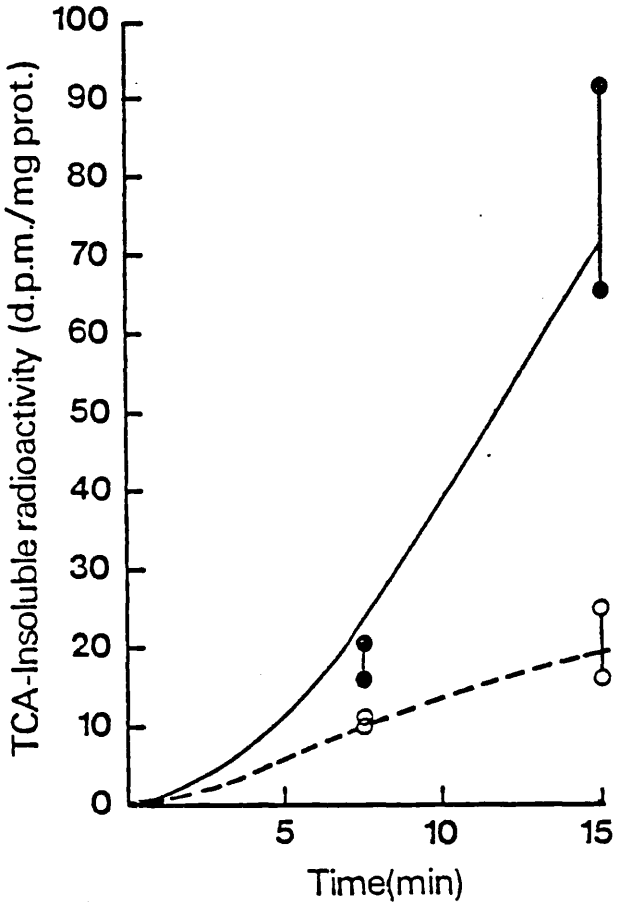
——————→ flow common to both control and L-dopa-treated animals

Fig. 4.5 Continued.....

The cellular model of compartmentation assumes that there are two distinct protein compartments in brain, each supplied by a separate precursor lysine pool. One protein compartment (7) is assumed to derive its lysine from a compartment (9) that equilibrates rapidly with the plasma pool. In the absence of L-dopa, the other protein compartment (5) derives its lysine from the large intracellular compartment (4). Lysine released from compartment 5 through proteolysis enters the large intracellular pool via a nonexchanging compartment (8). It is assumed that in L-dopa-treated rats the flow of lysine from the large intracellular compartment into protein is inhibited. In order that protein synthesis may continue in the presence of L-dopa, lysine released by proteolysis is recycled via the nonexchanging compartment directly back into protein.

For the purpose of performing the computer simulation the simplified scheme outlined in B was used. This scheme was derived from Scheme A by making the following approximations. It is assumed that precursor compartment 9 equilibrates with plasma lysine with sufficient rapidity that this precursor compartment may be neglected. It is also assumed that the two protein compartments are of equal size. However, the relative sizes of the two protein compartments can be varied over a considerable range without significant effect, providing the incorporation period is sufficiently short that the specific activity of protein-bound lysine remains low relative to that of the two precursor compartments. For example, if the duration of the incorporation period is 15 min, then even in a situation in which compartment 5 represents only 5% of total protein the specific activity of this compartment is still less than 2% that of compartment 4. If, on the other hand, compartment 7 represents 5% of total protein, the specific activity of compartment 7 at 15 min is less than 1% that of compartment 1. Providing the specific activity of compartment 5 is low the specific activity of compartment 8 will also be low, irrespective of its size, and may therefore be neglected. Pool sizes are as in Fig. 4.2 and flow rates are expressed in terms of umole/min/100g body weight. See Fig. 4.2 for further details.

FIG. 4.6 THE INCORPORATION OF RADIOACTIVE LYSINE INTO TCA-INSOLUBLE MATERIAL. A COMPARISON OF EXPERIMENTAL AND THEORETICAL DATA--



● , Brain TCA-insoluble radioactivity in control rats; o, Brain TCA-insoluble radioactivity in L-dopa-treated rats (from Tables 3.2.5, 3.2.6, 3.2.11 and 3.2.12. NB. The amount of radioactive lysine administered per kg body weight in the 7½min experiments was twice that used in the 15min experiments. The 7½min experimental data have therefore been halved.). Simulated incorporation curves generated using the models of lysine compartmentation* outlined in Figs 4.4 and 4.5 are also shown.——, simulated control; – – – –, simulated L-dopa-induced inhibition. *The two models yield theoretical incorporation data that differ by less than 0.8% during a 15min incorporation period.

lysine incorporation data are 85% or more of the average of the rates determined using valine, tyrosine, lysine and histidine.) Assuming that any nonexchanging lysine compartments represent only a small fraction of total intracellular lysine (this assumption is examined in Section 4.2) this is consistent with there being a low percentage of direct lysine reutilization in the brain.

2) Does the empirically determined rate of lysine exchange between the plasma and brain soluble pools agree with experimentally determined exchange rates?

The computer simulation was performed using an empirically determined rate of lysine uptake from plasma into brain of 1.21 ug/min/g tissue, which is equivalent to a half-life for the brain soluble pool of 17min. (NB. This figure for $t_{1/2}$ neglects the uptake of lysine into brain protein, see equation (4.2), Table 4.2.) In order to determine whether this uptake rate is physiologically meaningful, a comparison was made with literature data. In some instances this led to an examination of the reliability of the method adopted in the measurement of this parameter.

OLDENDORF (1971) measured rates of amino acid exchange between the plasma and brain soluble pools during a 15sec period and found that the rate of equilibration was very much faster than that previously reported by LAJTHA et al. (1957). An examination of the procedure adopted by Lajtha and co-workers indicates, however, that their estimates are likely to be in error (see Table 4.2 for details). According to the results obtained by OLDENDORF (1971) the half-life of the cerebral soluble lysine pool is considerably less than 1min, which does not agree with the half-life of 17min calculated on the basis of the simulated data. In contrast, the turnover rates obtained by TOTH and LAJTHA (1977) are in good agreement with the empirically determined rate (Table 4.3). Further, it is shown in Table 4.3 that the procedure adopted by the latter researchers should yield reasonable estimates of the true turnover rate. They pointed out that the discrepancy between their results and those of OLDENDORF (1971) may be

TABLE 4.2 A COMPARISON OF THEORETICAL RATES OF LYSINE UPTAKE INTO MOUSE BRAIN WITH THE EXPERIMENTAL DATA OBTAINED BY LAJTHA et al. (1957).

Time after precursor administration (min)	Apparent rates of lysine uptake (ug/min/g tissue)		Apparent half-life of brain soluble lysine (min)	
	Theoretical	Experimental	Theoretical	Experimental
2	1.11 (92%)	2.3	18.4 (108%)	11
3	1.05 (87%)	0.7	19.6 (115%)	43
5	0.86 (71%)	1.3	23.9 (141%)	24
10	-0.59			
20	5.93 (490%)	5.2	3.5 (21%)	5
30	9.91 (819%)	1.5	2.1 (12%)	17

LAJTHA et al. (1957) calculated rates of lysine uptake from plasma into brain using the equation

$$F_{14} = (dS_4/dt)\, Q_4/(S_1-S_4) \qquad (4.1a)$$

$$= (dR_4/dt)/(S_1-S_4) \qquad (4.1b)$$

(NB. The notation has been changed to be consistent with Fig. 4.2.) where S_4 and Q_4 are the specific activity and size of the brain soluble lysine pool, respectively, S_1 is the specific activity of plasma lysine and R_4 is the brain TCA-soluble radioactivity. Half-lives ($t_{\frac{1}{2}}$) were calculated using

$$t_{\frac{1}{2}} = Q_4 \ln 2/F_{14}. \qquad (4.2)$$

Equation (4.1) may yield erroneous results for two reasons. Firstly, as mentioned in Section 1.2, the error in dS_4/dt is expected to be greater than the error in S_4 itself, and secondly this equation, which applies to a two-compartment system, does not take into account the loss of radioactivity from the brain soluble lysine pool as a result of its incorporation into protein.

Table 4.2 continued.....

The consequences of error in either the numerator or denominator of equations of the form of (4.1) may be exemplified by considering the two-compartment model shown in Fig. 1.1. Assume that radioactivity is introduced into compartment 1 at zero time. As t increases both dS_2/dt and (S_1-S_2) tend to zero, and at the time when S_2 is at a maximum, $dS_2/dt = S_1-S_2 = 0$. $(dS_2/dt)/(S_1-S_2)$ is therefore undefined. Experimental error in dS_2/dt in the region of time during which (S_1-S_2) is very small may, however, cause $F_{12} = F_{21}$ to become very large (positive or negative).

Regarding the second problem, an examination of either the experimental results given in Fig. 1 of LAJTHA et al. (1957) or the simulated data given in Fig. 4.3 shows that equation (4.1) cannot yield sensible results when applied to the present multi-compartment system. Thus it can be seen that after the specific activity of the brain soluble lysine pool reaches a maximum, flow rates calculated using (4.1) become negative, and that at the point in time when $S_1 = S_4$ F_{14} is infinite and negative. Afterwards F_{14} becomes finite and positive. In order to make a quantitative examination of this second problem, equation (B.13c) was substituted into (4.1b) to obtain

$$F_{14} = \left(\sum_{j=1}^{n} \frac{d}{dt} D_{4j} e^{-\lambda_j t} \right) / (S_1-S_4). \qquad (4.3)$$

The eigenvalues and eigenvector components defining the lysine specific activity curves shown in Fig. 4.3 were substituted into (4.3) in order to calculate theoretical apparent rates* of lysine uptake. The results show that only for very short incorporation periods (up to 3min) does (4.1) yield reasonable approximations to the true rate of uptake (ie., 1.21 ug/min/g, which is equivalent to $t_{\frac{1}{2}}$ = 17min). The figures given in parenthesis in the above table are the theoretical apparent data expressed as a percentage of the theoretical true data. The experimental data are from LAJTHA et al. (1957). *See footnote on page 213.

TABLE 4.3 A COMPARISON OF THEORETICAL RATES OF LYSINE UPTAKE INTO MOUSE BRAIN WITH THE EXPERIMENTAL DATA OBTAINED BY TOTH AND LAJTHA (1977)

Time period (min)	Apparent rate of uptake (umole/min/100g)		Apparent half-life (min)	
	Theoretical	Experimental	Theoretical	Experimental
1 - 2	0.79 (95%)	0.98 ± 0.06	12.8 (105%)	14.8
2 - 4	0.74 (89%)	0.70 ± 0.04	13.6 (111%)	20.7
4 - 7	0.63 (76%)	0.92 ± 0.04	16.0 (131%)	15.8

In order to determine rates of amino acid exchange between the plasma and brain soluble pools, TOTH and LAJTHA (1977) gave mice intraperitoneal injections of radioactive amino acids and measured the increase in brain soluble radioactivity during various intervals of time. Apparent rates of uptake were calculated from the increase in brain soluble radioactivity during a given period of time assuming that the specific activity of amino acid entering the brain was equal to the average plasma specific activity for that time period. The authors emphasise that this procedure does not yield absolute rates of uptake. The apparent half-lives of the soluble amino acid pools in the brain were also calculated. Half-life was defined as the time required for the entry of labelled amino acid amounting to 50% of the endogenous brain content (see Table 1 of LAJTHA and TOTH, 1977). In order to determine what order of discrepancy might be expected between the true and apparent rates of uptake, theoretical apparent uptake rates were calculated as described above using simulated data. Simulated data were obtained using the 'subcutaneous' model outlined in Fig. 4.2 although experimentally the radioactive lysine was administered intraperitoneally. (Simulation of intraperitoneal administration was precluded for lack of the data required to determine the rate of lysine exchange between the peritoneal cavity and the plasma pool.) The figures given in parenthesis in the above table are the theoretical apparent rates of uptake and half-lives expressed as a percentage of the theoretical true uptake rate and half-life. The theoretical true rate of lysine uptake is 0.83 umole/min/100g which is equivalent to a half-life (as defined above) of 12.2 min. It should be noted that some discrepancy between the experimental and theoretical true rates of uptake is expected since the brain lysine content found experimentally was 0.29 umole/g compared with 0.20 umole/g used in the simulation (Table 4.1). The experimental and theoretical true half-life should, however, be directly comparable.

Table 4.3 continued.....

The following conclusions are made. (1) The observation that the theoretical apparent and true half-lives differ by 31% or less over the first 7min indicates that the procedure used by Toth and Lajtha is capable of yielding reasonable estimates of the true half-life. (2) The observed similarity between the theoretical figures for $t_{\frac{1}{2}}$ and those calculated from experimental data indicates that the experimentally determined half-life of 14-21 min reflects the average turnover rate of the total brain soluble lysine pool. (NB. This follows because the model outlined in Fig. 4.2 assumes the brain soluble lysine pool to be homogeneous.) This suggests that the rapidly exchanging pool detected by OLDENDORF (1971) represents a small rapidly equilibrating pool that does not significantly affect the average turnover rate.

explained on the basis of amino acid compartmentation. Thus, measurements made 15sec after the administration of the tracer will yield the turnover rates of the most rapidly exchanging compartments, while turnover rates based on measurements made after a longer time period will be slower due to the decreased contribution from those rapidly exchanging compartments that will have reached equilibrium. The rapidly equilibrating compartment detected by Oldendorf may represent the rapidly exchanging compartment that is a feature of the two models outlined in Figs. 4.4 and 4.5.

3) The incorporation of radioactive lysine into protein

The two models outlined in Figs. 4.4 and 4.5 yield protein specific activity curves that would be experimentally indistinguishable during a 2h incorporation period. For example, the total brain protein-bound radioactivity at 2h, as predicted by the two models, differ by less than 2.5%. Consequently, the following calculations were carried out using only the subcellular model (Model 1).

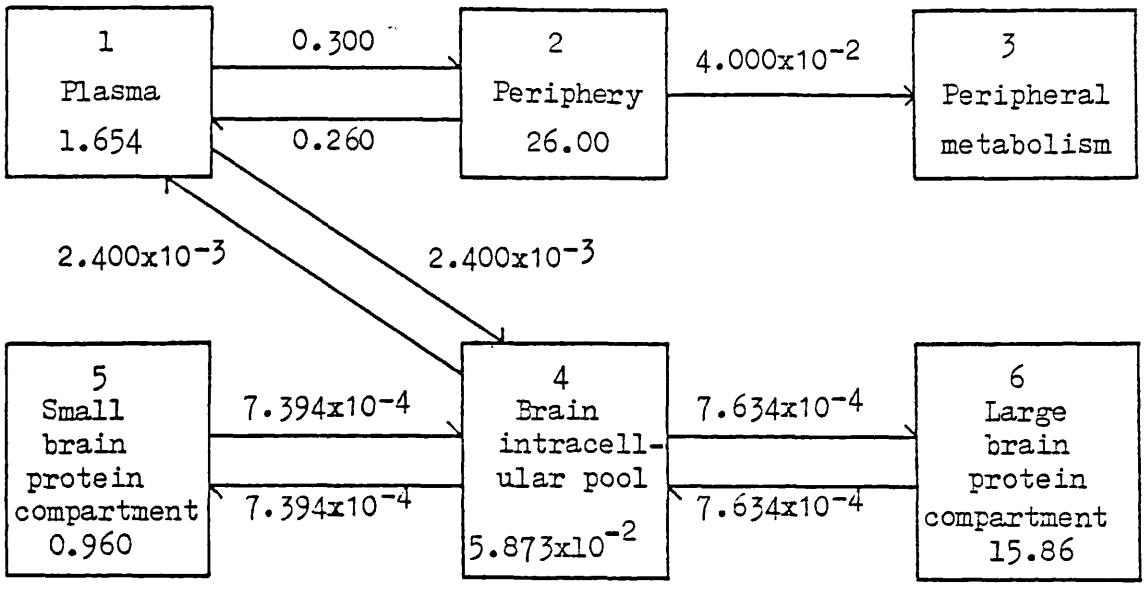
LAJTHA and co-workers (1957) reported that rates of brain protein turnover calculated on the basis of their lysine incorporation data decreased as the duration of the incorporation period was increased. They suggested that heterogeneity of brain protein turnover rates might be the cause of this phenomenon. They pointed out that after a short incorporation period, the majority of radioactivity will be present in those proteins turning over at the fastest rates. As the incorporation period is increased, the radioactivity present in rapidly synthesized protein will not increase proportionately because a percentage will be lost through protein degradation (see Section 1.2). Consequently, *apparent turnover rates are expected to

* Throughout this thesis the term "apparent" is used to indicate that the parameter in question is an index of, and has the same dimensions as some quantity that cannot be measured directly. The term "theoretical apparent" is assigned to the results obtained when a given calculation is performed using simulated data. These theoretical apparent data may be compared with the corresponding theoretical true value in order to determine whether a given procedure can, in principle, yield data that are reliable indices of the parameter in question.

decrease with increasing incorporation time. During their later studies on protein metabolism in the mouse, Lajtha and co-workers found that the specific activity of free tyrosine could be maintained at a constant level over a period of days by implanting [^{14}C]tyrosine pellets. They measured the rate of incorporation of radioactivity into brain protein following [^{14}C]tyrosine pellet implantation and found that a best theoretical fit of their incorporation data was obtained if it was assumed that two brain-protein compartments were present, one compartment accounting for 5.7% of total protein and having a half-life of 15h, the other compartment representing 94.3% of total protein and having a half-life of 10days (LAJTHA et al., 1976). The model shown in Fig.4.2 was modified in accordance with these results, making the assumption that lysine entering the two protein compartments was of the same specific activity (Fig.4.7). Using theoretical data derived from this modified model, apparent turnover rates were calculated. The results (Table 4.4) show that heterogeneity of protein turnover rates of the magnitude indicated by LAJTHA et al. (1976) does not account for the earlier observation that the apparent rate of lysine incorporation decreased by over 80% as the duration of the incorporation period was increased from 2 to 60min (see Table 6 of LAJTHA et al., 1957).

GARLICK and MARSHALL (1972) have given this matter their attention and they also argued that it is unlikely that the apparent difference in rates of protein synthesis calculated on the basis of data obtained using different incorporation times (between 1min and 1h) is caused by a rapidly turning over component in a mixed population of proteins. They pointed out that if this were the case, then this component would have to be present in a sufficient quantity to make a substantial contribution to the incorporation of radioactivity and have a half-life of less than 1h. They suggested that the problem of defining the precursor pool for protein synthesis might be the true cause of the phenomenon. This is consistent with the report that the rate of incorporation of valine into rat brain protein remained constant during at least the first 4h period following the administration of [^{14}C]valine at a dose level sufficient to maintain the specific activity

FIG. 4.7 A SCHEME FOR THE SIMULATION OF BRAIN PROTEIN HETEROGENEITY



The homogeneous precursor lysine model outlined in Fig. 4.2 was modified in accordance with the data obtained by LAJTHA et al. (1976). They measured the incorporation of radioactivity into brain protein in mice in which the specific activity of free tyrosine was maintained at a constant level, and found that a best theoretical fit of their experimental data was obtained if it was assumed that there are two brain protein compartments. One protein compartment (5) accounts for 5.7% of total protein and has a half-life of 15h, and the other (6) accounts for 94.3% of total protein and has a half-life of 10 days. It is assumed that the distribution of lysine between the two protein compartments is the same as that of tyrosine. All other parameters are as in Fig. 4.2. Flow rates are expressed in terms of umole/min/100g body weight and the lysine content of the compartments in umole/100g body weight.

TABLE 4.4 A SIMULATION STUDY OF THE EFFECT OF BRAIN PROTEIN HETEROGENEITY ON APPARENT TURNOVER RATES

Incorporation time (min)	Apparent rate of incorporation (umole/min)	Apparent half-life (days)
2	1.50×10^{-3} (100%)	5.40 (100%)
20	1.49×10^{-3} (99%)	5.43 (101%)
200	1.36×10^{-3} (91%)	5.95 (110%)

LAJTHA et al. (1957) found that apparent rates of lysine incorporation into mouse brain protein calculated using

$$F = \frac{Q_b(dS_b/dt)}{S_a - S_b}, \qquad (4.4)$$

where the subscripts a and b denote the precursor and protein-bound lysine pools, respectively, became progressively slower as the duration of the incorporation period was increased. They attributed this to heterogeneity of brain protein turnover rates. In order to obtain estimates of the error that might be expected to arise when equation (4.4) is applied to a heterogeneous protein system, theoretical apparent incorporation rates were calculated using theoretical data derived from the model outlined in Fig. 4.7. The following substitutions were made.

$$\frac{dR_{protein}}{dt} = \frac{d}{dt}(R_5 + R_6) \qquad (4.5a)$$

$$= \sum_{j=1}^{n} \left(\frac{d}{dt} D_{5j} e^{-\lambda_j t} + \frac{d}{dt} D_{6j} e^{-\lambda_j t}\right) \qquad (4.5b)$$

(see equation (B.13c) and

$$S_{protein} = (R_5 + R_6)/(Q_5 + Q_6). \qquad (4.6)$$

Half-life ($t_{\frac{1}{2}}$) was calculated using

Table 4.4 continued.....

$$t_{\frac{1}{2}} = \frac{\ln 2\ (Q_5 + Q_6)}{F} \qquad (4.7)$$

The theoretical true rate of lysine incorporation is 1.503×10^{-3} umole/min (Fig. 4.7) which is equivalent to a half-life of 5.39 days. The figures given in parenthesis are the apparent rates and half-lives expressed as a percentage of the true rate and half-life. The results indicate that heterogeneity of brain protein turnover rates of the magnitude reported by LAJTHA *et al.* (1976) does not account for the earlier observation that the apparent rate of lysine incorporation decreased by more than 80% as the duration of the incorporation period was increased from 2 to 60min (see Table 6 of LAJTHA *et al.*, 1957).

of the soluble valine pool at a relatively constant level during the incorporation period (see Fig. 2 of DUNLOP et al., 1975a).

Theoretical data for the incorporation of radioactivity into cerebral protein was obtained using the subcellular model of precursor lysine compartmentation (Model 1, Fig. 4.4) and this in turn was used to calculate a theoretical apparent half-life of brain protein for different incorporation times (Table 4.5). Similar calculations were performed using data obtained from the homogeneous precursor lysine model shown in Fig. 4.2. As expected, the simple model fails to account for the apparent time dependence of the turnover rate, while Model 1 yields values of $t_{\frac{1}{2}}$ which agree reasonably well with the experimental data for the first 10min. The failure of Model 1 to account for the apparent time dependence of the turnover rate in the time interval 10 to 60min probably indicates that the assumed presence of only two precursor compartments is not a good approximation to the in vivo multi-compartmental situation and that additional compartments must be added if a good agreement between experimental and theoretical data is to be expected over an extended period of time. Nevertheless, the results are consistent with the supposition that the problem of defining the precursor lysine pool and measuring its specific activity is the cause of the apparent time dependence of protein turnover rates.

4.1.4 THE EFFECT OF L-DOPA ON THE INCORPORATION OF RADIOACTIVE LYSINE INTO BRAIN PROTEIN

4.1.4.1 The effect of an expansion of the brain soluble lysine pool

ROEL et al. (1974) found that L-dopa caused a 34% increase in brain soluble lysine, although this increase was not statistically significant. They therefore suggested that "about half" of their observed 64-67%* reduction in protein-bound radioactivity in L-dopa-treated rats was due to a reduction in the rate of lysine incorporation, the

*These figures are based on data in which TCA-insoluble radioactivity is expressed as a percentage of total homogenate radioactivity.

TABLE 4.5 THE INCORPORATION OF RADIOACTIVE LYSINE INTO MOUSE BRAIN PROTEIN. A COMPARISON OF THEORETICAL AND EXPERIMENTAL DATA.

Time (min)	Experimental data		Theoretical data			
			Heterogeneous precursor lysine model (Fig. 4.4)		Homogeneous precursor lysine model (Fig. 4.2)	
	Protein specific activity (S_5) (c.p.m./ug)	Half-life ($t_{\frac{1}{2}}$) (min)	Protein specific activity (S_5) (c.p.m./ug)	Half-life ($t_{\frac{1}{2}}$) (min)	Protein specific activity (S_5) (c.p.m./ug)	Half-life ($t_{\frac{1}{2}}$) (min)
2	0.08	2.8	0.08	2.4	0.03	4.21
5	0.23	3.5	0.24	3.5	0.14	
10	0.51	5.5	0.52	4.0	0.40	
20	0.75	6.2	0.98	4.3	0.85	

Experimental data are from Tables 5 and 6 of LAJTHA et al. (1957). Theoretical specific activity data were obtained by computer simulation using the subcellular model of precursor lysine compartmentation outlined in Fig. 4.4 and the homogeneous precursor lysine model outlined in Fig. 4.2. (N.B. Simulation was performed for intravenous administration. See Fig. 4.3 for further details.) Half-lives ($t_{\frac{1}{2}}$) were calculated using the equation

$$t_{\frac{1}{2}} = Q_5 \ln 2/(F \times 1440) \qquad (4.8)$$

where Q_5 is the size of the brain protein-bound lysine pool and the factor 1440 converts units of time from minutes to days. Theoretical apparent rates of incorporation (F) were calculated using the equation

$$F = \left(\sum_{j=1}^{n} \frac{d}{dt} D_{5j} \, e^{-\lambda_j t} \right)/(S_4 - S_5) \qquad (4.9)$$

(see equation (B.13c) where the subscripts 4 and 5 denote the brain soluble and brain protein-bound lysine pools, respectively.

other half being the consequence of a dilution of the injected [^{14}C] lysine. This would, however, be true only if there was an accompanying 34% increase in total body soluble lysine. Computer simulation experiments were performed to investigate a) the effect of a brain-specific 34% expansion of the soluble lysine pool, assuming no precursor lysine compartmentation and b) the effect of a 34% expansion of the main intracellular pool in brain as predicted by the models of lysine compartmentation.

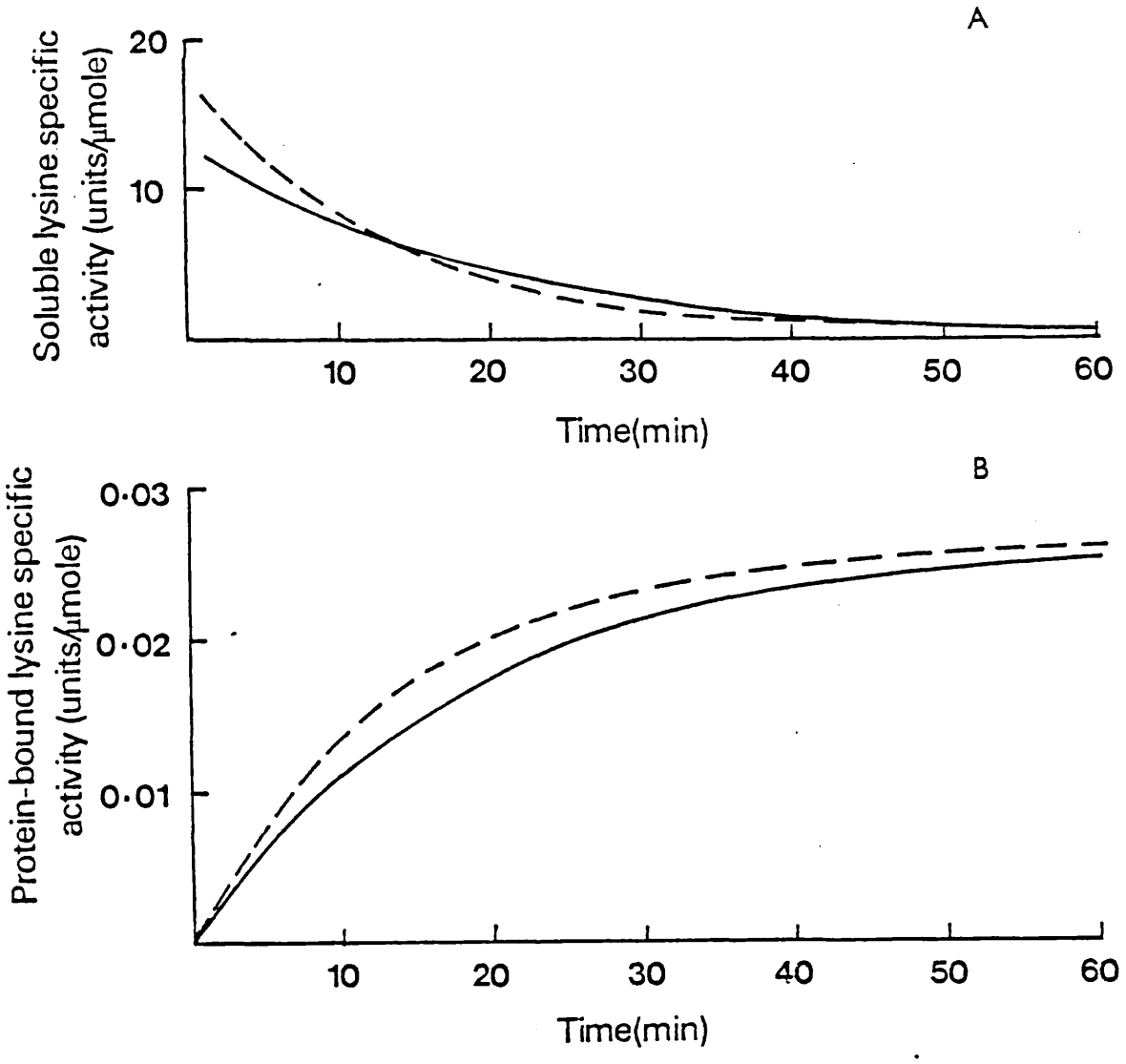
a) The effect predicted by the homogeneous precursor lysine model

The model outlined in Fig.4.2 was used to study the effect of a brain-specific increase in soluble lysine levels and the dependence of this effect on the route of tracer administration. The results indicate that the magnitude of the perturbation caused by an expansion of the brain soluble lysine pool is relatively independent of the mode of administration. Fig.4.8 shows the simulated effect of a 34% increase in brain soluble lysine (peripheral pool sizes remaining unchanged) in a hypothetical situation in which the administered radioactive lysine equilibrates instantaneously with the cerebral intracellular pool. The percentage decrease in protein-bound radioactivity is time dependent, but is always considerably less than 34%. A simulation of subcutaneous tracer administration yielded a 22.8%, 19.5% and 13.4% reduction in the radioactive content of the brain protein compartment at 7, 15 and 30min, respectively. Roel and co-workers administered their radioactive amino acids via the cisterna magna. It was not possible to simulate intracisternal administration because the cerebrospinal fluid is not represented in the model. Nevertheless, the above results indicate that if the 34% expansion of the soluble lysine pool was brain specific, the resulting decrease in TCA-insoluble radioactivity would have been considerably less than 34%.

b) The effect predicted by the models of precursor lysine compartmentation

Both models 1 and 2 predict that an L-dopa-induced decrease in the specific activity of the main intracellular lysine compartment in brain will have no measureable effect on the rate of incorporation of radioactive lysine into cerebral protein, since, in the presence of L-dopa, lysyl-tRNA is not charged from the main intracellular pool. Consequently,

FIG. 4.8 THE SIMULATED EFFECT OF AN L-DOPA-INDUCED EXPANSION OF THE BRAIN SOLUBLE LYSINE POOL



ROEL et al. (1974) have suggested that an L-dopa-induced 34% expansion of the brain soluble lysine pool is responsible for "about half" of their observed 64-67%* decrease in the uptake of radioactive lysine into brain protein in L-dopa-treated rats. This assumes that the dopa effect is not brain specific, but that there is a corresponding 34% increase in peripheral lysine levels. The effect of a 34% expansion of the brain soluble lysine pool in the hypothetical situation in which the administered radioactivity equilibrates instantaneously with the brain intracellular pool was tested, assuming that peripheral lysine pool sizes remain unchanged. Simulation was carried out using the model shown in Fig. 4.2. (The zero-time radioactivity vector $\underline{R}(0)$ was (0,0,0,1,0,0).)

A. Specific activity of brain soluble lysine. B. Specific activity of brain protein-bound lysine. – – – –, control; ——, perturbated system (34% increase in Q4). *See Footnote on page 218.

an expansion of the main intracellular lysine compartment in brain will have no significant effect on the rate at which radioactivity is incorporated into brain protein.

4.1.4.2 The effect of L-dopa on the apparent incorporation of lysine

Simulated data generated by the subcellular model of lysine compartmentation were used to calculate theoretical values for the apparent incorporation of lysine (I_{app}) in control and L-dopa-treated rats. The results are shown in Table 4.6 together with the experimental apparent incorporation data. The observation that the theoretical results agree at least qualitatively with those obtained experimentally lends support for the model from which the theoretical data were derived. The cellular model of lysine compartmentation yields the same theoretical results.

4.1.5 A SUMMARY OF THE RESULTS OF THE SIMULATION OF IN VIVO TRACER EXPERIMENTS

An explanation was sought for the observation that in L-dopa-treated rats the apparent incorporation of brain [^{3}H]lysine was not significantly lower than the control level 7½ min after tracer administration but was reduced by 55-59% after a 15 min incorporation period. Two models of brain lysine compartmentation are outlined both of which yield theoretical data that are consistent with these results. The main feature of these models is that the brain intracellular soluble lysine pool is heterogeneous and that brain protein-bound lysine is not derived from a single precursor compartment. According to these models, brain protein turnover rates calculated from lysine incorporation data will decrease significantly as the duration of the incorporation period is increased in the time interval 0 to 20min. This apparent time dependence in brain protein turnover rates was observed by LAJTHA et al. (1957) but they attributed the phenomenon to a heterogeneity of brain protein turnover rates. Computer simulation was used to show that this explanation may not be correct since heterogeneity of turnover rates of the magnitude reported by LAJTHA et al. (1976) will not cause a significant decrease in the turnover

TABLE 4.6 A COMPARISON OF THEORETICAL AND EXPERIMENTAL APPARENT LYSINE INCORPORATION DATA

Time (min)	Apparent incorporation (nmole/mg prot.*)					
	Control		L-dopa-treated		$\frac{(\text{L-dopa-treated})100}{\text{Control}}$ (%)	
	Theo-retical	Experi-mental	Theo-retical	Experi-mental	Theo-retical	Experi-mental
$7\frac{1}{2}$	0.321	1) 0.364 2) 0.327	0.241	1) 0.311 2) 0.324	75%	1) 85% 2) 99%
15	0.681	1) 0.75 2) 0.662	0.323	1) 0.31 2) 0.301	47%	1) 41% 2) 45%

According to the models of lysine compartmentation outlined in Figs. 4.4 and 4.5 a change in the specific activity of the large brain intracellular lysine pool, resulting from either an expansion of its size or a reduction in the rate of uptake of radioactivity into the brain, will have no significant effect on the rate at which radioactivity is incorporated into brain protein in L-dopa-treated rats, since, in the presence of L-dopa, lysine does not enter brain protein via the main intracellular pool. A change in the specific activity of the large intracellular pool will, however, affect the apparent incorporation (I_{app}) as defined by equation (1.13). Thus protein synthesis may appear to be affected by L-dopa. This has been demonstrated using simulated data obtained from the model outlined in Fig. 4.4. Theoretical values of I_{app} were calculated using (1.13), assuming that the specific activity of the brain soluble lysine pool in L-dopa-treated rats was 58% that of the control specific activity throughout the incorporation period (from Table 3.2.14). The experimental data are from Tables 3.2.9 - 3.2.12.
*Calculations were performed using a figure of 113mg/g tissue for the protein content of mouse brain (LONG, 1961 page 641).

rates calculated from incorporation data as the duration of the labelling period is increased up to 200 min.

An important feature of the models of lysine compartmentation is the presence of a sequestered intracellular lysine pool in brain tissue. The experimental evidence for the existence of such a compartment is examined in the following section.

4.2 EVIDENCE FOR AN INTRACELLULAR NONEXCHANGING LYSINE COMPARTMENT IN THE BRAIN

The two models of precursor lysine compartmentation outlined in the previous section assume the presence of an intracellular lysine compartment that does not exchange readily with the main intracellular pool. Some of the experimental evidence for the existence of sequestered intracellular amino acid compartments is reviewed in Section 1.3. Of particular interest is the observation that when rats or mice are infused with radioactive amino acids, the specific activity of the brain soluble amino acid pool remains significantly lower than that of the plasma throughout the infusion period (SETA et al., 1973; GARLICK and MARSHALL, 1972). Similarly, when brain slices are incubated in a medium containing radioactive amino acids, the specific activity of the tissue pool remains lower than that of the medium (DUNLOP et al., 1974; NEIDLE et al., 1975). It is not known to what extent the presence of nonexchanging intracellular compartments is the cause of this intracellular dilution, since the continual release of cold amino acids from degraded protein is expected to make some contribution. A computer simulation study was carried out in an attempt to answer this question.

4.2.1 COMPUTER SIMULATION OF INFUSION EXPERIMENTS

SETA et al. (1973) performed a series of experiments in which they infused rats with L-[U-^{14}C]lysine in order to maintain plasma lysine at a constant specific activity for a period of hours. They found that under these conditions the brain soluble lysine pool did not equilibrate with the plasma pool. For example, the specific activity

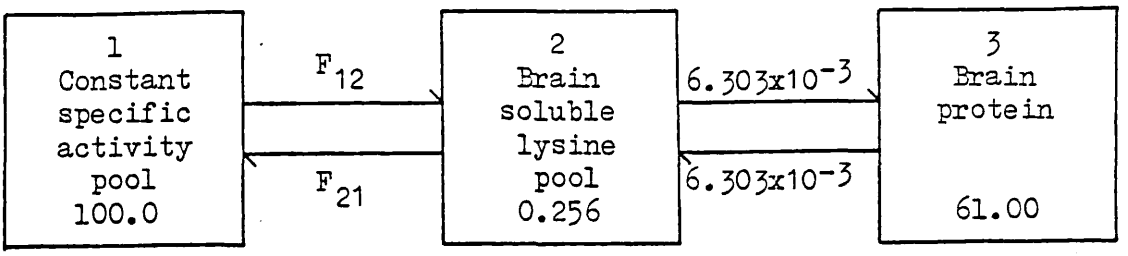
of cerebral lysine was 77% and 86% of the plasma specific activity at 3h and 6h, respectively. In order to determine whether this level of intracellular dilution might be accounted for by the release of [^{12}C]lysine from degraded protein, theoretical brain : plasma lysine specific activity ratios were determined using a model which assumes that the intracellular soluble pool is not compartmented. Simulation was performed according to the scheme outlined in Fig. 4.9 using a series of uptake rates that was expected to encompass the physiological range. A series of uptake rates was used because of the uncertainty of the published data on the rate at which lysine exchanges between the plasma and brain soluble pools (see Section 4.1.3). The simulated plasma and brain soluble lysine specific activity-time curves are shown in Fig. 4.10.

Before attempting to answer the question as to whether a sequestered lysine compartment is the cause of the observed intracellular dilution some points relating to the general behaviour of the system deserve attention. An examination of the simulated data indicates that the specific activity of the intracellular lysine pool increases relatively rapidly until it reaches a quasi-steady level which is less than that of the plasma (Fig. 4.10). Once the system has attained this tracer quasi-steady state, the brain : plasma specific activity ratio increases only very slowly as the specific activity of the protein-bound lysine pool increases. It is shown in Table 4.7 that the ratio given by

$$S_2/S_1 = F_{12}/(F_{12} + F_{32}) \qquad (4.10)$$

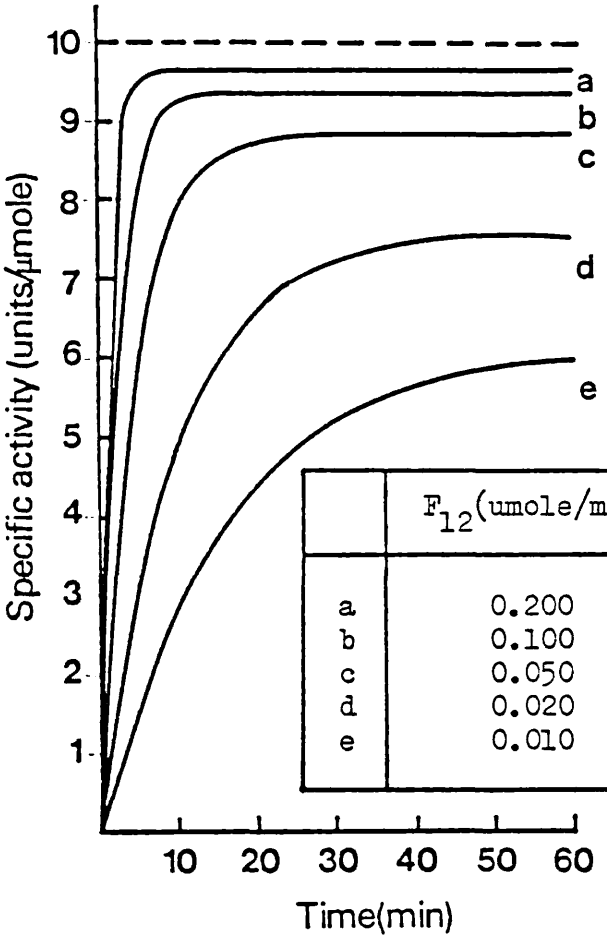
is a good approximation to this quasi-equilibrium brain : plasma specific activity ratio. This indicates that if the intracellular soluble pool is homogeneous, the specific activity ratio is directly determined by the rate at which the intracellular pool is supplied with amino acid from the plasma relative to the rate at which the amino acid is released from protein. In the absence of intracellular compartmentation, equation (4.10) is expected to apply to any essential amino acid providing the incorporation period is not of a sufficient

FIG. 4.9 A SCHEME FOR THE SIMULATION OF L-[U-^{14}C]LYSINE INFUSION EXPERIMENTS



In order to simulate a constant plasma specific activity, compartment (1) is large compared with (2). Flow rates and pool sizes, which are expressed in terms of umole/min/100g body weight and umole/100g body weight, respectively, were calculated from the following data. Rat brain soluble lysine content, 21 umole/100g tissue (Table 14-1 of MAKER et al., 1976); rat brain weight, 1.22g/100g body weight (page 639 of LONG, 1961); lysine content of rat brain protein, 50 umole/g tissue (Table IV of LAJTHA and TOTH, 1974); rate of lysine incorporation into adult rat brain protein, 0.62%/h (DUNLOP et al., 1975a).

FIG. 4.10 SIMULATED SPECIFIC ACTIVITY-TIME CURVES FOR THE BRAIN AND PLASMA SOLUBLE LYSINE POOLS IN THE INFUSED RAT



	F_{12}(umole/min)	U_{12}(min^{-1})
a	0.200	0.7813
b	0.100	0.3906
c	0.050	0.1953
d	0.020	0.0781
e	0.010	0.0391

Simulation was performed according to the model outlined in Fig. 4.9. The rate of lysine uptake into the brain (F_{12}) was varied in the range 0.01 to 0.20 umole/min/100g body wt as indicated. The fractional rate of uptake (U_{12}) is defined by equation (4.11) in the main text. The specific activity of the plasma lysine pool is indicated by the broken line.

TABLE 4.7 THEORETICAL BRAIN : PLASMA LYSINE SPECIFIC ACTIVITY RATIOS

Rate of uptake (F_{12}) (umole/min)	*Fractional rate of uptake (U_{12}) (min^{-1})	** Brain : plasma specific activity ratio at 3h	*** $\frac{F_{12}}{F_{12} + F_{32}}$
0.01	3.91×10^{-2}	0.62	0.61
0.02	7.81×10^{-2}	0.76	0.76
0.05	0.195	0.89	0.89
0.10	0.391	0.94	0.94
0.20	0.781	0.97	0.97

Computer simulation of L-[^{14}C] lysine infusion experiments was carried out according to the model outlined in Fig. 4.9 using a range of uptake rates.

* The fractional rate of uptake (U_{12}) is defined by

$$U_{12} = F_{12}/Q_2. \quad ^{***} \qquad (4.11a)$$

** SETA *et al.* (1973) observed a lysine specific activity ratio of 0.77 at 3h.

***$F_{32} = 6.303 \times 10^{-3}$ umole/min; $Q_2 = 0.256$ umole (from Fig. 4.9).

duration for the specific activity of the amino acid released from degraded protein to become significant.* In the case of nonessential amino acids the intracellular pool will be further diluted as a result of de novo amino acid synthesis.

To return to the question concerning the contribution that [^{12}C]lysine released from degraded protein might be expected to make towards the dilution of the intracellular soluble pool in brain, simulation of infusion experiments was performed using lysine uptake rates in the range 0.01 to 0.20 umole/min (NB. All data are expressed relative to 100g body wt, which is equivalent to 1.22g of brain tissue, see Fig.4.9) in order to find a rate of uptake that yields theoretical data consistent with the experimental observation that the specific activity of the brain soluble pool was "close to the 6h value at 30min" (SETA et al., 1973). According to this criterion an uptake rate of about 0.02 umole/min might be selected as being physiological (Fig.4.10), in which case the release of lysine through protein degradation accounts for the observed intracellular dilution (Fig. 4.10 and Table 4.7).

TOTH and LAJTHA (1977) measured rates of lysine uptake into mouse brain, and it is shown in Table 4.3 that their uptake rates are expected to be reasonable estimates of the true rate. In order to make use of their data it is convenient to define a new variable, the fractional rate of uptake (U_{ij}),

$$U_{ij} = F_{ij}/Q_j \,. \qquad (4.11)$$

According to the data given in Table 1 of TOTH and LAJTHA (1977), the fractional rate of lysine uptake from plasma into brain is in the

*If the protein compartment is heterogeneous, the specific activity of the amino acid released into the soluble pool through proteolysis could become significant while the average specific activity of the protein-bound amino acid compartment remains insignificant (see Section 1.2). Heterogeneity of brain protein turnover rates of the magnitude reported by LAJTHA et al. (1976) increases the lysine specific activity ratios given in Table 4.7 to 0.66 with F_{12} = 0.01 and 0.98 with F_{12} = 0.20.

range 2.4×10^{-2} to 3.4×10^{-2} min^{-1} which is in reasonable agreement with the figure of 4.1×10^{-2} min^{-1} obtained from the computer simulation of the experiments of LAJTHA et al. (1957) (see Fig. 4.2), but somewhat less than the empirically determined figure of 7.8×10^{-2} min^{-1} (equivalent to $F = 2 \times 10^{-2}$ umole/min) obtained on the basis of the above criterion. It can be seen from Table 4.7, Fig. 4.10 or equation (4.10) that with a fractional rate of uptake of about 4×10^{-2} min^{-1}, the theoretical lysine specific activity ratio is about 60%, which again is somewhat lower than the experimentally observed 77-86%. A specific activity ratio of 80% is expected with a fractional uptake rate of about $0.1 min^{-1}$ (equivalent to $F = 2.52 \times 10^{-2}$ umole/min). Clearly, without precise data for the rate of lysine exchange between plasma and brain it is not possible to obtain an accurate figure for the specific activity ratio. Although the results indicate that the release of [^{12}C]lysine from degraded protein makes a major contribution to the dilution of the intracellular lysine pool, it is neither possible to confirm nor rule out the existence of a sequestered intracellular lysine compartment in the in vivo brain.

4.2.2 COMPUTER SIMULATION OF BRAIN SLICE EXPERIMENTS

DUNLOP et al. (1974) measured rates of L-[U-^{14}C]lysine uptake into rat brain slices and its subsequent incorporation into protein. They also measured absolute rates of incorporation using flooding concentrations (1mM) of L-[^{14}C]lysine. The results of their trace experiments are shown in Fig. 4.11, together with the computer simulated data obtained using the model shown in Fig. 4.12. Also included in Fig. 4.11 is the protein specific activity curve that is generated by a model in which protein-bound lysine is derived directly from the incubation medium.* The similarity between the experimental protein specific activity data and the theoretical data generated by the model in which protein-bound

*It has been reported that protein-bound amino acids in the isolated rat extensor digitorum longus muscle and in fragments of rat pancreas are derived directly from the medium rather than from the intracellular pool (HIDER et al., 1969, 1971; VAN VENROOIJ et al., 1972; see Section 1.3 for further details).

FIG. 4.11 THEORETICAL AND EXPERIMENTAL DATA FOR THE UPTAKE OF L-[U-^{14}C]LYSINE INTO RAT BRAIN SLICES AND ITS SUBSEQUENT INCORPORATION INTO PROTEIN

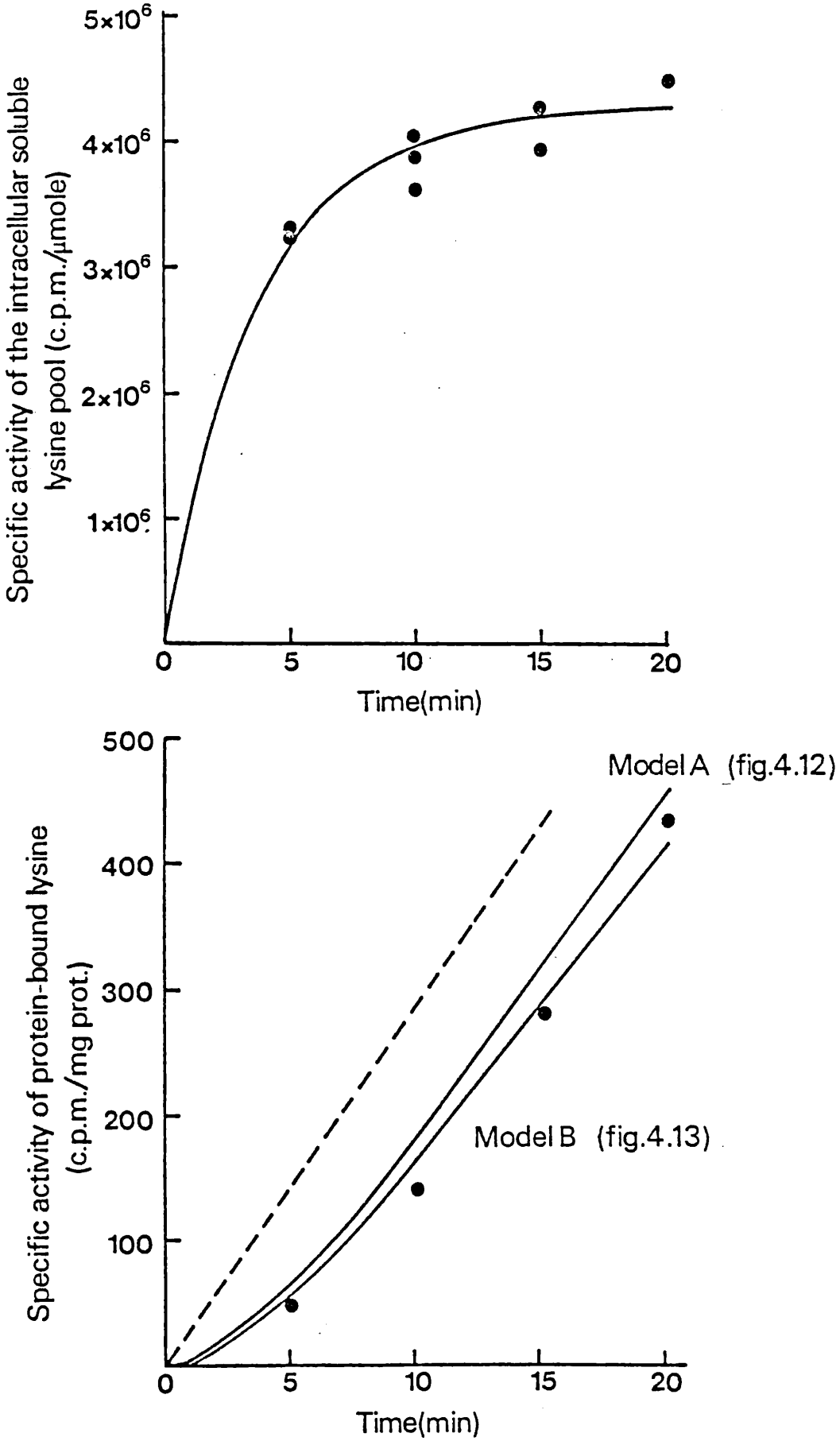
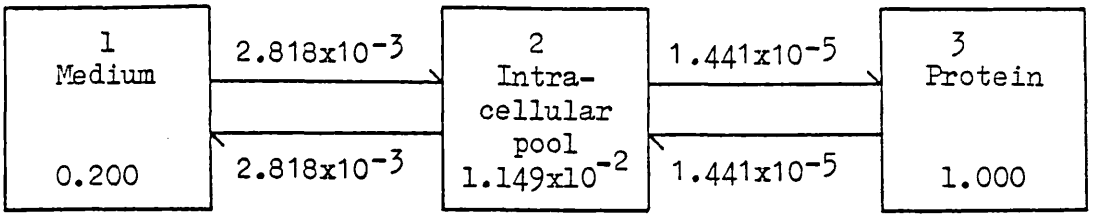


Fig. 4.11 Continued.....(2)

The experimental data are from Figs. 2 and 3 of DUNLOP et al. (1974). Computer simulation was carried out using the models outlined in Figs. 4.12 and 4.13. The unknown flow rates and the scaler multiplyer (see Appendix B) were adjusted to obtain a least-squares fit of the experimental data. ● , experimental data; ——, theoretical data. The upper theoretical protein specific activity curve is that given by Model A (Fig. 4.12) and the lower protein specific activity curve that generated by Model B (Fig. 4.13); — — — —, the protein specific activity curve generated by a model in which lysine present in the medium is incorporated directly into protein at a rate of 1.441×10^{-5} umole/min.

FIG. 4.12 MODEL A. A BASIC MODEL FOR THE SIMULATION OF RAT BRAIN SLICE EXPERIMENTS

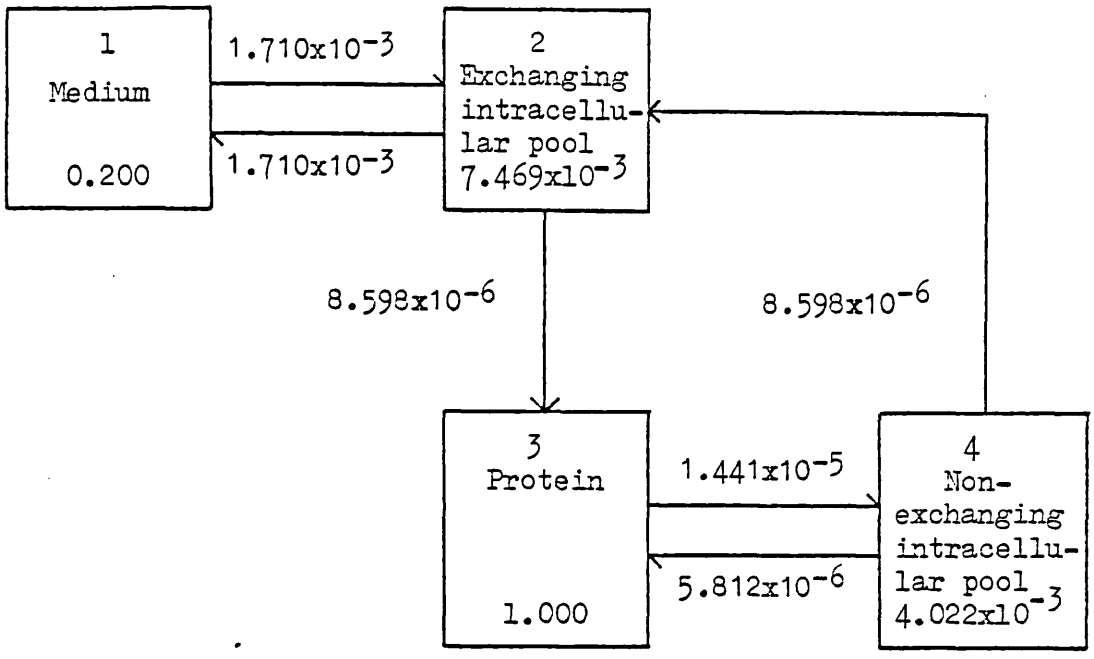


DUNLOP et al. (1974) measured rates of lysine incorporation into protein in rat brain slices. The slices were incubated in 2ml of medium containing 0.1mM L-[U-^{14}C]lysine. Simulation was performed assuming a tissue concentration of 10mg per ml of incubation medium. Flow rates (umole/min) and pool sizes (umole) were calculated using the following literature data on the rat. Tissue-medium concentration gradient, 7.18 (Table IX of KANDERA et al., 1968, taking an average of the data given for the right and left hemispheres and midbrain); protein-bound lysine content of rat brain, 50 umole/g tissue (Table IV of LAJTHA and TOTH, 1974); water content of rat brain slices, 80% (Table V of LEVI et al., 1967); protein content of rat brain, 110 mg/g tissue (page 641 of LONG, 1961); rate of lysine incorporation into rat brain slice protein, 0.393 nmole/mg prot./h (Table 5 of DUNLOP et al., 1974, measured at a 1mM lysine concentration.). Flow rates $F_{12} = F_{21}$ were adjusted to obtain a least-squares fit of the intracellular soluble lysine specific activity data given in Fig. 3 of DUNLOP et al. (1974). Steady state approximation. The above model assumes that the rate of lysine incorporation into protein is equal to the rate of release through proteolysis. NEIDLE et al. (1975) report that the rate of amino acid release from protein in mouse brain slices is about 0.3-0.7%/h which is approximately 3-7 times faster than the rate of incorporation. This imbalance would not result in a significant expansion of the soluble lysine pools in the present case. (0.7%/h is equivalent to a release of 7nmole/h compared with a total soluble lysine pool size at zero-time of 211 nmole.) Thus the failure to take the imbalance into account does not significantly affect the results. (An 'open' model in which the rate of protein degradation is about $3\frac{1}{2}$ times the rate of synthesis, ie., about 0.3%/h, yields tissue: medium specific activity ratios that differ from those given by the above model by less than 2%.)

lysine is derived from the intracellular pool indicates that in the time interval during which there is a rapid increase in tissue radioactivity, (ie., 0 to 15min) the specific activity of the precursor lysine compartment closely follows that of the total intracellular pool. However, according to the model, the medium and intracellular pools should rapidly equilibrate to attain a tracer quasi-steady state in which the tissue : medium specific activity ratio is greater than 99%, whereas a ratio of about 65% was observed experimentally (Fig. 4 of DUNLOP et al., 1974). This discrepancy may be explained if the presence of a nonexchanging intracellular compartment amounting to about 35% of intracellular lysine is assumed. In order to account for the experimental incorporation data it is then necessary to postulate that at a low medium lysine concentration (0.1mM), 60% of lysine incorporated into protein is derived from the exchanging intracellular compartment and the remainder from the nonexchanging pool (this modified model is outlined in Fig. 4.13). The modified model also assumes that when the medium lysine concentration (and therefore the intracellular concentration) is high, either lysine enters protein only from the exchanging intracellular compartment, or radioactive lysine is forced into the nonexchanging pool so that the specific activity of all compartments is the same. The theoretical protein specific activity curve predicted by the modified model (Fig. 4.13), given a 0.1mM lysine concentration in the medium, is included in Fig. 4.11.

NEIDLE et al. (1975) pointed out that if brain slices are incubated in a medium containing elevated levels of amino acids, there will be a net uptake of these amino acids into the tissue and that this will tend to obscure the presence of nonexchanging pools. For this reason they carried out a series of experiments in which medium and tissue amino acid specific activities were measured after incubating mouse brain slices in the presence of trace quantities of radioactive amino acids. At two hours the tissue : medium specific activity ratios for threonine, valine, leucine and tyrosine were in the range 80% to 94%, while the ratio for lysine was only 59%. The authors suggested that sequestered amino acid compartments were the cause of the observed

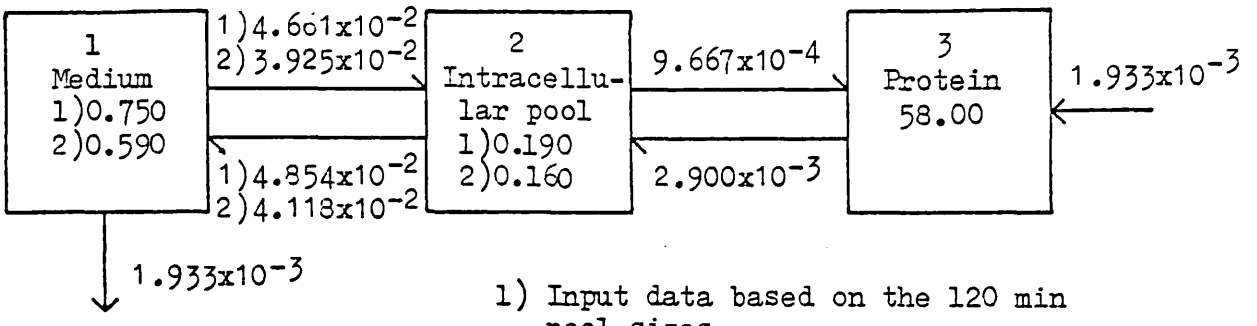
FIG. 4.13 MODEL B. A SUBCELLULAR MODEL OF LYSINE COMPARTMENTATION IN THE SLICED RAT BRAIN



The model shown in Fig. 4.12 was modified to account for the experimental observation that the quasi-equilibrium tissue: medium lysine specific activity ratio was about 0.65 (Fig. 4 of DUNLOP et al., 1974). Flow rates $F_{12} = F_{21}$ and $F_{23} = F_{42}$ ($F_{43} = 1.441 \times 10^{-5} - F_{23}$) were adjusted to obtain a least-squares fit of the protein and intracellular soluble lysine specific activity data given in Figs. 2 and 3 of DUNLOP et al. (1974) (see the example program given in Appendix B). Other parameters are as in Fig. 4.12. The flow rates are given in terms of umole/min and the pool sizes in umole.

intracellular dilution. They calculated that the lysine sequestered pool accounted for 0.078 umole/g tissue at 120min (see Table VII of NEIDLE et al., 1975) compared with a total tissue content of 0.19 umole/g, but this calculation assumes that the release of [^{12}C]lysine from degraded protein did not contribute to the intracellular dilution. This assumption may not be valid when applied to experiments in which incubations are carried out using only trace quantities of amino acids. In order to test the validity of this assumption, a computer simulation of the trace experiments of NEIDLE et al. (1975) was performed using the model outlined in Fig.4.14. The results indicate that the release of cold lysine from degraded protein probably causes a measurable but small dilution of the intracellular pool since, according to the model, a tissue : medium specific activity ratio of about 95% is expected in the absence of a nonexchanging intracellular lysine compartment. Consequently, although the sequestered lysine pool size given in Table VII of NEIDLE et al. (1975) is probably an overestimate of the true size, the discrepancy is not expected to be large. However, in order to obtain a figure for the size of the sequestered lysine pool which is not based upon a neglect of the effect of proteolysis, the simple model outlined in Fig. 4.14 was modified by the addition of a nonexchanging compartment (Fig. 4.15) the size of which was adjusted in order to obtain a quasi-equilibrium specific activity ratio of 58 - 59%. This requirement was met when the size of the nonexchanging compartment was about 36% of total intracellular lysine, that is about 0.069 umole/g tissue at 2h. This is somewhat smaller than the figure of 0.2 umole/g obtained from the previous simulation (see Fig. 4.13). In both cases, however, the sequestered pool accounts for 35-36% of total intracellular lysine, which suggests that the size of the sequestered pool may be determined by the lysine concentration of the exchanging compartment. This is consistent with the data given in Tables I and VII of NEIDLE et al. (1975) according to which the 19% increase in tissue soluble lysine levels that occurred during the second hour of incubation was accompanied by a 26% increase in the apparent sequestered lysine pool size. Other essential amino acids did not exhibit this behaviour.

FIG. 4.14 A BASIC MODEL FOR THE SIMULATION OF MOUSE BRAIN SLICE EXPERIMENTS



1) Input data based on the 120 min pool sizes
2) Input data based on the 60 min pool sizes

NEIDLE et al. (1975) observed that when mouse brain slices were incubated for 2h in a medium containing trace quantities of amino acids, the total soluble amino acid content of the system increased with time. This was attributed to an imbalance between the rate at which amino acids were incorporated into protein and their rate of release through proteolysis. They also found that the rate of amino acid release from protein was not constant, but was greater during the first hour of incubation than during the second hour. A computer simulation study of lysine compartmentation in mouse brain slices was performed using an open steady-state model to approximate to the nonsteady-state closed experimental system. The rate of lysine flow through the system ($F_{03} = F_{10}$) was set equal to the difference between the rate of lysine incorporation into protein and its rate of release through proteolysis. Simulation was performed only for the second hour of incubation, since the net release of lysine from protein and the consequent rate of expansion of the soluble lysine pool was slower during the second hour. Thus it was assumed that radioactivity was introduced at 60min and not at zero time. For a given rate of proteolysis, the quasi-equilibrium tissue: medium specific activity ratio is expected to be the same regardless of the time at which the tracer is introduced. Simulation for the second hour of incubation was performed using both the 60min and 120min pool sizes.

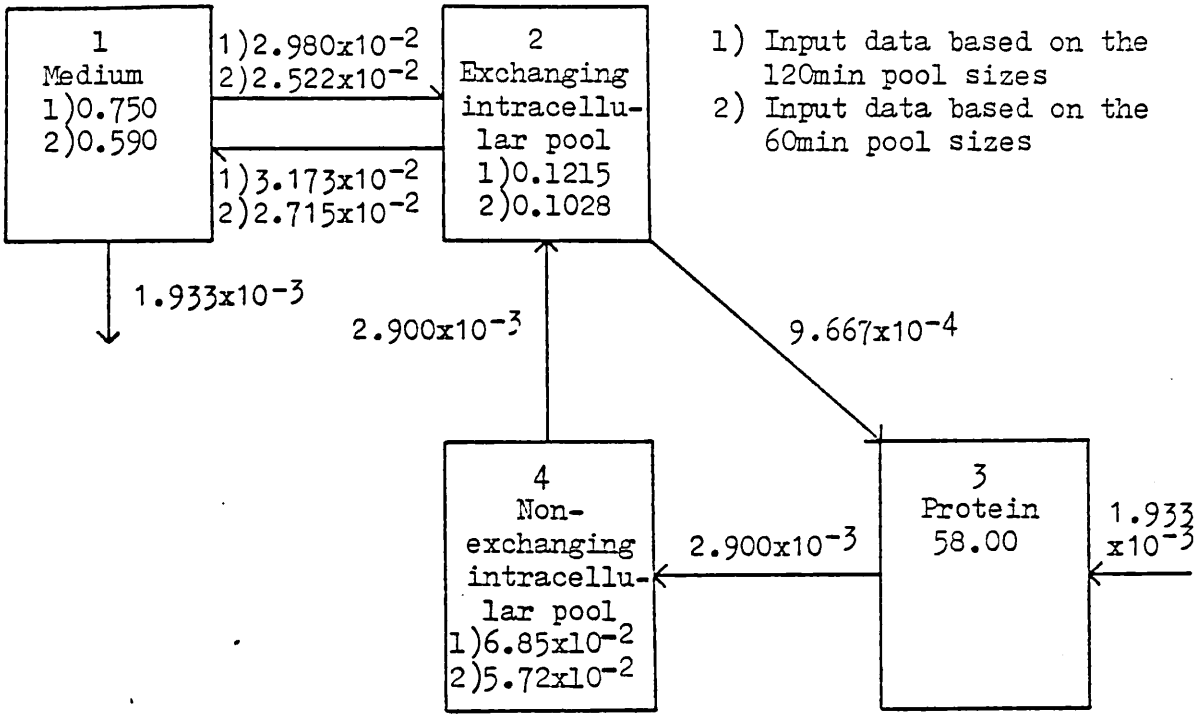
Input data were calculated per gramme of tissue on the basis of the following. Lysine content of mouse brain protein, 58 umole/g tissue (Table III of NEIDLE et al., 1975); soluble lysine content of the medium, 0.59 umole/g tissue at 1h and 0.75 umole/g tissue at 2h (Table I, ibid.); soluble lysine content of the tissue, 0.16 umole/g tissue at 1h and 0.19 umole/g tissue at 2h (Table I, ibid.); rate of lysine incorporation into protein, 0.1%/h (9.667×10^{-4} umole/min/g tissue) (ibid.); rate of lysine release from protein, 0.3%/h (2.9×10^{-3} umole/min/g tissue) (ibid.). The steady state fixes $F_{03}=F_{10}=F_{32}=F_{23}$.

Fig. 4.14 Continued.....

The rate of lysine uptake into the tissue was calculated assuming that the fractional rate of lysine uptake was the same as in rat brain slices (U_{12} = 0.2453 min^{-1}, see Fig. 4.12).

The theoretical quasi-equilibrium tissue: medium specific activity ratios obtained using the two sets of pool size data were 1) 95.3% for the 120min data and 2) 94.5% for the 60min data.

FIG. 4.15 A SUBCELLULAR MODEL OF LYSINE COMPARTMENTATION IN THE SLICED MOUSE BRAIN



The model shown in Fig. 4.14 was modified by the introduction of a nonexchanging intracellular lysine pool, the size of which was adjusted empirically to obtain a quasi-equilibrium tissue: medium lysine specific activity ratio of 58-59%. Flow rates F_{12} and $F_{21} = F_{12} + 1.933 \times 10^{-3}$ were calculated on the basis of a fractional uptake rate of 0.2453 min^{-1} (from Fig. 4.12). Other parameters are as in Fig. 4.14.

It is important to emphasise that because the simulation of mouse brain slice experiments was performed using a steady-state approximation to the nonsteady-state experimental system (see Figs. 4.14 and 4.15) the results must be regarded as approximate. The specific activity ratios obtained from the computer simulated data are, however, in agreement with those calculated using an equation of the form of (4.10) (data not given here). This indicates that, in the absence of nonexchanging compartments, the tissue : medium specific activity ratio is determined primarily by the relative rates at which lysine is supplied from the medium and from degraded protein, respectively. (This matter has been discussed in Section 4.2.1 in relation to the results of the in vivo infusion experiments, see Table 4.7). If this is the case, then other parameters used in the simulation will be of secondary importance, and so, the slow time-dependent expansion of the soluble pools will not significantly affect the results. It follows, however, that the computed specific activity ratios will be sensitive to error in the amino acid uptake rates. The present computations were performed using uptake rates based on measurements made in separate experiments and using rat brain slices. Clearly, a simulation based on specific activity ratios and uptake rates determined in the same experiment would be more reliable.

4.2.3 CONCLUDING REMARKS ON THE SIMULATION STUDY OF BRAIN SLICE AND ISOTOPIC LYSINE INFUSION EXPERIMENTS

In this section an attempt has been made to answer some questions concerning the compartmentation of lysine for protein synthesis in the brain. Some of these questions have been debated in the literature but, despite speculation, no reliable answers have been provided. These answers may be obtained only through some kind of kinetic analysis, and the work presented here represents a preliminary attempt at such an analysis. It has been emphasised that in some instances the mathematical treatment is far from refined, and the limitations of each model have been discussed. Although a more sophisticated treatment could have been employed it was felt that this was not justified. To take an example, in the simulation of the brain slice experiments

of Neidle and co-workers, a steady-state model is applied to a nonsteady-state system. If a more refined approach was sought two alternatives might be considered. Firstly, some nonsteady-state model might be employed, but this would necessitate making further approximations, since insufficient data are available to accurately define the time-dependent variables. Alternatively, the experiment could be repeated using conditions such that the steady-state model is applicable. Similarly, the simulation of the infusion experiments could be improved if additional experimental data were available. Accepting this to be the case, the work presented in this section represents an attempt to obtain preliminary answers using the available experimental data. It has been shown that the results obtained from brain slice experiments support the hypothesis that a nonexchanging intracellular lysine compartment is present in brain tissue. In contrast, the magnitude of the intracellular dilution observed by SETA et al. (1973) in the brain of the infused rat can be accounted for by a model that assumes the presence of only a single, exchanging intracellular lysine compartment. It should be noted that while the experimental confirmation of the presence of a nonexchanging intracellular lysine compartment in the intact brain would support the models of precursor lysine compartmentation outlined in Section 4.1, the models were formulated without specifying the size of the nonexchanging pool. The failure to obtain unequivocal evidence for its existence in vivo may merely indicate that the sequestered pool accounts for a small percentage of total intracellular lysine. One explanation for the apparent discrepancy between the results of the in vivo infusion experiments and the data obtained using brain slices is that the nonexchanging compartment does account for only a small percentage of total intracellular lysine in vivo, but that this compartment becomes grossly distorted in size when the tissue is sliced and incubated. Compatible with this hypothesis is the observation that mechanisms involved in the regulation of amino acid transport in the intact central nervous system are impaired in sliced tissue (NEIDLE et al., 1973).

4.3 AN ASSESSMENT OF THE METHODS USED FOR ESTIMATING COMPARATIVE RATES OF PROTEIN SYNTHESIS

In Section 1.2 a brief review is made of some of the methods most frequently used in the measurement of rates of *in vivo* protein synthesis. Although techniques have been devised for obtaining reliable estimates of absolute rates of amino acid incorporation into protein the procedures are relatively time consuming and in many studies absolute rates are not determined. Instead some parameter that may be used as an index of the absolute incorporation rate is measured. In the present study two parameters have been used, the relative incorporation and the apparent incorporation. Various properties of these two parameters are examined in this section making use of simulated lysine incorporation data generated using the homogeneous precursor lysine model outlined in Fig. 4.2. Although specific reference is made to the incorporation of lysine into brain protein, many of the following comments apply to any amino acid and any tissue.

The relative incorporation

The relative incorporation (I_{rel}) of an amino acid is defined by

$$I_{rel}(t=a) = R_j(t=a)/R_i(t=a) \qquad (1.14)$$

where R_j and R_i are the TCA-insoluble radioactivity and the TCA-soluble radioactivity, respectively, both measured at the end of the incorporation period (i.e., when t=a). The following examination of the properties of I_{rel} indicates why misleading results may be obtained when this parameter is used as an index of absolute rates of amino acid incorporation.

It is shown in Section 3.2.2.3 that I_{rel} is a reliable index of the absolute rate of amino acid incorporation only if within the group of m experimental animals

$$\overline{S_{pk}(t=a)} = C(t=a)\, R_{ik}(t=a) \qquad k = 1,2,\ldots,m \qquad (3.2.6)$$

where $\overline{S_{pk}(t=a)}$ is the time-average precursor specific activity for a given incorporation period and k denotes the kth animal of the group. (For the sake of clarity the subscript k is dropped from the equations which follow.) In the model outlined in Fig. 4.2 the precursor pool for brain protein synthesis is the main intracellular pool, ie., compartment 4. Now since

$$\overline{S_4(t=a)} = (1/Q_4 a)\int_o^a R_4 dt \tag{4.12}$$

(compare with equation 1.8) and

$$R_4 = \sum_{j=1}^{n} D_{4j} e^{-\lambda_j t} \tag{B.13c}$$

it follows that

$$C(t=a) = \frac{(1/Q_4 a)\left(\sum_{j=1}^{n}\int_o^a D_{4j} e^{-\lambda_j t} dt\right)}{\sum_{j=1}^{n} D_{4j} e^{-\lambda_j a}} \tag{4.13}$$

Substitution of (4.13) into (3.2.8.) yields

$$I_{rel}(t=a) = \frac{(F_{45}/Q_4)\left(\sum_{j=1}^{n}\int_o^a D_{4j} e^{-\lambda_j t} dt\right)}{\sum_{j=1}^{n} D_{4j} e^{-\lambda_j a}} \tag{4.14}$$

where F_{45} is the rate at which the amino acid is incorporated into brain protein.

Two points emerge from an examination of equation (4.14). Firstly, it is evident that regardless of whether or not the absolute rate of incorporation is time independent, no simple relationship can be expected between the numerical value of I_{rel} obtained at different times after the administration of the tracer and that the relative incorporation rate defined by

$$R_{rel} = I_{rel}/a \qquad (3.1.1)$$

will be time dependent.* For this reason, although I_{rel} (or R_{rel}) may be a useful index of comparative rates of protein synthesis in a given tissue within well matched groups of animals, if measurements are made at intervals of time it is not meaningful to present the data as I_{rel} versus time plots. The same argument applies to incorporation data expressed as a percentage of total tissue radioactivity, although plots of 100(TCA-insoluble radioactivity/total homogenate radioactivity) versus time are not infrequently seen in the literature.

*In one experiment in which TCA-insoluble and TCA-soluble radioactivity measurements were made 15,30 and 45min after the subcutaneous administration of L-[G-^{3}H]valine the relative incorporation rate was found to increase throughout the 45min incorporation period (Table 3.1.3). The same time-dependent increase is observed in the relative incorporation rate calculated from the theoretical data generated by the model outlined in Fig. 4.2. This phenomenon may be explained as follows. From equations (3.1.1), (4.14), (4.12) and (B.13c) it can be shown that

$$R_{rel} = \frac{F_{45}\bar{S}_4}{R_4} . \qquad (4.15)$$

(This equation can also be derived from (1.9), (1.14) and (3.1.1).) An examination of the theoretical data given in Table 4.8 reveals that in the time interval during which both $\bar{S}_4$ and R_4 increase with time, the rate of increase of $\bar{S}_4$ is greater than that of R_4 and hence R_{rel} increases. After both $\bar{S}_4$ and R_4 have reached their maxima and start to decline, R_4 decreases with the greater rapidity and hence R_{rel} continues to increase. It is clear that although TCA-soluble radioactivity may be a reliable index of the time-average precursor specific activity in a situation in which the duration of the incorporation period is kept constant R_4 cannot be used as an index of $\bar{S}_4$ if TCA-insoluble radioactivity measurements are made at intervals of time. This assertion does, of course, follow directly from equations (3.2.6) and (4.13).

The second point which must be made concerning equation (4.14) is that the eigenvalues λ_j, $j = 1,2,\ldots,n$ and the eigenvector components D_{4j}, $j = 1,2,\ldots,n$ are sensitive to changes in any part of the system. Consequently, I_{rel} will be affected by altered amino acid transport rates and pool sizes, even at some remote site, and must not therefore be treated as a linear function of the amino acid incorporation rate (F_{45}) unless it is known that other parameters do not change significantly. In fact, a change in F_{45} will itself be accompanied by a change in the above eigenvalues and eigenvector components although the effect is not expected to be significant. I_{rel} will be particularly sensitive to changes in the size of the soluble amino acid pool in the tissue in question and the rate of tracer uptake into that pool. This may be demonstrated by computer simulation. To this end the model outlined in Fig. 4.2 was used to simulate the effect of (a) a 50% decrease in the rate of lysine uptake into the brain and (b) a 100% expansion of the brain soluble lysine pool. In both cases the rate of lysine incorporation into brain protein was kept constant. Theoretical lysine relative incorporation data calculated from the simulated TCA-insoluble and TCA-soluble radioactivity data are given in Table 4.8. The 100% expansion in the size of the brain soluble lysine pool is accompanied by a decrease in the time-average specific activity of this pool but, because this is not reflected by a corresponding decrease in $R_4(t=a)$, the expansion causes a decrease in I_{rel} (relative to the control level), the magnitude of which increases as the incorporation time increases. A considerable error would therefore result from the use of this parameter as an index of the absolute lysine incorporation rate, the error becoming greater as the duration of the incorporation period increases. A 50% decrease in the rate of lysine uptake into the brain is accompanied by a decrease in the time-average specific activity of the brain soluble lysine pool that is of a similar magnitude to that resulting from a 100% expansion in its size. In this case, however, the reduced time-average specific activity is partially reflected by a reduction in $R_4(t=a)$ and consequently I_{rel} is a better index of the absolute incorporation rate.

TABLE 4.8 AN ASSESSMENT OF THE METHODS USED FOR ESTIMATING COMPARATIVE RATES OF PROTEIN SYNTHESIS

A. Control

Time (a) (min)	Radioactive content of protein (R_5) (units)	Final soluble radioactivity (R_4) (units)	Final precursor specific activity (S_4) (units/umole)	Time-average precursor specific activity ($\bar{S}_4$) (units/umole)	Relative incorporation (R_5/R_4)	Apparent incorporation (R_5/S_4) (umole)	Absolute incorporation ($R_5/\bar{S}_4$) (umole)
2	8.03×10^{-5}	2.25×10^{-3}	3.84×10^{-2}	2.09×10^{-2}	3.57×10^{-2}	2.09×10^{-3}	3.84×10^{-3}
5	3.95×10^{-4}	3.90×10^{-3}	6.64×10^{-2}	4.12×10^{-2}	0.101	5.95×10^{-3}	9.59×10^{-3}
10	1.10×10^{-3}	4.39×10^{-3}	7.47×10^{-2}	5.71×10^{-2}	0.251	1.47×10^{-2}	1.93×10^{-2}
20	2.35×10^{-3}	3.20×10^{-3}	5.44×10^{-2}	6.13×10^{-2}	0.734	4.32×10^{-2}	3.83×10^{-2}
30	3.21×10^{-3}	2.14×10^{-3}	3.64×10^{-2}	5.58×10^{-2}	1.50	8.82×10^{-2}	5.75×10^{-2}
40	3.80×10^{-3}	1.56×10^{-3}	2.65×10^{-2}	4.96×10^{-2}	2.44	0.143	7.66×10^{-2}

Table 4.8 Continued.....(2)

B. The effect of a 50% decrease in the rate of lysine uptake into the brain

Time (a) (min)	Radioactive content of protein (R_5) (units)	Final soluble radioactivity (R_4) (units)	Final precursor specific activity (S_4) (units/umole)	Time-average precursor specific activity ($\bar{S}_4$) (units/umole)	Relative incorporation (R_5/R_4)	Apparent incorporation (R_5/S_4) (umole)	Absolute incorporation ($R_5/\bar{S}_4$) (umole)
2	4.07×10^{-5}	1.15×10^{-3}	1.96×10^{-2}	1.06×10^{-2}	3.54×10^{-2} (99)	2.08×10^{-3} (>99)	3.84×10^{-3}
5	2.05×10^{-4}	2.07×10^{-3}	3.52×10^{-2}	2.13×10^{-2}	9.90×10^{-2} (98)	5.82×10^{-3} (98)	9.62×10^{-3}
10	5.88×10^{-4}	2.48×10^{-3}	4.22×10^{-2}	3.06×10^{-2}	0.237 (94)	1.39×10^{-2} (95)	1.92×10^{-2}
20	1.35×10^{-3}	2.07×10^{-3}	3.52×10^{-2}	3.51×10^{-2}	0.652 (89)	3.84×10^{-2} (89)	3.85×10^{-2}
30	1.93×10^{-3}	1.55×10^{-3}	2.64×10^{-2}	3.36×10^{-2}	1.25 (83)	7.31×10^{-2} (83)	5.74×10^{-2}
40	2.38×10^{-3}	1.20×10^{-3}	2.05×10^{-2}	3.10×10^{-2}	1.98 (81)	0.116 (81)	7.68×10^{-2}

Table 4.8 Continued.....(3)

C. The effect of a 100% expansion of the brain soluble lysine pool

Time (a) (min)	Radioactive content of protein (R_5) (units)	Final soluble radioactivity (R_4) (units)	Final precursor specific activity (S_4) (units/umole)	Time-average precursor specific activity ($\overline{S}_4$) (units/umole)	Relative incorporation (R_5/R_4)	Apparent incorporation (R_5/S_4) (umole)	Absolute incorporation ($R_5/\overline{S}_4$) (umole)
2	4.11×10^{-5}	2.34×10^{-3}	1.99×10^{-2}	1.07×10^{-2}	1.76×10^{-2} (49)	2.07×10^{-3} (99)	3.84×10^{-3}
5	2.10×10^{-4}	4.32×10^{-3}	3.67×10^{-2}	2.19×10^{-2}	4.86×10^{-2} (48)	5.72×10^{-3} (96)	9.59×10^{-3}
10	6.22×10^{-4}	5.45×10^{-3}	4.64×10^{-2}	3.24×10^{-2}	0.114 (45)	1.34×10^{-2} (91)	1.92×10^{-2}
20	1.50×10^{-3}	5.10×10^{-3}	4.34×10^{-2}	3.92×10^{-2}	0.294 (40)	3.46×10^{-2} (80)	3.83×10^{-2}
30	2.27×10^{-3}	4.25×10^{-3}	3.62×10^{-2}	3.94×10^{-2}	0.534 (36)	6.27×10^{-2} (71)	5.76×10^{-2}
40	2.90×10^{-3}	3.57×10^{-3}	3.04×10^{-2}	3.78×10^{-2}	0.812 (33)	9.54×10^{-2} (67)	7.67×10^{-2}

Table 4.8 Continued.....(4)

The following parameters were calculated using the theoretical data given by the homogeneous precursor lysine model outlined in Fig. 4.2 (intravenous injection). 1) The relative incorporation as defined by equation (1.14), 2) the apparent incorporation as defined by (1.13), 3) the time-average precursor specific activity ($\overline{S}_4$) which was calculated using

$$\overline{S} = (1/Q_4 a) \int_o^a R_4(t)dt \qquad (4.19a)$$

$$= (1/Q_4 a) \sum_{j=1}^{n} \int_o^a D_{4j} e^{-\lambda_j t} dt) \qquad (4.19b)$$

and 4) the absolute incorporation ($R_5/\overline{S}_4$). A) Control data. B) The perturbation caused by a 50% reduction in the rate of lysine uptake into the brain soluble pool. C) The perturbation caused by a 100% increase in brain soluble lysine. The figures given in parenthesis in (B) and (C) are the relative and apparent incorporation data expressed as a percentage of the corresponding control data.

RICHARDSON and ROSE (1971) have stated that a partial correction for variations in the precursor pool size is expected if the incorporation of radioactivity is expressed in terms of the relative specific activity (RSA) defined as the TCA-insoluble radioactivity divided by the total homogenate radioactivity. An inspection of the figures given in Table 4.8 reveals that this assertion may be unfounded since, according to these simulated data, RSA is no better than TCA-insoluble radioactivity as an index of protein synthetic activity in a situation in which the size of the soluble amino acid pool in the tissue in question is the only variable. Inter-animal variation in tracer uptake rates is, however, partially accounted for by expressing the incorporation data in terms of RSA.

The apparent incorporation

The apparent incorporation is defined by

$$I_{app}(t=a) = R_j(t=a)/S_i(t=a) \qquad (1.13)$$

where R_j and S_i are the TCA-insoluble radioactivity and the specific activity of the TCA-soluble pool in the tissue in question, both measured at the end of the incorporation period. Theoretical brain lysine incorporation data generated by the model given in Fig. 4.2 are used below to demonstrate some of the properties of I_{app} and so it is again convenient to make the substitution i=4 and j=5. It follows from equations (B.13c) and (1.8) that the proportionality factor of (3.2.12) is given by

$$b(t=a) = (1/a)\,\frac{\left(\sum_{j=1}^{n} \int_0^a D_{4j} e^{-\lambda_j t} dt\right)}{\sum_{j=1}^{n} D_{4j} e^{-\lambda_j a}} \qquad (4.16)$$

Since from (3.2.14)

$$I_{app} = F_{45}{}^{ba} \tag{4.17}$$

it follows that

$$I_{app} = \frac{F_{45}\left(\sum_{j=1}^{n} \int_{o}^{a} D_{4j} e^{-\lambda_j t} dt\right)}{\sum_{j=1}^{n} D_{4j} e^{-\lambda_j a}} \tag{4.18a}$$

$$= I_{rel}/Q_4 . \tag{4.18b}$$

Equation (4.18b) may, of course, be derived directly from (1.13) and (1.14). Equation (4.18a) serves to remind us, however, that, as with I_{rel}, $I_{app}(t=a)$ can only be treated as a linear function of the absolute incorporation rate if all other parameters are constant, and that regardless of whether or not F_{45} is time independent, I_{app} is not a linear function of time. Secondly, it follows from (4.18b) (or from (4.14) and (4.18a)) that only if the size of the soluble amino acid pool in the tissue in question is variable is I_{app} better than I_{rel} as an index of the absolute incorporation rate, assuming, of course, that the soluble pool is not contaminated with radioactive amino acid metabolites. This point may again be demonstrated using computer simulated data. Thus it can be seen from the theoretical results given in Table 4.8 that although an expansion in the size of the soluble amino acid pool causes a time-dependent decrease in I_{app}, this parameter is better than I_{rel} as an index of the absolute rate of amino acid incorporation. In contrast, the percentage decrease in I_{app} caused by a decrease in the rate at which the tracer enters the soluble pool is the same as the resulting percentage decrease in I_{rel}.

To summarise, I_{rel} and I_{app} are reliable indices of the absolute

incorporation rate only if all parameters excepting the incorporation rate itself, are constant. The use of these parameters in situations in which this requirement is not met may lead to considerable error.

NOTE ADDED IN PROOF

COULSON and HART (1977) have outlined a procedure by which amino acid turnover and incorporation rates may be evaluated through a graphical analysis of the data obtained from tracer experiments. The method, which requires that the appearance of radioactivity in protein and the loss of radioactive amino acid from the tissue soluble pool is monitored following tracer administration, was applied to the analysis of the data obtained from a series of experiments in which rats were injected intraventricularly with a number of radioactive amino acids, including lysine. A half-life for the brain soluble lysine pool of 16-19 min was obtained.

A direct comparison of the above result with the $t_{\frac{1}{2}}$ data given in Section 4.1.3 is not meaningful because the half-lives calculated using equations of the form of (4.2) depend upon which routes of outflow are considered in the calculation. (The uptake of amino acid into the protein-bound pool is often neglected.) In contrast, the graphical method is expected to yield an "absolute" value for $t_{\frac{1}{2}}$. A comparison of the results obtained in different studies may, however, be made by calculating the fractional uptake rates (see equation (4.11)).

It is easily shown from equation (1) of COULSON and HART (1977) that

$$t_{\frac{1}{2}} = \ln 2 / K \qquad (4.20)$$

where

$$K = (K_{+l} + K_{+x} + K_{+p}) \qquad (4.21)$$

and K_{+l}, K_{+x} and K_{+p} are the rate constants for amino acid flow out of the brain, metabolism and incorporation into protein, respectively. Lysine is degraded at a relatively slow rate in the brain (CHANG, 1976) and hence K_{+x} may be neglected. Equation (4.21) may then be rewritten in accordance with the scheme outlined in Fig. 4.2 and equation (B.2).

Thus

$$K = (F_{41} + F_{45})/Q_4 \cdot \qquad (4.22)$$

It therefore follows that

$$F_{41} = KQ_4 - F_{45} \cdot \qquad (4.23)$$

According to equation (4.23) and the data given in Table 1 of COULSON and HART (1977), lysine is transported out of the brain at a rate of 5.4 - 6.8 nmole/min/g tissue. Given a steady state this must equal the rate of lysine uptake and compares with the simulated uptake rate of 8.3 nmole/min/g tissue. Substitution into equation (4.11) yields the following fractional uptake rates: 4.1×10^{-2} min^{-1}, based on the simulated uptake rate; 2.6×10^{-2} - 3.3×10^{-2} min^{-1}, based on the data of COULSON and HART (1977); 2.4×10^{-2} - 3.4×10^{-2} min^{-1}, from the data given in Table 1 of TOTH and LAJTHA (1977).

Substitution into (4.22) of the lysine data given in Fig. 4.2 yields a theoretical value for K of 7.36×10^{-2} min^{-1} which, on substitution into (4.20), gives a theoretical half-life of 9.4min. The experimentally determined values of K^{lysine} and $t_{\frac{1}{2}}^{lysine}$ are 3.6×10^{-2} - 4.3×10^{-2} min^{-1} and 16-19 min, respectively. The discrepancy between the theoretical and experimental data arises from the difference between the lysine incorporation rate used in the simulation and that observed by Coulson and Hart. The simulation was performed using an incorporation rate of 6.6 nmole/min/g tissue (equivalent to a replacement rate of 0.685 %/h, which is taken from Table 4 of DUNLOP et al., 1975a) while according to the above researchers lysine is incorporated into protein at a rate of 2.2 nmole/min/g tissue (equivalent to a replacement rate of 0.26 %/h, assuming the lysine content of rat brain protein to be 50 umole/g tissue (Table IV of LAJTHA and TOTH, 1974)).

5 DISCUSSION

5.1 THE MEASUREMENT OF RATES OF CEREBRAL PROTEIN SYNTHESIS

The purpose of the present study was to determine whether L-dopa has an effect on the protein synthetic activity of the brain, as indicated by the tissue's capacity for incorporating radioactive amino acids into TCA-insoluble material. Despite the apparent simplicity of the problem, the measurement of rates of brain protein synthesis with amino acid tracers is fraught with difficulties some of which are discussed in Section 1.2. Absolute rates may be calculated only if the precursor amino acid specific activity is known throughout the incorporation period, and since little is known concerning the compartmentation of amino acids for protein synthesis in brain, rates calculated from radioactive incorporation data must always be intrinsically uncertain. This problem is accentuated as a result of the structural heterogeneity of the brain. Thus amino acids may be compartmented at the subcellular level, the cellular level and the regional level. In order to simplify matters, methods have been devised for maintaining the specific activity of soluble amino acid pools at a constant level over prolonged periods of time. This may be achieved either by the continuous infusion of radioactive amino acids (GARLICK and MARSHALL, 1972; SETA et al., 1973) or through the injection of a quantity of the labelled precursor sufficient to swamp the endogenous pools (GAITONDE and RICHTER, 1956; DUNLOP et al., 1975a). It is still necessary to assume, however, that all amino acid pools equilibrate with sufficient rapidity that the specific activity of the precursor amino acid pool and the average soluble amino acid specific activity are the same. This may not always be the case. For example, it is shown in Section 4.2 that during the continuous infusion of radioactive lysine, the release of cold lysine from degraded protein probably causes a significant dilution of the intracellular pool. Consequently, if the rate at which protein is degraded is not the same in different cell types, then the magnitude of the intracellular dilution will vary, which might in turn, cause the precursor specific activity to vary between different cell types. Even assuming that using the above techniques reliable rates of amino

acid incorporation may be obtained, it must be remembered that these rates represent averages of a spectrum of incorporation rates into different protein fractions in a heterogeneous population of cells.

Measurement of time-average amino acid specific activity ($\bar{S}$) following the pulse administration of tracers is a very time consuming business which must involve the use of large numbers of animals. Consequently it is seldom attempted. Instead, the final specific activity of the soluble amino acid pool is often used as an index of the time-average specific activity. Alternatively, TCA-soluble radioactivity is sometimes used as an index of this parameter. Thus two variables were defined in Section 1.2, firstly the relative incorporation (I_{rel}) which is the final TCA-insoluble radioactivity divided by the final TCA-soluble radioactivity and secondly, the apparent incorporation (I_{app}) which is the final TCA-insoluble radioactivity divided by the final specific activity of the soluble amino acid pool. Providing the requirements outlined below are met, these two parameters provide a means by which inter-animal variability in precursor specific activity may be partially corrected for, and it has been shown experimentally that this correction procedure is useful as a means of reducing scatter in the incorporation data (see Section 3.1.2). On the other hand, if absolute incorporation rates are calculated on the basis of the time-average specific activity determined from measurements made in a group of animals, no correction is made for inter-animal variation.

According to equations (3.2.6) - (3.2.8) I_{rel} can be a reliable index of the absolute incorporation rate only if the time-average precursor specific activity ($\bar{S}_p$) and the final TCA-soluble radioactivity are linearly correlated within the group of experimental animals. Assuming that inter-animal variability in $\bar{S}_p$ is greater than the variability in the rate at which amino acids are incorporated into protein, TCA-insoluble and TCA-soluble radioactivity should be correlated within a group of matched animals. It is therefore suggested that the experimental data be subjected to some kind of analysis to ensure that such

a correlation does exist. If no such correlation is observed I_{rel} should not be used as an index of the absolute rate of amino acid incorporation. Similarly, I_{app} should be used as an index of incorporation rates only if a correlation between TCA-insoluble radioactivity and the specific activity of the soluble amino acid pool is demonstrable. Although it is possible to test the validity of using I_{rel} and I_{app} as indices of incorporation rates within a group of animals (eg., the control group), the assumption that differences in the mean relative and apparent incorporation data obtained for different groups is indicative of altered rates of protein synthesis is only verifiable through further experimentation.

Some of the pitfalls that may accompany the use of I_{app} and I_{rel} have been demonstrated both theoretically (Section 4.3) and experimentally (Section 3.2.2). It is shown using simulated data that in a situation in which amino acid uptake rates are altered by the experimental procedure (eg. drug administration) but amino acid pool sizes remain constant I_{app} is no better than I_{rel} as an index of rates of protein synthesis. It is also shown that I_{rel} and I_{app} bear no simple relationship to any <u>single</u> physiological parameter. For that reason they can only be used as indices of rates of protein synthesis in closely matched groups of animals. They cannot, for example, be used to make comparison between rates of amino acid incorporation in different tissues, between the same tissue of animals of different ages, etc. Further, no simple relationship can be expected between the numerical value of I_{rel} determined at different times after the administration of the tracer. The same applies to apparent incorporation data.

RICHARDSON and ROSE (1971) and ROEL <u>et al.</u> (1974, see Fig. 2) use total homogenate radioactivity as an index of $\overline{S}_p$. Clearly all the above arguments apply in this situation. Roel and co-workers have commented on their observation that when brain incorporation data is expressed as a percentage of total homogenate radioactivity, linear incorporation-

time curves were obtained if radioactive lysine was used as a tracer but not when radioactive leucine was employed. They attributed this to leucine being rapidly metabolised in brain while lysine is not. Although this is clearly in part true, many other factors determine the shape of these curves not least of which is the mode of precursor administration. Thus the observation that brain lysine incorporation is a linear function of time when it is expressed as a percentage of total homogenate radioactivity is of no obvious physiological significance. This matter is discussed further in Section 5.2 in relation to the interpretation of the data obtained by Roel and co-workers.

When designing experiments in which I_{app} or I_{rel} are to be employed as indices of protein synthetic activity, it should be remembered that it is particularly desirable to keep incorporation periods short, since not only is the steady-state approximation more likely to be valid and the release of protein-bound radioactivity negligible, but the more likely it is that the final specific activity of the soluble amino acid pool and the final radioactive content of this pool are reliable indices of the time-average specific activity within the group of experimental animals (see Table 4.8). Often, however, some compromise must be accepted between the desirability of using short incorporation periods and other considerations. For example, following a short labelling period the level of radioactivity present in all but the most metabolically active of proteins may not be measureable.

Amino acid metabolism

It is important that the labelled amino acids used in the measurement of protein synthetic rates be chosen with care, since it is essential that radioactivity does not become incorporated into TCA-insoluble material in the form of amino acid metabolites. Further, if soluble radioactivity is used as an index of precursor specific activity a tracer amino acid should be chosen which does not give rise to soluble

radioactive metabolites. The position of labelling is as important as the amino acid itself. In the present study tritiated amino acids were used in the majority of experiments because they are relatively inexpensive. L-[G-^{3}H]valine and L-[4,5-^{3}H]lysine were selected on the basis of reports that their only major radioactive metabolite is water* (see Section 3.1.3). Having also decided upon the subcutaneous mode of administration and a maximum incorporation time of 15min, the brain and liver content of radiochemical impurities 15min after the subcutaneous administration of these two labelled amino acids was determined. The estimated radiochemical purity of dried TCA-soluble extracts of brain and liver was 92% and 76%, respectively, for L-[4,5-^{3}H]lysine, and 85% and 84% for L-[G-^{3}H]valine.

5.2 THE EFFECT OF L-DOPA ON THE INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO BRAIN AND LIVER TCA-INSOLUBLE MATERIAL

Preliminary experiments were performed to substantiate the report that L-dopa inhibits the incorporation of radioactive amino acids into brain protein *in vivo* (ROEL *et al.*, 1974). L-dopa (500mg/kg, ip.) was administered 45min before the subcutaneous injection of L-[^{3}H]lysine or L-[^{3}H]valine and the animals killed after a 15min incorporation period. In the first instance the incorporation of radioactivity was expressed in terms of I_{rel}. In order to obtain some indication as to whether the response was brain specific the effect of L-dopa on the incorporation of radioactive amino acids into liver TCA-insoluble material was also determined. It was recognised, however, that while brain amino acid levels are relatively constant, this is certainly not true of liver, and that I_{rel} cannot therefore be regarded as a

*The appearance of $^{3}H_2O$ after the administration of tritiated amino acids is often attributed to tritium exchange (see, for example, ROSE and SINHA, 1974) but this is probably not so, since the rate of chemical exchange between nonacidic aliphatic protons and water under physiological conditions is not expected to be significant (<1% over a period of months). The formation of $^{3}H_2O$ is presumably the result of amino acid metabolism. If, however, during the course of this metabolism a given tritium atom becomes acidic (eg. adjacent to a carbonyl group) then a percentage will be lost through exchange.

reliable index of absolute rates of amino acid incorporation into liver protein (see Section 4.3). In two experiments L-dopa caused a 70-76% decrease in the relative incorporation of brain lysine. The observation that the drug also caused a 44-80% decrease in the relative incorporation of lysine in the liver indicates that the effect was not brain specific.

In those experiments in which L-[^{3}H]valine was used as the tracer, variable responses in the brain were observed when the incorporation was expressed in terms of I_{rel}. In one experiment L-dopa had no effect, while in two other experiments the drug caused decreases of 22 and 47%, respectively, although neither decrease was statistically significant. In the liver L-dopa caused decreases in I_{rel} of 6, 43 and 14% in the three experiments, respectively, the second of which was statistically significant.

It is suggested above (Section 5.1) that if no correlation exists between TCA-insoluble and TCA-soluble radioactivity within a group of matched animals then I_{rel} cannot be a reliable index of the absolute rate of amino acid incorporation. No correlation was observed between brain TCA-insoluble and soluble radioactivity within the control groups of rats administered [^{3}H]valine, indicating that the valine relative incorporation rates cannot be relied upon as reflecting the absolute rates of incorporation into protein. This would explain the variability between the three replicate experiments. In order to obtain a more reliable indication of the effect of L-dopa on the protein synthetic activity of the brain, amino acid specific activity measurements were made and the incorporation data expressed in terms of I_{app}. No significant discrepancy was observed between the brain apparent incorporation data obtained from two separate valine experiments, which is consistent with I_{app} being more reliable than I_{rel} as an index of absolute amino acid incorporation rates. The observed correlation between TCA-insoluble radioactivity and the final specific activity of the brain soluble valine pool within the control groups of

rats substantiates this conclusion and suggests that inter-animal variability in the size of the soluble valine pool contributed to the variability in the relative incorporation data. L-dopa had no significant effect on the apparent incorporation of cerebral valine.

L-dopa caused a 55-59% decrease in the apparent incorporation of brain lysine compared with the 70-76% decrease in I_{rel}. Inspection of the data given in Tables 3.2.9 and 3.2.10 indicates that the drug-induced decrease in the final specific activity of the brain soluble lysine pool was not reflected by a similar decrease in soluble radioactivity, hence the discrepancy between the relative and apparent incorporation data. It is argued in Section 3.2.2.7 that this may have resulted from an accumulation of involatile radioactive lysine metabolites in the brains of L-dopa-treated rats. However, no experiments were performed to substantiate this proposal.

The discrepancy between the results obtained with valine and lysine is not eradicated when the incorporation data is expressed in terms of I_{app}, indicating that the apparent incorporation of one or both amino acids does not give a true indication of the absolute rate of protein synthesis. As explained in Section 3.2.2.6 the use of incorporation periods of different durations was expected to through some light on the problem and additional lysine experiments were therefore performed using a $7\frac{1}{2}$min incorporation period. In these experiments L-dopa had no significant effect on the apparent incorporation of brain lysine. On the basis of this observation and the results of the valine experiments it is proposed that L-dopa has little or no effect on the rate of brain protein synthesis. The 55-59% L-dopa-induced decrease in the apparent incorporation of lysine observed after a 15min incorporation period is explained in terms of precursor lysine compartmentation. Two models of lysine compartmentation are outlined in detail in Section 4.1. Essentially these models assume that lysine enters cerebral protein via two precursor pools which equilibrate with plasma lysine at different rates. It is assumed that in the absence of L-dopa, one precursor compartment is the main intracellular pool, or exchanges

rapidly with that pool, but that L-dopa blocks this route of lysine entry into protein. It is postulated that in order that protein synthesis may continue unimpaired the deficit is derived from some sequestered intracellular lysine pool.

After the initial series of *in vivo* experiments had been completed MOSKOWITZ *et al.* (1977) published their work indicating that the effect of L-dopa on rat cerebral polysomes is dependent upon the temperature at which the animals are maintained after drug administration. Thus an L-dopa-induced disaggregation of cerebral polysomes was observed in animals kept at 26°C but not in those maintained at 10° or 18°C. L-dopa caused hyperthermia in rats kept at 26°C, had little effect on body temperature at 18°C and caused hypothermia at 10°C, indicating that body temperature might be important with respect to the drug effect on polysomes. Hyperthermia caused through an elevation of ambient temperature was not as effective as drug-induced hyperthermia in causing polysome disruption, and it was therefore suggested that drug-induced hyperthermia plays a "permissive role" in polysome disaggregation.

During the present series of *in vivo* experiments it was noticed that some rats failed to exhibit any definite behavioural response to L-dopa. Further, non-responding L-dopa-treated rats injected with L-[^{3}H]lysine failed to exhibit the same gross reduction in I_{rel} 15min after tracer administration that was observed in responding rats. Although it was originally assumed that L-dopa failed to have an effect as a result of its faulty intraperitoneal administration, an alternative explanation was later considered, namely, that there exists a critical temperature range, above 19°C but below 24°C, within which some L-dopa-treated rats become hyperthermic, exhibit behavioural changes and, providing the incorporation period is not too short, a reduced relative lysine incorporation rate, while other rats fail to become hyperthermic, fail to exhibit a behavioural response and incorporate radioactive lysine at the control rate. Support for the latter hypothesis was afforded by two observations. Firstly, subsequent experimentation confirmed that the L-dopa-induced behavioural response

is temperature dependent. Thus rats maintained at 10°C normally exhibited only moderate stereotypy with occasional slight hyperactivity, while animals kept at 26°C were intensely hyperactive for prolonged periods of time. Further, the '10°C rats' showed no permanent ill effects, while those maintained at 26°C died within 3½h of L-dopa administration. The latter hypothesis is also supported by the observation that L-dopa had no significant effect on the relative incorporation of lysine in rats kept at 10°C. It remains to be established, however, whether the apparent insensitivity of some rats to L-dopa was simply the result of faulty administration or whether the alternative hypothesis outlined above is correct. Clearly, information on body temperature and tissue L-dopa levels in responding and non-responding rats is required before this matter can be resolved.

The observation that L-dopa had no significant effect on the relative incorporation of brain lysine in rats maintained at 10°C appears to be consistent with the results obtained by MOSKOWITZ et al. (1977) and could be interpreted as indicating that both the polysome disaggregation and the reduced relative lysine incorporation seen in L-dopa-treated rats maintained at temperatures above say 23°C is due to an inhibition of protein synthesis. Although this interpretation is at variance with the observation that the apparent incorporation of [^{3}H] valine was not significantly affected by the drug, ambient temperature was not controlled during these experiments, and it could therefore be argued that L-dopa had no effect on valine incorporation because, by chance, the temperature at which these valine experiments were carried out was low. To explain the gross L-dopa-induced inhibition of lysine incorporation observed after a 15min incorporation period compared with the relatively smaller effect seen at 7½min it must be postulated that the temperature at which the 7½min lysine experiments were performed was also low. This was considered very unlikely because repeat experiments were carried out at different times of the year, and since consistent effects were observed in duplicate experiments this would imply a considerable coincidence. Nevertheless, additional experiments were performed to investigate the effect of L-dopa on valine incorporation in rats maintained at 24-26°C, the temperature at which the drug

is reported to consistently disaggregate cerebral polysomes. In order to obtain reliable estimates of absolute rates of cerebral protein synthesis the flooding technique of DUNLOP et al. (1975a) was adopted. In one experiment L-dopa had no effect on valine incorporation into brain protein while in a second experiment the drug caused a non-significant 30% inhibition. Experiments were also carried out to test the effect of L-dopa on the incorporation of radioactivity into protein 15min after the administration of trace quantities of L-[4,5-^{3}H]lysine to rats maintained at 23-25°C. Although the L-dopa-induced decrease in the relative incorporation of brain lysine at 23-25°C (50-59%) was not as great as that observed at room temperature (70-76%) the difference between the 25°C and the room temperature I_{rel} data was not statistically significant. Had the difference been significant this would not necessarily have indicated that the rate at which lysine was incorporated into brain protein was altered by a change in ambient temperature or that the compartmentation of brain precursor lysine was affected, since changes in a number of other variables, even at some remote site, may lead to altered relative incorporation rates in the brain (see Section 4.3).

The results of the 23-26°C experiments were qualitatively the same as those obtained at room temperature, and while the possibility that L-dopa causes a small inhibition of brain protein synthesis can neither be confirmed nor ruled out, the results do indicate that the gross L-dopa-induced decrease in the incorporation of radioactive lysine into rat brain protein must, at least in part, be caused by some other mechanism. This interpretation assumes, of course, that L-dopa does not specifically inhibit the turnover of a protein fraction that has a particularly large capacity for incorporating radioactive lysine and/or a low capacity for incorporating radioactive valine. Although this assumption may appear reasonable, it should be noted that in a study of the incorporation of tritiated amino acids into protein in neurons of the dorsal column nuclei, BERKLEY et al. (1977) found that while small cells incorporated [^{3}H]proline more efficiently than [^{3}H]leucine, larger cells incor-

porated [^{3}H]leucine more efficiently than [^{3}H]proline. Berkley and co-workers suggested a number of explanations for their data including 1) a difference in the amino acid composition of protein in different cell types, 2) cell type differences in free amino acid pools and their relation to the protein synthesizing machinery, 3) differences between cell types with respect to the turnover rate of protein rich (or deficient) in the amino acids in question and 4) differences between the permeability to amino acids of different cell types. On the basis of the observations of Berkley and co-workers the possibility that L-dopa inhibits protein synthesis in a population of cells which incorporate radioactive lysine efficiently but have little capacity for incorporating radioactive valine should be considered. It should be noted that if the first of the above mechanisms is to be implicated as a possible cause of the difference between the effect of L-dopa on the incorporation of radioactive valine and lysine, it is only necessary to postulate that the amino acid composition of rapidly synthesized protein varies between different cell types. This follows because a substantial percentage of a tissue's capacity for amino acid incorporation is expected to be accounted for by a small percentage of total protein that has a shorter than average half-life (see Fig. 4.7). The difference between the 7½min and 15min lysine experiments would be explained if the precursor lysine pool in those cells sensitive to L-dopa equilibrates with plasma lysine slowly compared with the rate of equilibration between plasma lysine and the precursor pool in L-dopa-insensitive cells.* No experiments were performed to investigate any of these possibilities, although

*The cellular model of lysine compartmentation outlined in Fig. 4.5 may be modified in accordance with these ideas. Thus it may be assumed that the interrupted flow of lysine from the main intracellular pool (4) into protein compartment 5 is not compensated for by the reutilization of lysine released from degraded protein and that the protein synthetic capacity of compartment 5 is therefore abolished. It may also be assumed that protein compartment 7 has a much larger capacity than compartment 5 for incorporating radioactive valine, hence the unaltered rate at which isotopic valine is incorporated into brain protein in the presence of L-dopa. The theoretical data generated by such a model is essentially the same as that given in Fig. 4.6 and Table 4.6.

autoradiographic methods could presumably be used to this end. Consequently the above explanation remains a feasible alternative to the models of lysine compartmentation outlined in Section 4.1.

At first sight the data obtained by ROEL et al. (1974) do not appear to be consistent with the present results since they observed a significant L-dopa-induced decrease in TCA-insoluble radioactivity 7min after the administration of [^{14}C] lysine. Roel and co-workers expressed their incorporation data as a percentage of total homogenate radioactivity. It is shown in Section 4.3, however, that TCA-insoluble and TCA-soluble radioactivity are complicated functions of time, and it therefore follows that no simple relationship can be expected between the numerical values obtained for the quotient

$$\frac{\text{TCA-insoluble radioactivity}}{\text{Total tissue radioactivity}}$$

at different times after tracer administration. Consequently, in order to carry out a more detailed examination of the results given in Table 2 of ROEL et al. (1974) it was convenient to express the data in terms of TCA-insoluble and TCA-soluble radioactivity (Table 5.1). There is a discrepancy between the 30min control lysine data given in Table 2 and Fig. 2A of their paper and this result was therefore ignored. According to the figures given in Table 5.1, Roel and co-workers observed a 74% L-dopa-induced inhibition of lysine incorporation into TCA-insoluble material at 7min compared with the 32-54% inhibition seen after a 7½min incorporation period in the present study. While experimental error may have contributed to this discrepancy, it is probable that it arises at least in part, from the difference in the mode of tracer administration adopted in the two sets of experiments. Roel and co-workers administered their radioactive amino acids by intracisternal injection, while in the present study sub-

TABLE 5.1 THE EFFECT OF L-DOPA ON THE INCORPORATION OF L-[^{14}C]AMINO ACIDS INTO RAT BRAIN TCA-INSOLUBLE MATERIAL

A

^{14}C-Labelled amino acid	Time after injection (min)	Total radioactivity per brain (A) (c.p.m. x 10^{-3})		Soluble radioactivity per brain (B) (c.p.m. x 10^{-3})		TCA-insoluble radioactivity per brain (A-B) (c.p.m. x 10^{-3})		Corrected soluble radioactivity (D) (c.p.m. x 10^{-3})		Relative incorporation ((A-B)/D)	
		Control group	L-dopa group	Control group	L-dopa group	Control group	L-dopa group	Control group	L-dopa group	Control group	L-dopa group
Leucine	7	140	111	62	60	78	51	37.8	31.8	2.06	1.60
	15	243	155	79	80	164	75	34.0	30.4	4.82	2.47
	30	152	79	39	36	113	43	12.9	8.6	8.76	5.00
Lysine	7	430	463	391	453	39	10	360	408	0.108	0.025
	15	429	415	375	395	54	20	338	352	0.160	0.057

Table 5.1 Continued.....

B

^{14}C-Labelled amino acid	Time after injection (min)	Percentage decrease	
		TCA-insoluble radioactivity	Relative incorporation
Leucine	7	35	22
	15	54	49
	30	62	43
Lysine	7	74	77
	15	63	64

The experimental data is taken from Table 2 of ROEL et al. (1974). The 30min lysine data given in Table 2 is not consistent with that given in Fig. 2 of ROEL et al. (1974) and has not been included here. A. The data were calculated as follows. 1) TCA-insoluble radioactivity = (total)-(soluble radioactivity). 2) Corrected soluble radioactivity = (soluble radioactivity) x (the fraction of radioactivity present in the form of the injected amino acid). 3) Relative incorporation = (TCA-insoluble radioactivity)/(corrected soluble radioactivity). B. The L-dopa-induced decrease in TCA-insoluble radioactivity and relative incorporation is expressed as a percentage of the control levels.

cutaneous injection was adopted. The effect of using different routes of administration was studied by computer simulation* using the subcellular model of lysine compartmentation outlined in Fig. 4.4. According to this model the magnitude of the reduction in TCA-insoluble radioactivity observed in L-dopa-treated rats after a short incorporation period (ie., before the tracer has reached a quasi-steady state) depends upon the relative rates at which the radioactive lysine equilibrates with the two precursor pools following its administration. Only if, in the control animals, a considerable percentage of protein-bound radioactivity has entered via the main intracellular compartment will a substantial level of inhibition be detected. This clearly depends upon which route of tracer administration is adopted. If, when the intracisternal mode of administration is employed the tracer equilibrates with the two precursor pools with comparable rapidity then, according to the model, a reduced level of incorporation will be observed after a short incorporation period. In contrast, when the tracer is introduced subcutaneously, the rate at which radioactivity enters the main intracellular pool is slower than the rate at which it enters precursor compartment 1 (see Fig. 4.4) and hence the onset of the effect of L-dopa on the incorporation of radioactivity into TCA-insoluble material is slower.

Roel and co-workers found that L-dopa inhibited the incorporation of L-[U-^{14}C]lysine more than that of L-[U-^{14}C]leucine, which is consistent with the results obtained with L-[^{3}H]lysine and isotopic valine in the present study. Roel et al. attributed the difference in the magnitude of the effect seen with [^{14}C]lysine and [^{14}C]leucine to an expansion of the brain soluble lysine pool in L-dopa-treated rats. This assumes, however, that the effect is not brain specific and that the drug causes a parallel increase in total body lysine. The effect of an expansion of the brain soluble lysine pool in the absence of an effect in peripheral tissue was studied by computer simulation. According to the models of lysine compartmentation, a brain specific increase in intracellular lysine levels has no significant effect on the rate of incorporation of radioactivity into

*The simulated data obtained from this study are not included in this thesis.

brain protein since, in the presence of L-dopa, radioactive lysine does not enter the protein-bound pool via the main intracellular compartment. It is, of course, recognised that these models may be incorrect. Even so, an isolated increase in the size of the brain soluble lysine pool is not necessarily expected to cause a corresponding decrease in the incorporation of radioactive lysine into protein. For example, according to the homogeneous precursor lysine model outlined in Fig. 4.2, if the administered radioactive lysine equilibrates rapidly with the main intracellular brain soluble lysine pool, a 34% increase in the size of this compartment (cf. Table 1 of ROEL et al., 1974) will cause a reduction in protein-bound radioactivity of the order of 21, 16 and 9% at 7, 15 and 30min after tracer administration, respectively. Finally, the hypothesis that a significant percentage of the reduction in TCA-insoluble radioactivity is caused by an increase in brain soluble lysine levels is not substantiated by other researchers who failed to observe an increase in cerebral lysine in response to L-dopa (WEISS et al., 1971; LIU et al., 1972). It should be noted that the 34% increase observed by Roel and co-workers was not statistically significant.

The effect of L-dopa on the incorporation of L-[^{3}H]lysine into protein in vitro

WEISS et al. (1975) postulated that L-dopa disrupts cerebral polysomes via the formation of dopamine and its subsequent binding to some cellular receptor. They also postulated that this cellular dopamine receptor is common to the majority of brain cells. The data obtained in the present study do not rule out the possibility that L-dopa causes a small inhibition of protein synthesis in the brain. Furthermore, as mentioned above, the possibility that the gross L-dopa-induced decrease in protein-bound radioactivity observed 15min after the administration of [^{3}H]lysine is caused by an inhibition of protein synthesis in a population of cells that incorporate radioactive lysine particularly efficiently is not ruled out by the in vivo experimental data. If the L-dopa-induced decrease in the rate of incorporation of radioactive lysine was mediated via a cellular mechanism then one might

expect to observe the effect in brain slices. Preliminary experiments were therefore carried out in which the *in vitro* effect of L-dopa was investigated. In a study using brain slices it would have been necessary to consider the interpretational problems that accompany the structural heterogeneity of the brain. The experiments were therefore performed using chopped brain tissue. The present author recognises that the experiments were extremely unrefined, mainly because of the extensive cell damage that must have occurred during the preparation of the chopped tissue. Nevertheless, the effect of L-dopa on the incorporation of radioactive lysine *in vivo* was marked, and consequently such an effect should be observable *in vitro*, even in a crude experimental system providing the mechanism operating *in vivo* remains intact. L-dopa (20-640 ug/ml) had no significant effect on the incorporation of [^{3}H]lysine into protein in chopped rat brain. Further, in one experiment, no difference was observed in the rate at which [^{3}H]lysine was incorporated into protein in cerebral slices prepared from control and L-dopa-treated rats maintained at 26^{o}C.

Although the above results are consistent with the hypothesis that neither L-dopa nor its *in vitro* metabolites inhibit the incorporation of radioactive lysine into brain protein via a direct cellular mechanism, it should be noted that the rate of protein synthesis in adult rat brain slices under optimum conditions is only 10-20% of the *in vivo* rate (DUNLOP *et al.*, 1974, 1975b). Furthermore, in the present study, in order to reduce the rate at which L-dopa underwent oxidation, chopped rat brain tissue was incubated in an atmosphere of air instead of oxygen. Consequently protein synthesis was probably even more severely impaired. It is therefore possible that the L-dopa-induced decrease in TCA-insoluble radioactivity seen *in vivo* is due to a reduced turnover rate of some protein fraction, the synthesis of which is so severely inhibited *in vitro* in the control preparations that no further inhibition is observed in the presence of L-dopa. *In vitro* experiments were carried out using tissue taken from 35-day-old rats to match the age of the animals used in the *in vivo* experiments (27-42 days of age). Rates of protein synthesis in brain slices prepared from 3-day-old rats

are, under optimum conditions, 70-80% of the *in vivo* rate (DUNLOP *et al.*, 1975b). The above hypothesis could therefore be tested using immature brain tissue. No such experiments were carried out, however, and any conclusions made on the basis of the preliminary work reported here must therefore remain tentative.

5.3 MODELS OF LYSINE COMPARTMENTATION

According to the models of lysine compartmentation outlined in Section 4.1 a nonexchanging intracellular lysine compartment is present in central nervous tissue. This compartment may be separated physically from the main intracellular pool or it could represent a chemical derivative of lysine. Lysine released from degraded protein is fed into this sequestered pool and, in the presence of L-dopa, its contents re-enter protein without passing through the main intracellular compartment. No indication of the size of this sequestered pool is given by the present experiments. The presence of two precursor lysine pools, one of which equilibrates rapidly with plasma lysine, is also suggested and it is assumed that L-dopa has no effect on brain protein synthetic activity, although a small percentage inhibition of protein synthesis could easily be incorporated into the models.

Few of the features of these models are new. The concept that the main intracellular amino acid compartment is not the exclusive source of amino acids for protein synthesis has been suggested by a number of researchers and so has the presence of sequestered intracellular amino acid pools (see Section 1.3).

The models of compartmentation assume that lysine released from degraded brain protein is not normally recyled directly back into the protein-bound pool. In contrast, it has been suggested that in the liver a considerable percentage (about 50%) of lysine released from degraded protein is directly recycled since the rate at which liver protein-bound lysine is replaced is slow compared with the rate of replacement of other amino acids (SWICK, 1958). The percentage replacement rate of protein-bound lysine in rat brain is 1.85%/h compared with

2.37%/h for valine, 2.15%/h for tyrosine and 2.35%/h for histidine (Table 3 of DUNLOP et al., 1975a). Without commenting on whether the lysine replacement rate is significantly less than that of the other amino acids, these results indicate that lysine reutilization normally occurs to a lesser extent in brain than in the liver, recognising, of course, that a comparison of percentage replacement rates is valid only if protein fractions turning over at different rates have a similar amino acid composition.

Evidence for a sequestered intracellular lysine compartment in the brain

A number of researchers have reported that when the specific activity of a plasma amino acid is maintained at a constant level by the continuous infusion of a radioactive tracer, tissue amino acid pools attain a constant specific activity, but at a level lower than that of the plasma (GAN and JEFFAY, 1967; GARLICK and MARSHALL, 1972; SETA et al., 1973). This has been attributed to the presence of nonexchanging tissue amino acid compartments and/or the continual release of cold amino acids from degraded protein. A computer simulation study was carried out in order to determine whether the release of lysine from degraded protein could account for the observed equilibrium brain: plasma lysine specific activity ratio of 77% to 86% (SETA et al., 1973), since if this is not the case the presence of a nonexchanging lysine compartment is implied. A reliable answer to this question may be obtained only if an accurate figure for the rate of lysine uptake from plasma into brain is available. Since there is a discrepancy between the uptake rates determined by different researchers the results of the simulation must be regarded with caution. Bearing this in mind, the simulated data indicate that not only does the release of cold lysine from degraded protein make a substantial contribution, but that it might entirely account for the brain-plasma specific activity gradient observed in the continuous infusion experiment. This observation is not, however, incompatible with the hypothesis that a nonexchanging lysine compartment does exist in brain, but it does indicate that sequestered lysine may only represent a small percentage of the total intracellular pool.

NEIDLE et al. (1975) have suggested that the presence of nonexchanging intracellular compartments accounts for their observation that when mouse brain slices were incubated in media containing radioactive amino acids the specific activities of tissue amino acid pools were significantly less than that of the media. A simulation study of their experiments indicated that the release of cold lysine from degraded protein probably does not account for the magnitude of the tissue-medium lysine specific activity gradient. The apparent discrepancy between the in vivo and in vitro situations may indicate that a non-exchanging compartment present in vivo becomes grossly distorted in size when brain tissue is sliced and incubated, possibly as a result of an impairment of some lysine transport system. In this connection it is interesting to note that NEIDLE et al. (1973) have suggested that a derangement of the mechanisms involved in the regulation of amino acid transport occurs when central nervous tissue is sliced.

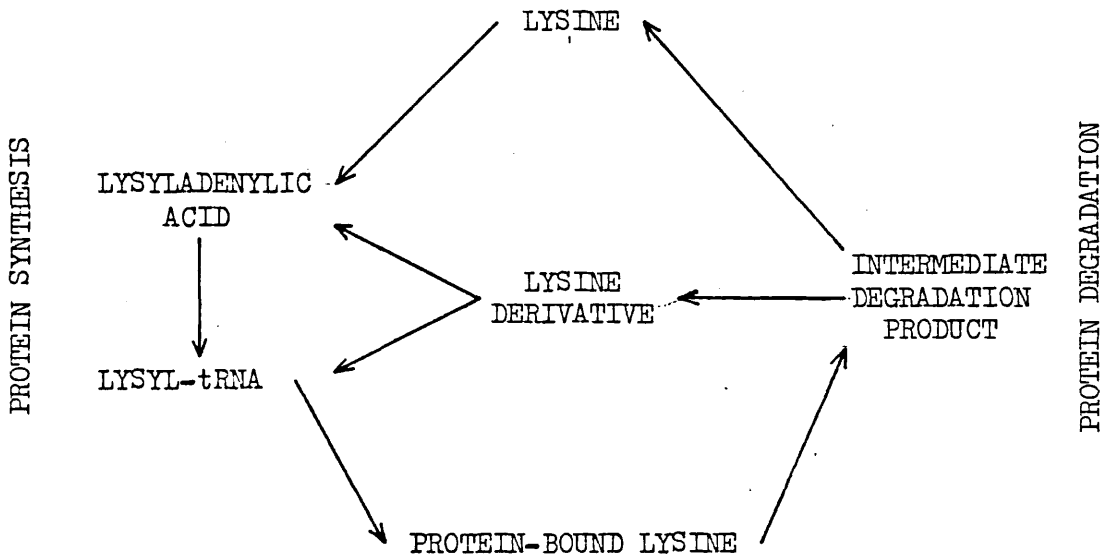
If a sequestered lysine pool is present in nervous tissue the question concerning the nature of this compartment is automatically raised. Providing the tracer experiments are not of a long duration, degraded protein is expected to be a source of cold amino acid, and since lysosomes are probably an important site of protein degradation (SEGAL, 1976 ; DEAN and BARRETT, 1976; BALLARD, 1977), the nonexchanging pool could be of lysosomal origin. This hypothesis, which assumes that the rate of exchange between the lysosomal and main intracellular pools is slow, is consistent with the report that the specific activity of a crude mitochondrial fraction prepared from the liver of rats perfused with radioactive lysine was lower than that of any other subcellular fraction (PORTUGAL et al., 1970). Further lysosomal lysine represents only a small fraction of the total tissue pool, which is in agreement with the suggestion that in vivo the nonexchanging lysine compartment is not sufficiently large to cause a significant dilution of the intracellular pool. As mentioned above in order to account for the results obtained from brain slice experiments it is necessary to postulate that when brain tissue is sliced and incubated the nonexchanging pool expands. This might be explained in terms of an impairment of the

transport system by which lysine leaves the nonexchanging compartment. According to the models of lysine compartmentation outlined in Section 4.1, a mechanism must exist through which lysine present in the non-exchanging pool may be re-incorporated into protein without mixing with the main intracellular lysine pool. This implies a considerable degree of spacial organisation.

An alternative hypothesis that might be considered is that the non-exchanging lysine compartment is not of a morphological nature but exists in the form of a chemical derivative of lysine. A number of researchers have suggested that free amino acids are not the direct product of protein degradation, but that some intermediate(s) is(are) involved (STEINBERG and VAUGHAN, 1956; WALTER, 1960; LAJTHA, 1964). If these intermediates were the same as, or were precursors of those involved in protein synthesis, not only would this be economical in terms of energy conservation, but it would provide a mechanism by which amino acids released from degraded protein could be re-incorporated without mixing with the main intracellular amino acid pool. (See Fig. 2 of LAJTHA, 1964 and Fig. 5.1). If sequestered lysine does exist in the form of some derivative, then this material must be sufficiently labile that free lysine is released during the preparation of tissue homogenates or during subsequent amino acid purification. This is consistent with the proposed intermediate being a mixed anhydride. For example, α-amino acyladenylates undergo rapid hydrolysis at neutral pH (MOLDAVE et al., 1959).

The involvement of intermediates in the breakdown of protein was proposed on the basis of the observation that although peptide hydrolysis is an exothermic process, protein degradation is both energy dependent and is inhibited by the same agents that inhibit protein synthesis (SIMPSON, 1953; STEINBERG and VAUGHAN, 1956). Opponents of this theory have argued, however, that energy may be required either for the maintenance of some structure essential for protein degradation or for the transport of materials across membranes (BROSTROM and JEFFAY, 1970; BALLARD, 1977). The observation that inhibitors of protein synthesis also inhibit protein degradation has been used in support of

FIG. 5.1 A MODEL FOR THE CHEMICAL COMPARTMENTATION OF LYSINE BASED ON THE SCHEME OF LAJTHA (1964).



the hypothesis that some intermediate(s) may be common to the synthetic and degradative pathways (STEINBERG and VAUGHAN, 1956; WALTER, 1960). This phenomenon has, however, also been explained in terms of an inhibition of protein degradation through either the accumulation of precursors of protein synthesis or the reduced rate of synthesis of some labile protein that is required to facilitate degradation (BALLARD, 1977).

The present author recognises that the existence of amino acid derivatives as intermediate products of protein degradation is not generally accepted. Nevertheless, the hypothesis that the nonexchanging lysine compartment represents either some intermediate common to the synthetic and degradative pathways, or an intermediate which can be converted to lysyl-tRNA without the prior release of free lysine provides an explanation for the apparent expansion of the nonexchanging lysine compartment in brain slices. Thus an accumulation of sequestered lysine could arise from a reduction in the rate at which the proposed derivative is removed *in vitro* together with an increase in its rate of formation, the latter of which is the result of excessive proteolytic activity in freshly sliced brain tissue. If the premise that the direct reutilization of lysine occurs to a minimal extent in the *in vivo* brain is not strictly correct and a small percentage of the derivatised lysine is re-incorporated into protein, then a reduced rate of removal may, in part, be a consequence of the impaired protein synthetic activity of brain slices. Concerning the question of lysine reutilization *in vivo*, although it is argued above that the level of reutilization observed in the liver does not occur in the brain, the percentage replacement rate of brain lysine is 19% less than the average percentage replacement rate of valine, tyrosine and histidine (Table 3 of DUNLOP *et al.*, 1975a). Thus the reutilization of brain lysine *in vivo* is not ruled out but probably does not account for more than 20% of the total lysine incorporated into protein. It is shown in Section 4.2.2 that the experimental data of DUNLOP *et al.* (1974) is accounted for by a model in which 40% of lysine released from degraded protein in brain slices is recycled directly back into the protein-bound pool

(see Fig. 4.13). This apparent discrepancy between the *in vivo* and *in vitro* percentage reutilization levels may be explained as follows. Maximum rates of protein synthesis in adult rat brain slices are only 10-20% of the *in vivo* rate (DUNLOP *et al.*, 1974, 1975b). Thus if it is assumed that a small percentage of sequestered lysine is re-incorporated into protein *in vivo*, and that this process continues in sliced tissue, albeit at a slightly reduced rate, while the incorporation into protein of "exchangeable" lysine is grossly inhibited compared with its *in vivo* rate, then a greater level of reutilization *in vitro* is expected. This hypothesis is compatible with the model outlined in Fig. 5.1. According to this scheme, lysyl-tRNA is charged from both the exchangeable lysine pool and from an intermediate of protein degradation. If it is postulated that *in vivo*, protein-bound lysine is derived predominantly from tRNA charged from the main intracellular pool then little direct reutilization would occur. If, on the other hand, this pathway becomes so severely impaired *in vitro* that the alternative source of activated lysine becomes important, then reutilization would occur.

It must be emphasised that the above hypotheses should be regarded as highly speculative since not only is the presence of sequestered lysine far from established, but the existence of amino acid derivatives as products of protein degradation is not generally accepted. The model outlined in Fig. 5.1 is included here merely to indicate that some experimental observations can be explained in terms of a chemical lysine compartment.

The mathematical models used in the simulation of *in vivo* tracer experiments are somewhat unrefined. In particular, the structural heterogeneity of brain tissue is not taken into account, but the term 'rate of brain protein synthesis' itself implies a neglect of heterogeneity. Other limitations of the mathematical treatments are discussed in Section 4. Despite their unrefined nature, the models do account for

a number of experimental observations in addition to those of the present study. LAJTHA et al. (1957) calculated rates of brain protein turnover on the basis of the rate of uptake of radioactive lysine into protein, and found that these turnover rates decreased as the duration of the incorporation period was increased. This was attributed to heterogeneity of protein turnover rates (LAJTHA et al., 1957; LAJTHA, 1964, LAJTHA and MARKS, 1969). While it is true that the presence of protein fractions having different turnover rates can lead to time-dependent apparent turnover rates (see Section 1.2), it is not known a priori over what time period this will be observed. This will depend not only on the range of turnover rates, but also on what percentage of total protein is represented by the different protein fractions. The results of a computer simulation study indicate that heterogeneity of protein turnover rates of the magnitude reported by LAJTHA et al. (1976) does not account for the discrepancy in turnover rates obtained by LAJTHA et al. (1957) using incorporation periods of between 2 and 60 min. The latter data are, however, partly accounted for by models of lysine compartmentation which assume the presence of two precursor pools for protein synthesis, one of which equilibrates rapidly with plasma lysine. This is consistent with the proposition that the problem of defining the precursor pool and measuring its specific activity is the cause of the apparent time dependence in rates of protein synthesis calculated from tracer incorporation data (GARLICK and MARSHALL, 1972). In contrast, ENTINGH and DAMSTRA (1976) put forward the interesting idea that the decreased apparent turnover rates observed after longer incorporation periods might reflect a return to a baseline rate of protein synthesis after a stress-induced increase following precursor administration. The explanation offered by the present author is, however, consistent with the data given in Table IV of SETA et al. (1973) and in Fig. 2 of DUNLOP et al. (1975a) since no decrease in incorporation rates was observed with increasing incorporation time in experiments in which precursor specific activity was maintained at a constant level during the incorporation period. The premise that the precursor lysine pool is heterogeneous is consistent

with the results obtained by OLDENDORF (1971) in experiments in which rates of labelled amino acid uptake from plasma into brain were measured during a 15 sec time interval. He obtained rates of lysine uptake much faster than those reported by other researchers (TOTH and LAJTHA, 1977; COULSON and HART, 1977). Measurements made only 15 sec after tracer administration may yield rates of uptake into compartments which are exchanging very rapidly with the plasma pool and which represent only a fraction of the total soluble amino acid present in the tissue. The rapidly exchanging brain lysine compartment detected by Oldendorf may represent the rapidly exchanging precursor pool that is a feature of the two models of lysine compartmentation outlined in Section 4.1.

5.4 CONCLUDING REMARKS

The effect of L-dopa on rat brain protein synthesis has been re-examined by studying the effect of the drug on the incorporation of L-[^{3}H]lysine, L-[^{3}H]valine and L-[^{14}C]valine into TCA-insoluble material. Incorporation is expressed in terms of 1) the relative incorporation (I_{rel}) defined as the final TCA-insoluble radioactivity divided by the final TCA-soluble radioactivity, and 2) the apparent incorporation (I_{app}) defined as the final TCA-insoluble radioactivity divided by the final specific activity of the soluble amino acid pool. L-dopa had no significant effect on the apparent incorporation of [^{3}H]valine measured 15 min after the injection of a trace quantity of the label, and no significant effect on the apparent incorporation of [^{14}C]valine measured 35 min after its co-administration with a flooding quantity of carrier valine. In the latter experiments it was assumed that the specific activity of the soluble valine pool was the same as that of the administered amino acid (cf. DUNLOP et al., 1975a). These results are interpreted as indicating that L-dopa had no significant effect on rates of brain protein synthesis. However, because the errors associated with the group means were large, the possibility that the drug had a small inhibitory effect cannot be ruled out. In order to resolve this matter the scatter in the data must be reduced. Using a simple but sensitive technique for detecting

small changes in polysome aggregation, JOHNSON et al. (1975) were able to demonstrate a statistically significant 9% decrease in active brain polysome levels in rats given phenylalanine (1g/kg). It is doubtful whether the reduction in amino acid incorporation rates that might accompany such a small decrease in active polysome levels would be detected using amino acid tracers and consequently the technique of Johnson and co-workers may be the method of choice in this kind of study and should perhaps be applied to the present problem. If, using this method, a significant decrease in the brain concentration of active polysomes was observed in L-dopa-treated rats the question concerning the involvement of a soluble dopamine receptor as proposed by WEISS et al. (1975) would remain.

L-dopa caused a significant 55-59% decrease in the apparent incorporation of [^{3}H]lysine measured after a 15 min labelling period but had no significant effect on that measured 7½ min after tracer administration. This apparent discrepancy is explained in terms of models of lysine compartmentation (see Section 4.1). According to these models brain protein-bound lysine is normally derived from two precursor pools, one of which exchanges rapidly with the main intracellular lysine compartment, the other exchanging rapidly with plasma lysine. In the presence of L-dopa, the main intracellular compartment ceases to be a source of lysine for protein synthesis and the deficit is made up from a sequestered pool.

Additional experiments should be carried out with a view to substantiating the data presented in this thesis and testing the models of lysine compartmentation. It is particularly important to obtain unequivocal evidence for an L-dopa-induced reduction in the rate of incorporation into brain protein of radioactive lysine in the absence of a reduction in the valine incorporation rate. For example, the demonstration in the same animal of a reduced [^{3}H]lysine incorporation rate in the absence of an effect on [^{14}C]valine incorporation would lend considerable weight to the hypothesis that the former effect

is not due to a general inhibition of protein synthesis. Secondly, the observation that the magnitude of the L-dopa-induced decrease in I_{app} increased with time during the first 15 min following the subcutaneous administration of radioactive lysine should be substantiated. In particular, it would be desirable to obtain more data points. If the observation was confirmed it would be of interest to test the effect of L-dopa on the incorporation of other amino acids in order to determine whether the effect is specific to lysine, specific to the basic amino acids or observable using amino acids belonging to other classes. Finally, an alternative explanation is offered in Section 5.2 for the observed difference between the effect of L-dopa on the incorporation of [^{3}H]lysine and isotopic valine. Thus a specific inhibition of protein synthesis in a population of brain cells that has a particularly large capacity for incorporating radioactive lysine could give rise to the same effect. Although this explanation is considered unlikely, the presence or absence of such a population of cells could be demonstrated using autoradiographic techniques.

Assuming that the results presented in this thesis were verified through further experimentation, one might proceed to investigate the mechanism by which the L-dopa effect is mediated. The failure of L-dopa to affect the rate of incorporation of radioactive lysine into protein in rat brain slices may indicate that the drug does not act directly via a cellular mechanism. However, because protein synthesis in adult brain slices occurs at only a fraction of the *in vivo* rate (10-20% under optimum conditions, DUNLOP *et al.*, 1974, 1975b), the possibility remains that the *in vivo* response is due to an effect on some protein fraction which has a particularly large capacity for incorporating radioactive lysine, the synthesis of which is severely impaired *in vitro*. This could be investigated using tissue taken from immature rats since protein synthetic rates in immature rat brain slices incubated under optimum conditions are comparable with the *in vivo* rate (DUNLOP *et al.*, 1975b). If no effect was observed in immature brain slices this might indicate that the *in vivo* response

is mediated via a mechanism that is dependent upon either an intact brain or an intact animal. The observation that the L-dopa response is affected by the temperature at which the animals are maintained after drug administration may indicate that the response is mediated indirectly via some system that is intact only in the whole animal. An in vivo study using carefully selected dopaminergic agonists and antagonists might lead to some understanding of the mechanisms involved. Preliminary results indicate that L-dopa has an effect on the incorporation of radioactive lysine into liver protein similar to that seen in the brain. However, no serious attempt was made to investigate this since the methods used in this study were totally inadequate for that purpose. Liver perfusion is extensively used in the study of hepatic protein metabolism and might be adopted in order to further investigate the effect of L-dopa in this tissue. Should it turn out that L-dopa inhibits the incorporation of radioactive lysine into liver protein, but has no effect on valine incorporation rates, then liver may be the tissue of choice for further investigations into the mechanisms mediating the response.

Computer simulation was used as an aid to the interpretation of the results obtained from tracer experiments. In particular an attempt was made to answer some questions concerning the compartmentation of lysine in relation to its incorporation into protein. The experimental evidence for the existence of a sequestered lysine pool in brain was also examined. The procedure adopted in this simulation study was in some instances extremely unrefined, mainly because of a lack of the experimental data required to fix some of the variables. Consequently, the work merely represents an attempt to obtain some preliminary answers using the available experimental data.

Simulation of in vivo experiments was performed using amino acid exchange rates and pool sizes calculated from literature data wherever possible. The remaining variables were determined empirically. To this end particular use was made of the lysine specific activity data given

in Table 3 of LAJTHA et al. (1957). However, this data was compiled from measurements made in the mouse and it would clearly be desirable to base the simulation on lysine specific activity measurements made in the rat. This would involve the compilation of lysine specific activity-time curves for the plasma and brain soluble pools as well as the brain protein-bound pool. Providing sufficient data points were obtained, reliable estimates of the variable parameters could be obtained using an optimization routine.

The phenomenon of intracellular dilution in amino acid tracer experiments is widely reported, but the cause remains a matter for debate. Computer simulation of in vivo radioactive lysine perfusion experiments was performed to determine what level of intracellular dilution might be expected in the brain as a result of the continual release of cold lysine from degraded protein . Had the results shown that protein degradation causes no significant intracellular dilution, the unrefined nature of the model used in the simulation would have been of little consequence since its refinement would only change the quantitative result but not the qualitative conclusion. The results indicate, however, that the release of lysine from degraded protein probably makes a significant contribution to the observed intracellular dilution. Consequently, because the result is quantitatively uncertain, it is not possible to comment on whether or not protein degradation is the sole cause of the phenomenon.

A simulation of the experiments of NEIDLE et al. (1975) indicated that the release of lysine from degraded protein is not the only cause of the dilution of the intracellular pool in brain slices incubated in a medium containing radioactive lysine, although it does make a contribution. While this is consistent with the assumed presence of a sequestered lysine pool in the brain, the theoretical data must again be viewed with caution since a steady-state model was applied to a nonsteady-state system. (The imbalance between the rates of protein degradation and protein synthesis in incubated brain slices causes a continual expansion of the soluble amino acid pool (NEIDLE

et al., 1975; JONES and MCILWAIN, 1971).) While a nonsteady-state treatment might be adopted in order to obtain a more reliable result, this would necessitate making further approximations since insufficient data are available to accurately define the time-dependent variables. Alternatively, the experimental system could be modified to make the steady-state approximation more reasonable. Thus the experiments could be repeated using incubation mixtures containing the amino acid in question at a concentration sufficiently low so as not to obscure any sequestered pools, but using an incubation volume sufficiently large that the time-dependent expansion of the soluble pools is insignificant. Reliable data on the rate of exchange between the tissue and medium free amino acid pools at the concentration in question is also required. A more tenable simulation could then be performed using a steady-state model.

The results presented in this thesis indicate that an inhibition of protein synthesis may not be the major cause of the L-dopa-induced reduction in the incorporation of radioactive lysine into brain protein. It is suggested that an alteration in lysine compartmentation might be involved. Some of the problems associated with the measurement of rates of protein synthesis have been demonstrated, particularly in relation to the difficulty of obtaining reliable indices of the time-average precursor specific activity. The desirability of using incorporation periods of different durations and labelled amino acids belonging to different classes is also demonstrated.

TABLE A1

Animal no. (i)	Brain TCA-insoluble radioactivity (R_{2i}) (d.p.m./mg prot.)	Rank(R_{2i})	Brain TCA-soluble radioactivity (R_{1i}) (d.p.m./mg prot.)	Rank(R_{1i})	d_i = rank(R_{2i}) - rank(R_{1i})	d_i^2
1	707	6	247	6	0	0
2	513	2	174	2	0	0
3	478	1	169	1	0	0
4	515	3	206	4	-1	1
5	564	5	215	5	0	0
6	532	4	184	3	+1	1

The brain TCA-insoluble and TCA-soluble radioactivity data tabulated above were obtained from a group of rats injected with L-[4,5-^{3}H]lysine (30 min incorporation period, see Table 3.1.1 for details). d_i is the difference in the ranks for each pair of observations. The Spearman rank correlation coefficient (r_s) defined by

$$r_s = 1 - \frac{6 \sum_{i=1}^{n} d_i^2}{N (N_2-1)}$$

(see eg. page 275 of COLQUHOUN, 1971) is 0.943, $P < 0.05$ (two tail). (Critical values of r_s for $4 \leq n \leq 8$ are given in Table 12.9.2 of COLQUHOUN, 1971).

TABLE A2

Source of variation	Sum of squares	Degrees of freedom	Estimate of variance	F
Between experiments (E)	2376	1	2376	8.83*
Between dopa and control animals (A)	68211	1	68211	253.6***
Interaction EA	2040	1	2040	7.58*
Between replicates (R)	2044	4	511	1.90
Interaction ER	2450	4	612	2.28
Interaction AR	5404	4	1351	5.02
Residual (EAR)	1075	4	269	
Total	83600	19		

An analysis of variance was carried out on the relative incorporation data obtained from experiments 5 and 6 (Tables 3.2.5 and 3.2.6) in order to determine whether the two sets of data could be combined and treated as a single set of results. The twenty results for I_{rel} were classified into a 5x2x2 grouping (five replicates, control and dopa-treated groups, and two experiments). The calculations were performed on a computer using the IBM routine ANOVA (The Scientific Subroutine Package, Version III) (see acknowledgements). *, significant; ***, highly significant. (For $\nu_1=1$, $\nu_2=4$, the 5% and 1% points are 7.71 and 21.20, respectively. For $\nu_1=\nu_2=4$, the 5% and 1% points are 6.39 and 15.98, respectively.) Tables of the distribution of F are given in FISHER and YATES (1963).

TABLE A3 THE EFFECT OF L-DOPA ON THE RELATIVE INCORPORATION OF L-[3,4(n)-^{3}H]VALINE IN BRAIN. AN ANALYSIS OF COMBINED DATA USING THE WILCOXON SIGNED-RANKS TEST

Exp no.	Pair no. (i)	q_{rel_i}	$1-q_{rel_i}$	Signed rank
4	1	0.473	+0.527	+10
	2	0.520	+0.480	+ 8
	3	0.529	+0.471	+ 7
	4	0.584	+0.416	+ 5
2	5	0.713	+0.287	+ 3
	6	0.910	+0.090	+ 1
	7	1.179	-0.179	- 2
3	8	1.392	-0.392	- 4
	9	1.505	-0.505	- 9
	10	0.429	+0.571	+11
	11	0.322	+0.678	+12
	12	0.565	+0.435	+ 6

The set of relative incorporation quotients $q_{rel_i} = I^D_{rel_i}/I^C_{rel_i}$, $i = 1,2,\ldots,12$ (see equation 3.2.11) tabulated above was calculated using paired data taken from three separate experiments (exps. 2,3 and 4, Section 3.2.2) in which L-[3,4(n)-^{3}H]valine was employed as the radioactive precursor. The Wilcoxon signed-ranks test was used to determine whether the mean quotient ($\overline{q_{rel}}$) differs significantly from $\overline{q_{rel}} = 1$ (ie., no drug effect). The mean quotient is 0.76 and the sum of the negative ranks (T) is 15. For T=15 and n=12, $P \simeq 0.05$ (two tail) (Table A4 of COLQUHOUN, 1971).

TABLE A4 THE EFFECT OF L-DOPA ON THE RELATIVE INCORPORATION OF L-[3,4(n)-^{3}H]VALINE IN LIVER. AN ANALYSIS OF COMBINED DATA USING THE WILCOXON SIGNED-RANKS TEST

Exp no.	Pair no. (i)	q_{rel_i}	$1-q_{rel_i}$	Signed rank
4	1	0.413	0.587	+10
	2	0.606	0.394	+ 6
	3	1.278	-0.278	- 5
	4	1.178	-0.178	- 3
2	6	0.591	0.409	+ 8
	7	0.884	0.116	+ 1
3	8	0.779	0.221	+ 4
	9	1.165	-0.165	- 2
	10	0.433	0.567	+ 9
	11	0.191	0.809	+11
	12	0.604	0.396	+ 7

See Table A3 for details. The mean quotient ($\overline{q_{rel}}$) is 0.74 and the sum of the negative ranks is 10, $P<0.05$.

TABLE A5 A COMPARISON OF THE EFFECT OF L-DOPA ON THE RELATIVE INCORPORATION OF L-[4,5-^{3}H]LYSINE AND L-[3,4(n)-^{3}H]VALINE IN BRAIN

Lysine			Valine		
i^{LY}	$q^{LY}_{rel_i}$	Rank of $q^{LY}_{rel_i}$	i^{V}	$q^{V}_{rel_i}$	Rank of $q^{V}_{rel_i}$
1 }	0.404	9	1 }	0.473	11
2	0.034	2	2 } Exp 4	0.520	13
3 } Exp 5	0.032	1	3	0.529	14
4	0.176	4	4 }	0.584	16
5 }	0.641	17	5 }	0.713	18
6 }	0.321	7	6 } Exp 2	0.910	19
7	0.290	6	7 }	1.179	20
8 } Exp 6	0.483	12	8 }	1.392	21
9	0.172	3	9	1.505	22
10 }	0.237	5	10 } Exp 3	0.429	10
			11	0.322	8
			12 }	0.565	15

The relative incorporation data obtained from matched-paired L-dopa-treated and control rats of two lysine experiments (experiments 5 and 6) and three valine experiments (experiments 2,3 and 4) were used to calculate the relative incorporation quotients tabulated above. The Wilcoxon two-sample test was used to determine the significance of the difference between the results obtained with [^{3}H]valine and [^{3}H]lysine. $q^{LY}_{rel_i} = I^{D}_{rel}/I^{C}_{rel}$ for the ith lysine pair and similarly $q^{V}_{rel_i}$ refers to the ith valine pair (see equation 3.2.11). $\overline{q^{LY}_{rel}} = 0.279$ and $\overline{q^{V}_{rel}} = 0.760$. The sum of the ranks of $q^{LY}_{rel_i}$ is 66 and the sum of the ranks of $q^{V}_{rel_i}$ is 187. These results indicate that, in brain, the L-dopa-induced decrease in the relative incorporation of [^{3}H]lysine is significantly greater than that of [^{3}H]valine, $P<0.01$ (two tail) (Table A3 of COLQUHOUN, 1971). (Note that while there is a significant difference between the I_{rel} data obtained in the two replicate lysine experiments (5 and 6) (see the analysis of variance given in Table A2) there is no significant difference between experiments 5 and 6 with respect to the relative incorporation quotients (Wilcoxon two-sample test).

TABLE A6 A COMPARISON OF THE EFFECT OF L-DOPA ON THE RELATIVE INCORPORATION OF L-[4,5-^{3}H]LYSINE AND L-[3,4(n)-^{3}H]VALINE IN LIVER

Lysine			Valine		
i^{LY}	$q^{LY}_{rel_i}$	Rank of $q^{LY}_{rel_i}$	i^{V}	$q^{V}_{rel_i}$	Rank of $q^{V}_{rel_i}$
1	0.858	16	1	0.413	8
2	0.046	1	2 Exp 4	0.606	13
3 Exp 5	0.072	2	3	1.278	21
4	0.673	14	4	1.178	20
5	1.147	18	6 Exp 2	0.591	11
6	0.167	5	7	0.884	17
7	0.120	4	8	0.779	15
8 Exp 6	0.470	10	9	1.165	19
9	0.111	3	10 Exp 3	0.433	9
10	0.210	7	11	0.191	6
			12	0.604	12

See Table A5 for details. $\overline{q^{LY}_{rel}} = 0.387$ and $\overline{q^{V}_{rel}} = 0.738$. The sum of the ranks of $q^{LY}_{rel_i} = 80$ and the sum of the ranks of $q^{V}_{rel_i}$ is 151. These results indicate that, in liver, the L-dopa-induced decrease in the relative incorporation of [^{3}H]lysine is significantly greater than that of [^{3}H] valine, $P<0.05$.

TABLE A7

Exp no.	Animal no. (i)	TCA-insoluble radioactivity (R_{2i})	Rank of R^s_{2i}	Rank of R^c_{2i}	TCA-soluble radioactivity (R_{1i})	Rank of R^s_{1i}	Rank of R^c_{1i}	d^s_i = rank(R^s_{2i}) - rank(R^s_{1i})	d^c_i = rank(R^c_{2i}) - rank(R^c_{1i})	$(d^s_i)^2$	$(d^c_i)^2$
3	1	117	1	1	230	3	6	-2	-5	4	25
	2	158	5	7	366	5	9	0	-2	0	4
	3	119	2	2	210	2	5	0	-3	0	9
	4	151	4	5	277	4	8	0	-3	0	9
	5	128	3	3	186	1	3	+2	0	4	0
4	6	180	3	8	266	4	7	-1	1	1	1
	7	148	1	4	202	3	4	-2	0	4	0
	8	207	4	9	141	2	2	+2	7	4	49
	9	155	2	6	134	1	1	+1	5	1	25

A7 continued

The brain TCA-insoluble and TCA-soluble radioactivity data tabulated in Table A7 were obtained from the control groups of rats of experiments 3 and 4 (Section 3.2.2) in which L-[3,4(n)-^{3}H] valine was employed in the measurement of $I_{rel.}$ Spearman rank correlation analysis was carried out as outlined in Table A1. The analysis was performed on the two separate sets of results and on the combined data*.

Rank (R^{s}_{ji}) is the rank of R_{ji} (j = 1 or 2) when the two sets of data are treated separately and rank(R^{c}_{ji}) is the rank of (R_{ji}) in the combined data set.

Experiment 3, $\sum_{i}(d^{s}_{i})^{2} = 8$, $r_{s} = 0.6$, no correlation

Experiment 4, $\sum_{i}(d^{s}_{i})^{2} = 10$, $r_{s} = 0.0$, no correlation

Combined data, $\sum_{i}(d^{c}_{i})^{2} = 122$, $r_{s} = -0.017$, no correlation

(NB. When there are more than eight pairs of observations, P values (two tail) may be found by using

$$t = r_{s}\sqrt{(N-2)/(1-r_{s}^{2})}$$

and making reference to tables of Student's t-distribution for $\nu = n-2$ where n is the number of pairs.)

* It is not possible to reach a value of $P<0.1$ with four pairs of observations or a value of $P<0.05$ with five observations. Consequently, in order to increase the number of pairs, analysis was carried out using combined data. If, however, equations 3.2.6 and 3.2.7 were valid within each experiment, but c(a) varied between experiments then it is possible that no correlation would be observed in the combined data, although the correlation exists within experiments. For this reason the two sets of data were also treated separately.

TABLE A8

Exp no.	Animal no. (i)	TCA-insoluble radioactivity (R_{2i})	Rank of R^s_{2i}	Rank of R^c_{2i}	TCA-soluble radioactivity (R_{1i})	Rank of R^s_{1i}	Rank of R^c_{1i}	d^s_i = rank(R^s_{2i}) - rank(R^s_{1i})	d^c_i = rank(R^c_{2i}) - rank(R^c_{1i})	$(d^s_i)^2$	$(d^c_i)^2$
5	1	59	1	4	407	1	2	0	+2	0	4
	2	121	5	10	596	5	9	0	+1	0	1
	3	92	3	8	584	4	8	-1	0	1	0
	4	88	2	7	424	2	4	0	+3	0	9
	5	101	4	9	540	3	7	+1	+2	1	4
6	6	87	5	6	620	5	10	0	-4	0	16
	7	72	4	5	497	4	6	0	-1	0	1
	8	54	1	1	409	2	3	-1	-2	1	4
	9	58	3	3	398	1	1	+2	+2	4	4
	10	56	2	2	427	3	5	-1	-3	1	9

A8 continued

The brain TCA-insoluble and TCA-soluble radioactivity data tabulated in Table A8 were obtained from the control groups of rats of experiments 5 and 6 (Section 3.2.2) in which L-[4,5-^{3}H]lysine was employed in the measurement of $I_{rel.}$ Spearman rank correlation analysis was carried out as outlined in Table A1. See Table A7 for further details.

Experiment 5, $\sum_i (d_i^s)^2 = 2$, $r_s = 0.900$, $P = 0.1$

Experiment 6, $\sum_i (d_i^s)^2 = 6$, $r_s = 0.700$, No correlation

Combined data, $\sum_i (d_i^c)^2 = 52$, $r_s = 0.685$, $P < 0.05$

TABLE A9 A CORRELATION ANALYSIS OF BRAIN AND LIVER L-[4,5-^{3}H]LYSINE q_{rel} DATA

Exp no.	Pair no. (i)	Brain		Liver		$d = \text{rank}(q^B_{rel_i}) - \text{rank}(q^L_{rel_i})$	d^2
		$q^B_{rel_i}$	$\text{Rank}(q^B_{rel_i})$	$q^L_{rel_i}$	$\text{Rank}(q^L_{rel_i})$		
5	1	0.404	8	0.858	9	-1	1
	2	0.034	2	0.046	1	+1	1
	3	0.032	1	0.072	2	-1	1
	4	0.176	4	0.673	8	-4	16
	5	0.641	10	1.147	10	0	0
6	6	0.321	7	0.167	5	+2	4
	7	0.290	6	0.120	4	+2	4
	8	0.483	9	0.470	7	+2	4
	9	0.172	3	0.111	3	0	0
	10	0.237	5	0.210	6	-1	1

The relative incorporation quotients tabulated above were calculated using paired data taken from two experiments (experiments 5 and 6, Section 3.2.2) in which L-[4,5-^{3}H]lysine was employed as the radioactive precursor. Spearman rank correlation analysis was performed to test for a correlation between q_{rel} in liver and brain (see Tables A1 and A7 for details).

$\sum_i d_i^2 = 32$, $r_s = 0.806$, $P<0.01$.

TABLE A10 A CORRELATION ANALYSIS OF BRAIN AND LIVER L-[3,4(n)-^{3}H]VALINE q_{rel} DATA

Exp no.	Pair no. (i)	Brain		Liver		$d = rank(q^B_{rel_i}) - rank(q^L_{rel_i})$	d^2
		$q^B_{rel_i}$	$Rank(q^B_{rel_i})$	$q^L_{rel_i}$	$Rank(q^L_{rel_i})$		
4	1	0.473	3	0.413	2	+1	1
	2	0.520	4	0.606	6	-2	4
	3	0.529	5	1.278	11	-6	36
	4	0.584	7	1.178	10	-3	9
2	6	0.910	8	0.591	4	+4	16
	7	1.179	9	0.884	8	+1	1
3	8	1.392	10	0.779	7	+3	9
	9	1.505	11	1.165	9	+2	4
	10	0.429	2	0.433	3	-1	1
	11	0.322	1	0.191	1	0	0
	12	0.565	6	0.604	5	+1	1

The relative incorporation quotients tabulated above were calculated using paired data taken from three experiments (experiments 2, 3 and 4, Section 3.2.2) in which L-[3,4(n)-^{3}H]valine was employed as the radioactive precursor. Spearman rank correlation analysis was performed to test for a correlation between q_{rel} in liver and brain (see Tables A1 and A9 for details).

$\sum_i d_i^2 = 82$, $r_s = 0.627$, $P<0.05$.

TABLE A11

Exp no.	Animal no. (i)	TCA-insoluble radioactivity (R_{2i})	Rank of R^s_{2i}	Rank of R^c_{2i}	Specific activity of soluble valine (S_{1i})	Rank of S^s_{1i}	Rank of S^c_{1i}	d^s_i = rank(R^s_{2i}) - rank(S^s_{1i})	d^c_i = rank(R^c_{2i}) - rank(S^c_{1i})	$(d^s_i)^2$	$(d^c_i)^2$
3	1	117	1	1	218	4	5	-3	-4	9	16
	2	158	5	7	308	5	6	0	+1	0	1
	3	119	2	2	190	2	2	0	0	0	0
	4	151	4	5	191	3	3	+1	+2	1	4
	5	128	3	3	122	1	1	+2	+2	4	4
4	6	180	3	8	311	2	7	+1	+1	1	1
	7	148	1	4	209	1	4	0	0	0	0
	8	207	4	9	510	4	9	0	0	0	0
	9	155	2	6	312	3	8	-1	-2	1	4

All continued

The brain TCA-insoluble radioactivity and soluble valine specific activity data tabulated in Table A11 were obtained from the control groups of rats of experiments 3 and 4 (Section 3.2.2). Spearman rank correlation analysis was carried out as outlined in Tables A1 and A7.

Experiment 3, $\sum_i (d_i^s)^2 = 14$, $r_s = 0.300$, No correlation

Experiment 4, $\sum_i (d_i^s)^2 = 2$, $r_s = 0.800$, $P>0.1$

Combined data, $\sum_i (d_i^c)^2 = 30$, $r_s = 0.750$, $P\simeq 0.02$

TABLE A12

Exp no.	Animal no. (i)	TCA-insoluble radioactivity (R_{2i})	Rank of R^s_{2i}	Rank of R^c_{2i}	Specific activity of soluble valine (S_{1i})	Rank of S^s_{1i}	Rank of S^c_{1i}	d^s_i = rank(R^s_{2i}) - rank(S^s_{1i})	d^c_i = rank(R^c_{2i}) - rank(S^c_{1i})	$(d^s_i)^2$	$(d^c_i)^2$
3	1	117	6	8	218	9	13	-3	-5	9	25
	2	158	10	15	308	10	14	0	+1	0	1
	3	119	7	9	190	7	10	0	-1	0	1
	4	151	9	13	191	8	11	+1	+2	1	4
	5	128	8	10	122	5	5	+3	+5	9	25
	10	93	4	6	127	6	6	-2	0	4	0
	11	104	5	7	114	4	4	+1	+3	1	9
	12	38	2	2	98	3	3	-1	-1	1	1
	13	33	1	1	86	1	1	0	0	0	0
	14	66	3	3	91	2	2	+1	+1	1	1
4	6	180	6	16	311	5	15	+1	-1	1	1
	7	148	4	12	209	4	12	0	0	0	0
	8	207	7	17	510	7	17	0	0	0	0
	9	155	5	14	312	6	16	-1	-2	1	4
	15	75	1	4	178	3	9	-2	-5	4	25
	16	78	2	5	170	2	8	0	-3	0	9
	17	134	3	11	146	1	7	+2	+4	4	16

A12 continued

Results obtained from L-dopa-treated rats were combined with the control data given in Table A11 and new correlation coefficients calculated.

Experiment 3, $\sum_i (d_i^s)^2 = 26$, $r_s = 0.842$, $P<0.01$

Experiment 4, $\sum_i (d_i^s)^2 = 10$, $r_s = 0.821$, $P<0.05$

Combined data, $\sum_i (d_i^c)^2 = 122$, $r_s = 0.850$, $P<0.001$

TABLE A13

Exp no.	Animal no. (i)	TCA-insoluble radioactivity (R_{2i})	Rank of R^s_{2i}	Rank of R^c_{2i}	Specific activity of soluble lysine (S_{1i})	Rank of S^s_{1i}	Rank of S^c_{1i}	d^s_i = rank(R^s_{2i}) - rank(S^s_{1i})	d^c_i = rank(R^c_{2i}) - rank(S^c_{1i})	$(d^s_i)^2$	$(d^c_i)^2$
5	1	59	1	4	93	1	3	0	+1	0	1
	2	121	5	10	136	4	9	+1	+1	1	1
	3	92	3	8	117	3	8	0	0	0	0
	4	88	2	7	115	2	7	0	0	0	0
	5	101	4	9	150	5	10	-1	-1	1	1
6	6	87	5	6	112	5	6	0	0	0	0
	7	72	4	5	110	4	5	0	0	0	0
	8	54	1	1	97	3	4	-2	-3	4	9
	9	58	3	3	92	2	2	+1	+1	1	1
	10	56	2	2	81	1	1	+1	+1	1	1

A13 continued

The brain TCA-insoluble radioactivity and soluble lysine specific activity data tabulated in Table A13 were obtained from experiments 5 and 6 (Section 3.2.2). Spearman rank correlation analysis was carried out as outlined in Tables A1 and A7.

Experiment 5,	$\sum_i (d_i^s)^2$	2,	r_s = 0.900,	P=0.1
Experiment 6,	$\sum_i (d_i^s)^2$	6,	r_s = 0.700,	No correlation
Combined data,	$\sum_i (d_i^c)^2$	14,	r_s = 0.915,	P<0.001

TABLE A14 THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[3,4(n)-^{3}H]VALINE IN BRAIN. AN ANALYSIS OF DATA USING THE WILCOXON SIGNED-RANKS TEST

Exp no.	Pair no. (i)	q_{app_i}	$1-q_{app_i}$	Signed rank
4	1	0.731	+0.269	+1
	2	0.651	+0.349	+3
	3	2.269	-1.269	-8
3	8	1.361	-0.361	-4
	9	1.783	-0.783	-7
	10	0.622	+0.378	+5
	11	0.479	+0.521	+6
	12	0.689	+0.311	+2

The set of apparent incorporation quotients $q_{app_i} = I^D_{app_i}/I^C_{app_i}$, (see equation 3.2.19) tabulated above was calculated using paired data taken from two experiments in which L-[3,4(n)-^{3}H] valine was employed as the radioactive precursor. The pair numbers correspond to those in Table A3. The Wilcoxon signed-ranks test was used to determine whether the mean quotient ($\overline{q_{app}}$) differs significantly from $\overline{q_{app}} = 1$ (i.e., no drug effect). The mean quotient is 1.073 and the sum of the positive ranks (T) is 17, NS (see Table A3 for details).

TABLE A15 A COMPARISON OF THE EFFECT OF L-DOPA ON THE APPARENT INCORPORATION OF L-[4,5-^{3}H]LYSINE AND L-[3,4(n)-^{3}H]VALINE IN BRAIN

Lysine			Valine		
i^{LY}	$q^{LY}_{app_i}$	Rank of $q^{LY}_{app_i}$	i^{V}	$q^{V}_{app_i}$	Rank of $q^{V}_{app_i}$
1	0.820	14	1	0.731	13
2	0.132	2	2 Exp 4	0.651	10
3 Exp 5	0.063	1	3	2.269	18
4	0.263	3	8	1.361	16
5	0.964	15	9	1.783	17
6	0.408	6	10 Exp 3	0.622	9
7	0.506	8	11	0.479	7
8 Exp 6	0.718	12	12	0.689	11
9	0.283	4			
10	0.374	5			

The apparent incorporation data obtained from matched-paired L-dopa-treated and control rats of two lysine experiments (experiments 5 and 6) and two valine experiments (experiments 3 and 4) were used to calculate the apparent incorporation quotients tabulated above. The Wilcoxon two-sample test was used to determine the significance of the difference between the results obtained with [^{3}H]lysine and [^{3}H]valine.

$q^{LY}_{app_i} = I^{D}_{app}/I^{C}_{app}$ for the ith lysine pair and similarly $q^{V}_{app_i}$ refers to the ith valine pair (see equation 3.2.19). The pair numbers (i^{LY} and i^{V}) correspond to those in Tables A3 - A6. $\overline{q^{LY}_{app}} = 0.453$ and $\overline{q^{V}_{app}} = 1.073$. The sum of the ranks of q^{LY}_{app} is 70 and the sum of the ranks of q^{V}_{app} is 101. These results indicate that, in brain, the L-dopa-induced decrease in the apparent incorporation of [^{3}H] lysine is significantly greater than that of [^{3}H]valine, $P<0.05$ (two tail).

TABLE A16 A COMPARISON OF THE EFFECT OF L-DOPA ON THE RELATIVE INCORPORATION OF L-[4,5-^{3}H]LYSINE IN BRAIN AT 25°C AND ROOM TEMPERATURE

25°C				Room temperature			
i^{25}		$q^{25}_{rel_i}$	Rank of $q^{25}_{rel_i}$	i^{RT}		$q^{RT}_{rel_i}$	Rank of $q^{RT}_{rel_i}$
1	Exp 13	0.354	11	1	Exp 5	0.404	12
2		0.320	9	2		0.034	2
3		0.493	16	3		0.032	1
4		0.650	19	4		0.176	4
5		0.222	5	5		0.641	18
6	Exp 14	0.533	17	6	Exp 6	0.321	10
7		0.840	20	7		0.290	8
8		0.232	6	8		0.483	15
9		0.420	13	9		0.172	3
10		0.431	14	10		0.237	7

See Table A5 for details. $\overline{q^{25}_{rel}} = 0.450$ and $\overline{q^{RT}_{rel}} = 0.279$. The sum of the ranks of $q^{25}_{rel_i}$ is 130 and the sum of the ranks of $q^{RT}_{rel_i}$ is 80. $P>0.05$. It is concluded that the difference between the effect of L-dopa on the relative incorporation of lysine in brain at 25°C and at room temperature is not statistically significant.

TABLE A17

Source of variation	Sum of squares	Degrees of freedom	Estimate of variance	F
Between concentration levels (C)	277.16	5	55.43	1.47
Between replicates (R)	56.09	2	28.04	0.75
Residual (CR)	376.29	10	37.63	
Total	709.54	17		

An analysis of variance was carried out to determine whether there was a significant difference in the capacity of chopped rat brain tissue to incorporate L-[4,5-^{3}H]lysine into TCA-insoluble material in the presence of different concentrations of L-dopa (see Table 3.3.3). The TCA-insoluble radioactivity data were classified into a 6 x 3 grouping (6 concentration levels, three replicates). The calculations were performed on a computer using the IBM routine ANOVA (The Scientific Subroutines Package, Version III) (see acknowledgements). The difference between the levels of incorporation of radioactivity into TCA-insoluble material at different concentrations of L-dopa is not significant. (For $\nu_1 = 5$, $\nu_2 = 10$ the 5% point is 3.33. For $\nu_1 = 2$, $\nu_2 = 10$ the 5% point is 4.10). Tables of the distribution of F are given in FISHER and YATES (1963).

APPENDIX B. THE COMPARTMENTAL ANALYSIS OF TRACER KINETIC DATA

B1 A general mathematical model of metabolic compartmentation

MATSEN and FRANKLIN (1950) have outlined a general procedure for setting up and solving the rate equations defining a coupled set of first order reactions. The present treatment is essentially based on their method. In the case discussed by Matsen and Franklin the components of the system are interconverting chemical species while the present work deals with the distribution of radioactivity within a system of amino acid compartments following the introduction of a trace quantity of radioactive amino acid. The most general multi-compartment system is that in which every compartment exchanges with every other compartment (see Fig. 4.1). Any particular model may be fitted by setting the appropriate flow rates equal to zero. Although the analysis of nonsteady-state systems is possible, the problem is very much simplified if a steady state can be assumed. In the present treatment it is assumed that the system is in a steady state with respect to both the sizes of the compartments and the rates of exchange between them. It is also assumed that each compartment is homogeneous. This implies an instantaneous mixing of the material entering a compartment with the contents of that compartment. Compartments may, in fact, be defined in terms of instantaneous mixing. For example, although in the present work every cell in a given tissue may be regarded as a separate compartment, providing the tracer reaches and mixes with the contents of each cell simultaneously, then the population of cells may be treated as a single compartment.

The general steady-state system is defined by a set of n linear, first order, ordinary differential equations

$$\frac{dR_i}{dt} = -\sum_{j=1}^{n} F_{ij}S_i + \sum_{j=1}^{n} F_{ji}S_j, \quad i=1,2,\ldots,n. \qquad (B.1)$$

(NB. $F_{ii} = 0$, $i = 1,2,\ldots,n$. see Fig 4.1). Rate constants k_{ij} are defined by

$$k_{ij} = F_{ij}/Q_i \tag{B.2}$$

and therefore

$$F_{ij}S_i = k_{ij}Q_iS_i \tag{B.3a}$$

$$= k_{ij}R_i. \tag{B.3b}$$

Substitution of (B.3b) into (B.1) yields

$$\frac{dR_i}{dt} = -\sum_{j=1}^{n} k_{ij}R_i + \sum_{j=1}^{n} k_{ji}R_j, \quad i = 1,2,\ldots,n. \tag{B.4}$$

A set of new constants is defined by

$$K_{ij} = -k_{ji} \text{ for } i \neq j \tag{B.5a}$$

and

$$K_{ii} = \sum_{j=1}^{n} k_{ij}. \tag{B.5b}$$

Substitution of (B.5) into (B.4) gives

$$\frac{dR_i}{dt} = -\sum_{j=1}^{n} K_{ij}R_j, \quad i = 1,2,\ldots,n \tag{B.6a}$$

which using matrix notation is

$$\frac{d\underline{R}}{dt} = -K\underline{R} \tag{B.6b}$$

where $\underline{R}$ is a vector-valued function of time and K is a matrix of constant coefficients. Written in "standard" form* the set of n rate equations is

*Although it may appear unnecessarily repititious, a number of key equations have been written in both "standard" and matrix form in order to assist readers who may be unfamiliar with matrix and vector notation.

$$\begin{aligned}
\frac{dR_1}{dt} &= -K_{11}R_1 \cdots -K_{1j}R_j \cdots -K_{1n}R_n \\
&\vdots \\
\frac{dR_i}{dt} &= -K_{i1}R_1 \cdots -K_{ij}R_j \cdots -K_{in}R_n \\
&\vdots \\
\frac{dR_n}{dt} &= -K_{n1}R_1 \cdots -K_{nj}R_j \cdots -K_{nn}R_n
\end{aligned} \qquad \text{(B.6c)}$$

Matsen and Franklin have made an error in their paper since they have failed to recognise that the real matrix K will not always be symmetric (the models dealt with in Chapter 4 all yield unsymmetric matrices) and that the eigenvectors associated with the different eigenvalues (ie. the vectors $\underline{B}$ of their equation (7)) will not therefore necessarily be mutually orthogonal (compare with page 365 of SPIEGEL, 1971). The matrix B of their equation (2) can only be orthogonal if its column vectors (or row vectors) are orthonormal (see, for example, page 103 of AYRES, 1974) and so B is not, in general, an orthogonal matrix. It follows that the Q-basis defined by $\underline{Q} = \underline{BA}$ (see equation (2) of MATSEN and FRANKLIN, 1950) is not generally orthogonal (see, for example page 104 of AYRES, 1974). In their treatment, however, Matsen and Franklin have made the unnecessary restriction that an orthogonal Q-basis be selected.

A solution of the general system of linear differential equations (B.6) may be obtained as follows (compare with Chapter 6 of BUCK and BUCK, 1976). If λ is an eigenvalue of K and $\underline{B}$ is an associated eigenvector (ie., $K\underline{B} = \lambda \underline{B}$), then it is easily shown that the vector function

$$\underline{R} = \underline{B}e^{-\lambda t} \qquad \text{(B.7)}$$

is a solution of the equation

$$\frac{d\underline{R}}{dt} = -K\underline{R} \qquad \text{(B.6b)}$$

(compare with Theorem 6.1 of BUCK and BUCK, 1976). The proof is as follows.

$$\frac{d\underline{R}}{dt} = \frac{d}{dt}\underline{B}e^{-\lambda t} \tag{B.8a}$$

$$= -\lambda \underline{B}e^{-\lambda t}. \tag{B.8b}$$

Since

$$K\underline{R} = \lambda \underline{R} \tag{B.9a}$$

$$= \lambda \underline{B}e^{-\lambda t} \tag{B.9b}$$

it follows from (B.8b) that

$$\frac{d\underline{R}}{dt} = -K\underline{R}. \tag{B.6b}$$

The eigenvalues and eigenvectors are evaluated as follows. Since

$$K\underline{B} = \lambda \underline{B} \tag{B.10}$$

then

$$(K-I\lambda)\underline{B} = 0 \tag{B.11a}$$

where I is the unit matrix. Written without using matrix notation (B.11a) is

$$\sum_{j=1}^{n} (K_{ij}-\delta_{ij}\lambda)B_j = 0, \quad i = 1,2,\ldots,n \tag{B.11b}$$

where δ_{ij} is the 'Kronecker delta' defined by $\delta_{ij} = 1$ for $i = j$ and $\delta_{ij} = 0$ for $i \neq j$. Equation (B.11) has non-trivial solutions only if

$$|K-I\lambda| = 0 \tag{B.12}$$

(see Cramer's rule (Case 3) in Chapter 15 of SPIEGEL, 1971; also see Chapter 16 of STEPHENSON, 1966.). The above determinant is commonly called the secular determinant and its solution yields n distinct eigenvalues $(\lambda_1,\ldots,\lambda_j,\ldots,\lambda_n)$. The associated eigenvectors are then obtained from equation (B.11). The set of all solutions constitute a vector space called null space (N_τ) (see, for example, page 87 of AYRES, 1974 or page 290 of BUCK and BUCK, 1976). The n linearly-independent solution vectors $\underline{B}_j e^{-\lambda_j t}$, $j = 1,2,\ldots,n$ form a basis for N_τ and it therefore follows that the general solution is given by

$$\underline{R} = \sum_{j=1}^{n} C_j \underline{B}_j e^{-\lambda_j t} \qquad \text{(B.13a)}$$

$$= \sum_{j=1}^{n} \underline{D}_j e^{-\lambda_j t} \qquad \text{(B.13b)}$$

where C_j, $j = 1,2,\ldots,$ n are arbitrary scalers and $\underline{D}_j = C_j \underline{B}_j$. Equation (B.13) written without using vector notation is

$$R_i = \sum_{j=1}^{n} D_{ij} e^{-\lambda_j t}, \quad i = 1,2,\ldots,n. \qquad \text{(B.13c)}$$

In the present case the arbitrary scalers are fixed by the condition

$$\sum_{j=1}^{n} C_j \underline{B}_j = \underline{R}(t=o), \qquad \text{(B.14a)}$$

that is

$$B\underline{C} = \underline{R}(t=o) \qquad \text{(B.14b)}$$

where B is a matrix the column vectors of which are the n eigenvectors $\underline{B}_j$, $j = 1,2,\ldots,n$ and $\underline{C}$ is a column vector the components of which are the scalers C_j, $j = 1,2,\ldots,n$.

B2 Computational methods

The secular determinant (B.12) is not easily solved manually for a system of four or more compartments and a computer program (PROGRAM COMP) was therefore written which, given a set of flow rates, pool sizes and the initial conditions finds the required solution. A large number of algorithms have been developed for computing the eigenvalues and eigenvectors of real matrices, the present program making use of NAG library routines produced by the Nottingham Algorithm Group. A second program (PROGRAM OPT) was written for optimizing variable parameters (flow rates and/or pool sizes). A documentation of the library subroutines used is given in the NAG manual but, because a considerable knowledge of linear algebra is assumed, a brief nonmathematical description is included here. For completeness both programs are dealt with in their entirety although part of the algorithm follows directly from the theory outlined above. Listings and sample output of the two programs are given at the end of this appendix.

Program COMP

This program deals with a number of problems consecutively, the program being terminated by a "dummy" set of data in which $F_{12}=0$. The program first reads in the flow rates and pool sizes and checks for the sentinel. The rate constants are then calculated using (B.2) from which the matrix elements of K are evaluated according to equations (B.5a) and (B.5b). The program then proceeds to compute the eigenvalues and eigenvectors of K. If the elements of K vary considerably in size, then the computed eigensystem may not be of satisfactory accuracy. The error is reduced by transforming the matrix K to a matrix T, the eigensystem of which may be computed with accuracy. This procedure, which is referred to as balancing, is carried out by subroutine FOLATF which applies similarity transformations to the matrix K. The eigenvalues of T are the same as those of K. (It is easily shown that the eigenvalues of the similarity transform of K (ie., SKS^{-1}) are the same as those of K. Since $S\lambda IS^{-1}=\lambda I$, $\det(SKS^{-1}-\lambda I) = \det[(S(K-\lambda I)S^{-1}] = \det(K-\lambda I)$. See page 31 of WILKINSON, 1966). The amount of com-

putation involved in calculating the eigensystem of T is greatly reduced by transforming T to a matrix H which is of Upper Hessenberg form, that is a matrix such that $H_{ij} = 0$ for $i > j+1$ (see pages 39-40 of WILKINSON, 1966). This transformation is carried out by subroutine F01AKF. Subroutine F01APF uses output from F01AKF to set up a matrix V which defines the transformation of T to H. This is required by subroutine F02AQF to calculate the eigenvectors of T given the eigenvectors of H. Subroutine F02AQF first calculates all the eigenvalues of H and then calculates the eigenvectors by backsubstitution. Using the transformation matrix V given by F01APF the eigenvectors of T are then obtained. Subroutine F01AUF transforms the eigenvectors of the balanced matrix T to those of the real unsymmetric matrix K using information given by F01ATF. Subroutine LCPS employs library subroutine F04ATF to evaluate the vector $\underline{C}$ satisfying equation (B.14b) which is required by the main program to calculate the elements of the general solution matrix D (see equation B.13). The initial condition is specified by a DATA statement in LCPS. In the present application the zero-time radioactivity vector $\underline{R}(t=o)$ was given as $R_j=1$, $R_{i\neq j}=0$ where the jth compartment is that into which radioactivity is introduced at zero-time. The sum of the components of the radioactivity vector is therefore unity for all time and before the theoretical data can be compared with experimental results it is necessary to multiply $\underline{R}$ by some scaler. Subroutine SUBS calculates the radioactive content and specific activity of each compartment at intervals of time using equation (B.13b).

The program was written retaining the symbols used in the main text only where convenient. In some instances the notation was changed to comply with FORTRAN restrictions, while in other instances the retention of the same notation would have led to unnecessary programming complications. The variable names listed in the NAG documentation have not been changed in order that reference to the documentation may easily be made. For this reason in some instances the variable name allocated to a parameter changes as the parameter is passed to a subroutine. A list of variable names is given in Table B1.

Running instructions

Flow rates F_{12} to F_{1n} (n=7 in the example program) are punched on the first data card, F_{21} to F_{2n} on the second, etc., and the pool sizes on the (n+1)th card. A number of sets of (n+1) input cards may be placed one after the other, each of which will be processed in turn until the program is terminated by a set of dummy data in which $F_{12}=0$. It is therefore necessary to formulate every model in such a way that $F_{12}\neq 0$. Models consisting of m compartments with $m<n$ may be dealt with without changing the existing program simply by setting $F_{ij}=0$ for all $i>m$ and $j>m$, and $Q_i\neq 0$ for $i>m$.

Program OPT*

The above routine was incorporated into a second program which given a set of fixed parameters (flow rates and pool sizes) together with initial estimates of the variable parameters proceeds to obtain a least-squares fit of a set of experimental data points by iterative adjustment of the variable parameters. The program, a listing of which is given at the end of this appendix, employs NAG subroutine E04FAF to minimize the sum of the squares of the deviations. Details of the subroutines and a specification of the parameters are given in the NAG documentation (the variable names are listed in Table B2) but the following important points should be noted.

M specifies the number of residuals (ie., the number of experimental observations) and N the number of independent variables. X is an array of dimension N which, on entry, gives an estimate of the position of the minimum. On exit X contains the points which yielded the smallest sum of squares found by E04FAF. STEPMX(I), I=1,2,...,N specifies an estimate of the expected change in X(I) required to obtain the minimum. The routine works most efficiently if X(I) and X(I) + STEPMX(I) I=1,2,...,N straddle the minimum. On entry FTOL

*The variable names given below correspond to those used in the main program, some of which are modified on passing the parameters to the subroutines (see Table B2).

specifies the accuracy to which F (the smallest sum of squares) is required. A successful exit from E04FAF is dependent upon the condition that F is less than FTOL. In the present application the minimum sum of squares is expected to be nonzero and FTOL must therefore be set at a value equal to or greater than the machine accuracy. Machine accuracy is specified by X02AAF (NB. X02AAF returns the value of x, where x is the smallest positive real number satisfying 1.0+x> 1.0). On entry XTOL specifies the relative accuracy to which the position of the minimum is required. If Y is the best point obtained for one iteration and Z is the best point for the following iteration, the routine will terminate if for two successive iterations

$$|Y_I - Z_I| < \text{XTOL}*\text{STEPMX}(I),\ I=1,2,\ldots,N. \qquad \text{(B.15)}$$

XTOL must not be less than the value supplied by X02AAF. MAXCAL specifies the maximum number of iterations that will be performed. W is a real array of dimension at least IW which is used as working space. IW must specify the value of 2M+4N+MN+N(N+1)/2+P(M+2+2N) where P is the largest integer less than or equal to N+3+(N/3). The main program reads in the fixed parameters and initial estimates of the variable parameters using the format described above for PROGRAM COMP. The experimental data is supplied by DATA statements in subroutine RESID. RESID solves the secular equation (B.12) for the current set of flow rates and pool sizes as described above and calculates the required theoretical data points and residuals. Subroutine MONIT is used to monitor the progress of the minimization. The zero-time condition is supplied by a DATA statement in LCPS as described above so that the sum of the components of the radioactivity vector is unity. One parameter that must therefore be optimized is the scaler multiplyer. Finally it should be noted that in order to ensure that the minimization proceeds efficiently, F must be suitably scaled and should preferably be of the order of unity in the region of interest. Further, the problem should be formulated in such a way that the squares of the deviations are of the same order of magnitude in this region.

B3 A method for evaluating the rate constants of the exchange processes from the set of eigenvalues and eigenvectors

In Section 4.1 it was mentioned that two distinct approaches may be applied to the mathematical modelling of compartmental systems. In the present study a direct modelling procedure was employed. The alternative approach involves fitting the experimental data to linear combinations of exponential functions. Providing sufficient data is available to permit the evaluation of the complete set of eigenvalues and associated eigenvectors, the rate constants for the exchange processes may be determined. From the theory outlined in this appendix follows a simple procedure by which the matrix of rate constants may be obtained.

Let D be the matrix whose column vectors are the eigenvectors $\underline{D}_j$, j=1,2,...,n*, then since

$$K\underline{D}_j = \lambda_j \underline{D}_j, \quad j=1,2,\ldots,n \tag{B.16a}$$

that is

$$\begin{bmatrix} K_{11} & \cdots & K_{1n} \\ \vdots & & \vdots \\ K_{n1} & \cdots & K_{nn} \end{bmatrix} \begin{bmatrix} D_{11} \\ \vdots \\ D_{n1} \end{bmatrix} = \lambda_1 \begin{bmatrix} D_{11} \\ \vdots \\ D_{n1} \end{bmatrix}$$

$$\vdots$$

$$\begin{bmatrix} K_{11} & \cdots & K_{1n} \\ \vdots & & \vdots \\ K_{n1} & \cdots & K_{nn} \end{bmatrix} \begin{bmatrix} D_{1j} \\ \vdots \\ D_{nj} \end{bmatrix} = \lambda_j \begin{bmatrix} D_{1j} \\ \vdots \\ D_{nj} \end{bmatrix} \tag{B.16b}$$

$$\vdots$$

$$\begin{bmatrix} K_{11} & \cdots & K_{1n} \\ \vdots & & \vdots \\ K_{n1} & \cdots & K_{nn} \end{bmatrix} \begin{bmatrix} D_{1n} \\ \vdots \\ D_{nn} \end{bmatrix} = \lambda_n \begin{bmatrix} D_{1n} \\ \vdots \\ D_{nn} \end{bmatrix}$$

*Any scaler multiple of $\underline{B}_j$ is an eigenvector of K and since $\underline{D}_j = C_j \underline{B}_j$ where C_j is a scaler it follows that the vectors $\underline{D}_j$ j=1,2,...,n are eigenvectors of K.

and

$$KD = \begin{bmatrix} K_{11} & \cdots & K_{1n} \\ \vdots & & \vdots \\ K_{n1} & \cdots & K_{nn} \end{bmatrix} \begin{bmatrix} D_{11} & \cdots & D_{1n} \\ \vdots & & \vdots \\ D_{n1} & \cdots & D_{nn} \end{bmatrix} \tag{B.17}$$

it follows that

$$KD = \begin{bmatrix} \lambda_1 D_{11} & \cdots & \lambda_n D_{1n} \\ \vdots & & \vdots \\ \lambda_1 D_{n1} & \cdots & \lambda_n D_{nn} \end{bmatrix} \tag{B.18a}$$

$$= D\Lambda \tag{B.18b}$$

where Λ is the diagonal matrix with the eigenvalues λ_j as the diagonal elements (compare with page 367 of SPIEGEL, 1971). Multiplying (B.18b) on the right by the inverse of D (ie., D^{-1}) yields

$$KDD^{-1} = D\Lambda D^{-1}, \tag{B.19a}$$

that is

$$K = D\Lambda D^{-1} \tag{B.19b}$$

and thus the elements of K may be determined and the rate constants (k_{ij}) evaluated.

TABLE B1. PROGRAM COMP PARAMETERS*

Main program and subroutine SUBS	Subroutine LCPS	Text	Parameter
N	N	n	Number of compartments (max)
F(I,J)		F_{ij}	Flow rates
Q(I)		Q_i	Pool sizes
B(I,J)		k_{ij}	Rate constants
A		K	Matrix of constant coefficients
Z (on exit)	A	B	Eigenvector array
WR(I)		λ_j	Real part of eigenvalue
WI(I)			Imaginary part of eigenvalue**
	B	$\underline{R}(t=o)$	Zero-time radioactivity vector
C	C	$\underline{C}$	Vector of arbitrary coefficients
Y		D	General solution matrix
R(I)		R_i	Radioactive content of compartments
S(I)		S_i	Specific activity of compartments

*This list is not complete but contains only those parameters referred to in the main text. A complete list of parameters used in connection with the NAG library subroutines is given in the NAG documentation.

**In the present application all eigenvalues are real.

TABLE B2. PROGRAM OPT PARAMETERS*

Main program	Subroutines RESID and SUBS	Subroutine LCPS	Subroutine MONIT	Text	Parameter
NO	N	N		n	Number of compartments (max)
FR(I,J)	FR(I,J)			F_{ij}	Flow rates
Q(I)	Q(I)			Q_i	Pool sizes
M	MD		M	M	Number of experimental points (residuals)
N	ND		N	N	Number of variable parameters
X(I)	XC(I)		XC(I)	X(I)	Variable parameters
FTOL (on entry)				FTOL	Accuracy to which the sum of squares is required
XTOL				XTOL	Accuracy to which the minimum is required
STEPMX(I)				STEPMX(I)	Expected change in variable parameters
IPRINT			IPRINT		Frequency of call of MONIT
MAXCAL				MAXCAL	Maximum number of iterations
			ITERC		Number of iterations

*This list is not complete. In particular a number of parameters that pass information between the NAG library subroutines are omitted. A complete list of these parameters is given in the NAG documentation.

TABLE B2. Continued.....(2)

Main program	Subroutines RESID and SUBS	Subroutine LCPS	Subroutine MONIT	Text	Parameter
					Experimental data points
	T(IPT)				a) Time
	SOL(IPT)				b) Specific activity of soluble lysine pool
	INSOL(IPT)				c) Specific activity of protein-bound lysine
B(I,J)	B(I,J)			k_{ij}	Rate constants
	A			K	Matrix of constant coefficients
	Z (on exit)	A		B	Eigenvector array
WR(I)	WR(I)			λ_j	Real part of eigenvalue
WI(I)	WI(I)				Imaginary part of eigenvalue
		B		$\underline{R}(t=o)$	Zero-time radioactivity vector
	C	C		$\underline{C}$	Vector of arbitrary coefficients
Y	Y			D	General solution matrix
R(I)	RC(IPT/IPT+4)				Residuals
F			FC	F	Sum of squares
	R(I)			R_i	Radioactive content of compartments
	S(I)			S_i	Specific activity of compartments
	SCALER				Scaler multiplyer

```
      PROGRAM COMP (INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
      REAL A(7,7),D(7),Z(7,7),WR(7),WI(7),X02AAF,B(7,7),C(7),Y(7,7)
      REAL F(7,7),Q(7)
      INTEGER X02BAF,NIN,NOUT,I,N,J,IA,IT,K,L,IZ,IFAIL,M,INTGER(7)
      DATA NIN/5/,NOUT/6/
C
      N=7
C     N SPECIFIES THE NUMBER OF COMPARTMENTS
C     SPECIFY MAXIMUM NUMBER (NO) OF CALCULATIONS PER RUN
      DO 7 NO=1,100
C
      READ(NIN,99998) ((F(I,J),J=1,N),I=1,N)
C     CHECK FOR SENTINEL
      IF(F(1,2).EQ.0.0)STOP
      WRITE(NOUT,99984) ((F(I,J),J=1,N),I=1,N)
      READ(NIN,99987) (Q(I),I=1,N)
      WRITE(NOUT,99986) (Q(I),I=1,N)
C     CALCULATE RATE CONSTANTS
      DO 14 I=1,N
      DO15 J=1,N
      B(I,J)=F(I,J)/Q(I)
   15 CONTINUE
   14 CONTINUE
      WRITE(NOUT,99999) ((B(I,J),J=1,N),I=1,N)
C     EVALUATE MATRIX OF CONSTANT COEFFICIENTS
      DO 1 I=1,N
      DO 2 J=1,N
      IF(I.EQ.J)GO TO 3
      A(J,I)=-B(I,J)
      GO TO 2
    3 A(I,J)=B(I,1)+B(I,2)+B(I,3)+B(I,4)+B(I,5)+B(I,6)+B(I,7)
    2 CONTINUE
    1 CONTINUE
      WRITE(NOUT,99993) ((A(I,J),J=1,N),I=1,N)
      IA=7
C      BALANCE MATRIX
      CALL F01ATF(N, X02BAF(IT), A, IA, K, L, D)
C     REDUCE TO HESSENBERG FORM
      CALL F01AKF(N, K, L, A, IA, INTGER)
      IZ=7
C     ACCUMULATE TRANSFORMATIONS
      CALL F01APF(N, K, L, INTGER, A, IA, Z, IZ)
C     CALCULATE EIGENVALUES AND EIGENVECTORS
      IFAIL=1
      CALL F02AQF(N,K,L,X02AAF(IT),A,IA,Z,IZ,WR,WI,INTGER,IFAIL)
      IF (IFAIL.EQ.0)GO TO 20
      WRITE (NOUT,99996)IFAIL
      STOP
   20 M=N
C     EIGENVECTORS OF ORIGINAL MATRIX FROM THOSE OF BALANCED MATRIX
      CALL F01AUF(N,K,L,M,D,Z,IZ)
      WRITE (NOUT,99995) (WR(I),WI(I),I=1,N)
      WRITE (NOUT,99994) ((Z(I,J),J=1,N),I=1,N)
C     FIND THE GENERAL SOLUTION THAT SATISFIES THE INITIAL CONDITIONS
      CALL LCPS(Z,N,C,NOUT)
C     EVALUATE ELEMENTS OF THE GENERAL SOLUTION MATRIX
      DO 5 I=1,N
```



```
      DO 6 J=1,N
      Y(I,J)=Z(I,J)*C(J)
    6 CONTINUE
    5 CONTINUE
      WRITE (NOUT,99992) ((Y(I,J),J=1,N),I=1,N)
C     CALCULATE THE RADIOACTIVE CONTENT AND SPECIFIC ACTIVITIES OF POOLS
      CALL SUBS(Y,WR,N,Q,NOUT)
    7 CONTINUE
      STOP
99999 FORMAT (1H0,14HRATE CONSTANTS/(1H ,1P7E16.4))
99998 FORMAT (1P7E10.4)
99996 FORMAT (25H0ERROR IN F02AQF IFAIL= , I2)
99995 FORMAT (12H0EIGENVALUES/(2H (, 1PE10.4, 1H,, F5.2, 1H)))
99994 FORMAT (18H0EIGENVECTOR ARRAY/(1H ,7F12.6))
99993 FORMAT (32H0MATRIX OF CONSTANT COEFFICIENTS/(1H ,1P7E16.4))
99992 FORMAT (24H0GENERAL SOLUTION MATRIX/(1H ,1P7E16.4))
99987 FORMAT(1P7E10.4)
99986 FORMAT(22H0AMINO ACID POOL SIZES/(1H ,1P7E16.4))
99984 FORMAT(1H1,10HFLOW RATES/(1H ,1P7E16.4))
      END
```

```
      SUBROUTINE LCPS(A,N,C,NOUT)
C     FINDS THE GENERAL SOLUTION SATISFYING THE INITIAL CONDITIONS
      REAL A(7,7), B(7), C(7), AA(7,7),WKS1(7),WKS2(7)
      INTEGER NIN, NOUT, I,N,J,IA,IAA,IFAIL
C
C     SPECIFY THE INITIAL CONDITIONS
      DATA(B(I),I=1,7)/0.0,0.0,0.0,0.0,0.0,1.0,0.0/
C
      IA=7
      IAA=7
      IFAIL=1
C     CALCULATE COEFFICIENTS
      CALL F04ATF(A,IA,B,N,C,AA,IAA,WKS1,WKS2, IFAIL)
      IF (IFAIL.EQ.0) GO TO 20
      WRITE (NOUT,99990) IFAIL
99990 FORMAT (25H0ERROR IN F04ATF IFAIL= ,I2)
      STOP
   20 WRITE(NOUT,99991) (C(I),I=1,N)
99991 FORMAT (13H0COEFFICIENTS/(1H ,F10.6))
      RETURN
      END
```



```
      SUBROUTINE SUBS(Y,WR,N,Q,NOUT)
C     CALCULATES THE RADIOACTIVE CONTENT AND SPECIFIC ACTIVITIES OF THE
C     COMPARTMENTS
      REAL Y(7,7),S(7),R(7),WR(7),Q(7)
      WRITE(NOUT,99988)
99988 FORMAT(1H0,7X,4HTIME,16X,39H(A) RADIOACTIVE CONTENT OF COMPARTMENT
     1S,/,1H ,27X,37H(B) SPECIFIC ACTIVITY OF COMPARTMENTS)
C
C     SPECIFY RANGE OF TIME
      DO 12 L=1,2
      DO 8 K=10,200,10
      T=K*(10**L)/100.
C
C     CALCULATE THE RADIOACTIVE CONTENT OF THE COMPARTMENTS
      DO 11 I=1,N
   11 R(I)=0.0
      DO 9  I=1,N
      DO10 J=1,N
      X=WR(J)*T
C     ENSURE ARGUMENT IS NOT TOO LARGE
      IF(X.GT.600.0)GO TO 10
      R(I)=R(I)+Y(I,J)*EXP(-X)
   10 CONTINUE
    9 CONTINUE
      WRITE(NOUT,99989)T,(R(I),I=1,N)
99989 FORMAT(1H ,F15.5,10X,3H(A),2X,7F13.7)
C
C     CALCULATE SPECIFIC ACTIVITIES
      DO 13 I=1,N
   13 S(I)=R(I)/Q(I)
      WRITE(NOUT,99985) (S(I),I=1,N)
99985 FORMAT(1H ,25X,3H(B),2X,1P7E13.4)
    8 CONTINUE
   12 CONTINUE
      RETURN
      END
```

FLOW RATES

0.	3.0000E-01	0.	2.4000E-03	0.	2.0000E-04	1.5000E-04
2.6000E-01	0.	4.0000E-02	0.	0.	0.	0.
0.	0.	0.	0.	0.	0.	0.
2.4000E-03	0.	0.	0.	1.7710E-03	0.	0.
0.	0.	0.	1.7710E-03	0.	0.	0.
2.0000E-04	0.	0.	0.	0.	0.	0.
1.5000E-04	0.	0.	0.	0.	0.	0.

AMINO ACID POOL SIZES

1.6540E+00	2.6000E+01	1 0000E+00	5.8730E-02	8.4100E+00	1.0000E-03	8.4100E+00

RATE CONSTANTS

0.	1.8138E-01	0.	1.4510E-03	0.	1.2092E-04	9.0689E-05
1.0000E-02	0.	1.5385E-03	0.	0.	0.	0.
0.	0.	0.	0.	0.	0.	0.
4.0865E-02	0.	0.	0.	3.0155E-02	0.	0.
0.	0.	0.	2.1058E-04	0.	0.	0.
2.0000E-01	0.	0.	0.	0.	0.	0.
1.7836E-05	0.	0.	0.	0.	0.	0.

MATRIX OF CONSTANT COEFFICIENTS

1.8304E-01	-1.0000E-02	0.	-4.0865E-02	0.	-2.0000E-01	-1.7836E-05
-1.8138E-01	1.1538E-02	0.	0.	0.	0.	0.
0.	-1.5385E-03	0.	0.	0.	0.	0.
-1.4510E-03	0.	0.	7.1020E-02	-2.1058E-04	0.	0.
0.	0.	0.	-3.0155E-02	2.1058E-04	0.	0.
-1.2092E-04	0.	0.	0.	0.	2.0000E-01	0.
-9.0689E-05	0.	0.	0.	0.	0.	1.7836E-05

EIGENVALUES

(0. , 0.00)
(1.9097E-01, 0.00)
(2.0253E-01, 0.00)
(7.0695E-02, 0.00)
(1.4969E-03, 0.00)
(1.1779E-04, 0.00)
(1.7768E-05, 0.00)

EIGENVECTOR ARRAY

0.000000	-1.763730	-1.117855	.071622	.431913	-.020699	.003018
0.000000	1.782897	1.061570	-.219598	7.801515	-.328734	.047518
1.000000	-.014363	-.008064	.004779	-8.018305	4.293685	-4.114365
0.000000	.021342	.012337	.250312	.008417	-.012158	.000115
0.000000	-.003374	-.001839	-.107090	-.197320	-3.950863	.017992
0.000000	-.023610	.053350	.000067	.000263	-.000013	.000002
0.000000	.000838	.000501	-.000092	-.026483	.018781	4.045719

COEFFICIENTS

1.000000
-9.251969
14.649045
.062424
.122512
-.006723
.000938

GENERAL SOLUTION MATRIX

0.	1.6318E+01	-1.6376E+01	4.4709E-03	5.2914E-02	1.3917E-04	2.8300E-06
0.	-1.6495E+01	1.5551E+01	-1.3708E-02	9.5578E-01	2.2102E-03	4.4555E-05
1.0000E+00	1.3289E-01	-1.1813E-01	2.9832E-04	-9.8234E-01	-2.8868E-02	-3.8578E-03
0.	-1.9746E-01	1.8072E-01	1.5625E-02	1.0312E-03	8.1741E-05	1.0787E-07
0.	3.1214E-02	-2.6935E-02	-6.6850E-03	-2.4174E-02	2.6563E-02	1.6870E-05
0.	2.1844E-01	7.8152E-01	4.1809E-06	3.2233E-05	8.4189E-08	1.7112E-09
0.	-7.7500E-03	7.3332E-03	-5.7368E-06	-3.2445E-03	-1.2627E-04	3.7934E-03

TIME		(A) RADIOACTIVE CONTENT OF COMPARTMENTS (B) SPECIFIC ACTIVITY OF COMPARTMENTS						
1.00000	(A)	.1651972	.0159195	.0000084	.0001248	.0000013	.8187407	.0000080
	(B)	9.9877E-02	6.1229E-04	8.4358E-06	2.1249E-03	1.5493E-07	8.1874E+02	9.5022E-07
2.00000	(A)	.2731063	.0560121	.0000613	.0004298	.0000093	.6703529	.0000282
	(B)	1.6512E-01	2.1543E-03	6.1324E-05	7.3184E-03	1.1089E-06	6.7035E+02	3.3574E-06
3.00000	(A)	.3389181	.1111028	.0001884	.0008337	.0000282	.5488725	.0000563
	(B)	2.0491E-01	4.2732E-03	1.8843E-04	1.4195E-02	3.3538E-06	5.4887E+02	6.6893E-06
4.00000	(A)	.3742238	.1745226	.0004074	.0012793	.0000600	.4494182	.0000888
	(B)	2.2625E-01	6.7124E-03	4.0742E-04	2.1782E-02	7.1351E-06	4.4942E+02	1.0557E-05
5.00000	(A)	.3878163	.2415057	.0007272	.0017275	.0001054	.3679944	.0001235
	(B)	2.3447E-01	9.2887E-03	7.2723E-04	2.9414E-02	1.2527E-05	3.6799E+02	1.4682E-05
6.00000	(A)	.3863205	.3087228	.0011506	.0021527	.0001639	.3013309	.0001587
	(B)	2.3357E-01	1.1874E-02	1.1506E-03	3.6654E-02	1.9490E-05	3.0133E+02	1.8367E-05
7.00000	(A)	.3746871	.3739193	.0016761	.0025390	.0002347	.2467506	.0001922
	(B)	2.2653E-01	1.4382E-02	1.6761E-03	4.3231E-02	2.7910E-05	2.4675E+02	2.2977E-05
8.00000	(A)	.3565796	.4356382	.0022993	.0028775	.0003165	.2020624	.0002264
	(B)	2.1559E-01	1.6755E-02	2.2993E-03	4.8996E-02	3.7629E-05	2.0206E+02	2.6924E-05
9.00000	(A)	.3346765	.4930067	.0030143	.0031646	.0004076	.1654726	.0002578
	(B)	2.0234E-01	1.8962E-02	3.0143E-03	5.3884E-02	4.8468E-05	1.6547E+02	3.0653E-05
10.00000	(A)	.3109058	.5455741	.0038138	.0033998	.0005066	.1355128	.0002871
	(B)	1.8797E-01	2.0984E-02	3.8138E-03	5.7889E-02	6.0240E-05	1.3551E+02	3.4134E-05
11.00000	(A)	.2866282	.5931890	.0046904	.0035851	.0006119	.1109812	.0003142
	(B)	1.7329E-01	2.2815E-02	4.6904E-03	6.1044E-02	7.2763E-05	1.1098E+02	3.7355E-05
12.00000	(A)	.2627778	.6359068	.0056365	.0037240	.0007221	.0908938	.0003391
	(B)	1.5887E-01	2.4458E-02	5.6365E-03	6.3408E-02	8.5863E-05	9.0894E+01	4.0316E-05
13.00000	(A)	.2399717	.6739204	.0066446	.0038206	.0008358	.0744450	.0003618
	(B)	1.4509E-01	2.5920E-02	6.6446E-03	6.5053E-02	9.9382E-05	7.4445E+01	4.3025E-05
14.00000	(A)	.2185928	.7075098	.0077078	.0038796	.0009518	.0609755	.0003826
	(B)	1.3216E-01	2.7212E-02	7.7078E-03	6.6059E-02	1.1317E-04	6.0976E+01	4.5495E-05
15.00000	(A)	.1988542	.7370045	.0088195	.0039059	.0010690	.0499454	.0004015
	(B)	1.2023E-01	2.8346E-02	8.8195E-03	6.6505E-02	1.2712E-04	4.9945E+01	4.7743E-05
16.00000	(A)	.1808476	.7627570	.0099736	.0039039	.0011866	.0409126	.0004187
	(B)	1.0934E-01	2.9337E-02	9.9736E-03	6.6472E-02	1.4110E-04	4.0913E+01	4.9788E-05
17.00000	(A)	.1645795	.7851244	.0111647	.0038781	.0013038	.0335152	.0004344
	(B)	9.9504E-02	3.0197E-02	1.1165E-02	6.6032E-02	1.5502E-04	3.3515E+01	5.1648E-05
18.00000	(A)	.1499992	.8044549	.0123878	.0038325	.0014198	.0274572	.0004486
	(B)	9.0689E-02	3.0941E-02	1.2388E-02	6.5256E-02	1.6882E-04	2.7457E+01	5.3342E-05
19.00000	(A)	.1370188	.8210804	.0136386	.0037708	.0015341	.0224957	.0004616
	(B)	8.2841E-02	3.1580E-02	1.3639E-02	6.4205E-02	1.8242E-04	2.2496E+01	5.4887E-05
20.00000	(A)	.1255279	.8353107	.0149130	.0036962	.0016464	.0184323	.0004735
	(B)	7.5894E-02	3.2127E-02	1.4913E-02	6.2935E-02	1.9577E-04	1.8432E+01	5.6300E-05
10.00000	(A)	.3109058	.5455741	.0038138	.0033998	.0005066	.1355128	.0002871
	(B)	1.8797E-01	2.0984E-02	3.8138E-03	5.7889E-02	6.0240E-05	1.3551E+02	3.4134E-05
20.00000	(A)	.1255279	.8353107	.0149130	.0036962	.0016464	.0184323	.0004735
	(B)	7.5894E-02	3.2127E-02	1.4913E-02	6.2935E-02	1.9577E-04	1.8432E+01	5.6300E-05
30.00000	(A)	.0666867	.8965194	.0283764	.0027148	.0026114	.0025368	.0005545
	(B)	4.0318E-02	3.4482E-02	2.8376E-02	4.6225E-02	3.1051E-04	2.5368E+00	6.5932E-05
40.00000	(A)	.0531374	.8984344	.0422143	.0019366	.0032973	.0003728	.0006072
	(B)	3.2127E-02	3.4555E-02	4.2214E-02	3.2974E-02	3.9207E-04	3.7277E-01	7.2204E-05
50.00000	(A)	.0498790	.8881414	.0559630	.0014871	.0037991	.0000770	.0006535
	(B)	3.0157E-02	3.4159E-02	5.5963E-02	2.5320E-02	4.5174E-04	7.6954E-02	7.7709E-05
60.00000	(A)	.0486604	.8756275	.0695314	.0012475	.0041992	.0000360	.0006980
	(B)	2.9420E-02	3.3678E-02	6.9531E-02	2.1241E-02	4.9930E-04	3.6042E-02	8.3000E-05
70.00000	(A)	.0478372	.8628217	.0829041	.0011204	.0045450	.0000300	.0007417
	(B)	2.8922E-02	3.3185E-02	8.2904E-02	1.9077E-02	5.4043E-04	3.0027E-02	8.8187E-05
80.00000	(A)	.0471011	.8500934	.0960802	.0010505	.0048614	.0000288	.0007846
	(B)	2.8477E-02	3.2696E-02	9.6080E-02	1.7887E-02	5.7805E-04	2.8817E-02	9.3289E-05
90.00000	(A)	.0463937	.8375196	.1090616	.0010091	.0051609	.0000283	.0008268
	(B)	2.8049E-02	3.2212E-02	1.0906E-01	1.7183E-02	6.1366E-04	2.8280E-02	9.8313E-05
100.00000	(A)	.0457023	.8251189	.1218509	.0009820	.0054497	.0000278	.0008684
	(B)	2.7631E-02	3.1735E-02	1.2185E-01	1.6720E-02	6.4800E-04	2.7843E-02	1.0326E-04
110.00000	(A)	.0450233	.8128963	.1344508	.0009620	.0057309	.0000274	.0009094
	(B)	2.7221E-02	3.1265E-02	1.3445E-01	1.6380E-02	6.8144E-04	2.7426E-02	1.0813E-04
120.00000	(A)	.0443553	.8008523	.1468640	.0009456	.0060061	.0000270	.0009498
	(B)	2.6817E-02	3.0802E-02	1.4686ED01	1.6100E-02	7.1416E-04	2.7019E-02	1.1293E-04
130.00000	(A)	.0436978	.7889857	.1590933	.0009310	.0062761	.0000266	.0009895
	(B)	2.6419E-02	3.0346E-02	1.5909E-01	1.5853E-02	7.4626E-04	2.6618E-02	1.1766E-04
140.00000	(A)	.0430503	.7772947	.1711414	.0009175	.0065412	.0000262	.0010287
	(B)	2.6028E-02	2.9896E-02	1.7114E-01	1.5622E-02	7.7779E-04	2.6224E-02	1.2231E-04
150.00000	(A)	.0424125	.7657770	.1830110	.0009046	.0068019	.0000258	.0010672
	(B)	2.5642E-02	2.9453E-02	1.8301E-01	1.5403E-02	8.0879E-04	2.5835E-02	1.2690E-04
160.00000	(A)	.0417842	.7544302	.1947046	.0008921	.0070582	.0000255	.0011052
	(B)	2.5263E-02	2.9017E-02	1.9470E-01	1.5189E-02	8.3926E-04	2.5452E-02	1.3142E-04
170.00000	(A)	.0411653	.7432519	.2062251	.0008798	.0073102	.0000251	.0011426
	(B)	2.4888E-02	2.8587E-02	2.0623E-01	1.4981E-02	8.6923E-04	2.5075E-02	1.3587E-04
180.00000	(A)	.0405556	.7322396	.2175748	.0008678	.0075581	.0000247	.0011795
	(B)	2.4520E-02	2.8163E-02	2.1757E-01	1.4776E-02	8.9870E-04	2.4704E-02	1.4025E-04
190.00000	(A)	.0399549	.7213909	.2287563	.0008560	.0078018	.0000243	.0012158
	(B)	2.4157E-02	2.7746E-02	2.2876E-01	1.4575E-02	9.2768E-04	2.4338E-02	1.4456E-04
200.00000	(A)	.0393632	.7107033	.2397722	.0008443	.0080415	.0000240	.0012515
	(B)	2.3799E-02	2.7335E-02	2.3977E-01	1.4377E-02	9.5618E-04	2.3978E-02	1.4881E-04

```
      PROGRAM OPT (INPUT,OUTPUT,TAPE5=INPUT,TAPE6=OUTPUT)
C
C     IN THIS EXAMPLE PROGRAM THE OPTIMIZATION ROUTINE IS USED TO
C     OBTAIN A LEAST-SQUARES FIT OF THE DATA GIVEN IN FIGS.2 AND 3
C     OF DUNLOP ET AL. (1974) (J. NEUROCHEM. 22. 821-830) BY ADJUSTMENT
C     OF THE UNKNOWN FLOW RATES OF THE 4-COMPARTMENT MODEL SHOWN
C     IN FIG. 4.13.
C
C     NOTE. THE DIMENSION OF W MUST BE GREATER THAN OR EQUAL TO IW.
C     IW = N*(4+M)+2*M+P*(M+2+2*N)+N*(N+1)/2 WHERE P IS THE LARGEST
C     INTEGER LESS THAN OR EQUAL TO N+3+(N/3)
C
      INTEGER NOUT,M,N,IW,IPRINT,MAXCAL,IFAIL,I,NIN,NO,J
      REAL FTOL,RR,XTOL,F,X(3),X02AAF,STEPMX(3),R(8),W(170)
      REAL FR(5,5),Q(5),B(5,5),Y(5,5),WR(5),WI(5)
      COMMON FR,Q,Y,WR,WI,B
      EXTERNAL RESID, MONIT
      DATA NIN/5/,NOUT/6/
      NO=5
C     NO SPECIFIES THE NUMBER OF COMPARTMENTS. THE PRESENT EXAMPLE
C     PROGRAM DEALS WITH A 4-COMPARTMENT MODEL. COMPARTMENT 5 IS
C     THEREFORE ISOLATED
C
C     READ IN FIXED PARAMETERS AND INITIAL ESTIMATES OF VARIABLE
C     PARAMETERS
      READ(NIN,99994)((FR(I,J),J=1,NO),I=1,NO)
      WRITE(NOUT,99993)((FR(I,J),J=1,NO),I=1,NO)
      READ(NIN,99992)(Q(I),I=1,NO)
      WRITE(NOUT,99991)(Q(I),I=1,NO)
C     SPECIFY NUMBER OF RESIDUALS AND VARIABLE PARAMETERS
      M=8
      N=3
C     SPECIFY VARIABLE PARAMETERS
C     X(1) IS THE SCALER MULTIPLYER
      X(1)=1.4
      X(2)=FR(1,2)
      X(3)=FR(2,3)
C     SPECIFY ACCURACY TO WHICH THE SUM OF SQUARES AND THE POSITION OF
C     THE MINIMUM IS REQUIRED
      FTOL = X02AAF(RR)
      XTOL=5.0E-05
C     INDICATE THE EXPECTED CHANGE IN X(I) REQUIRED TO OBTAIN A MINIMUM
      STEPMX(1)=0.1
      STEPMX(2)=0.5E-03
      STEPMX(3)=2.0E-06
C
      IW = N+3+N/3
      IW =N*(4+M)+2*M+IW*(M+2+2*N)+N*(N+1)/2
C
C     SPECIFY THE FREQUENCY OF THE CALL OF MONIT
      IPRINT = 1
C     SPECIFY THE MAXIMUM NUMBER OF ITERATIONS
      MAXCAL=50
C
      IFAIL = 0
      CALL E04FAF(M,N,X,R,F,FTOL,XTOL,STEPMX,W,IW,RESID,
     1MONIT,IPRINT,MAXCAL,IFAIL)
```



```
C
C     PRINT OUT THE FINAL SUM OF SQUARES, THE FINAL FTOL AND THE FINAL
C     VALUES OF THE VARIABLE PARAMETERS AND RESIDUALS
C
      WRITE(NOUT,99998)F,FTOL
      WRITE(NOUT,99997)(X(I),I=1,N)
      WRITE(NOUT,99996)(R(I),I=1,M)
      WRITE(NOUT,99995)IFAIL
C
C     PRINT OUT THE COMPLETE SET OF FINAL FLOW RATES, RATE CONSTANTS
C     AND POOL SIZES AND THE ASSOCIATED EIGENVALUES AND GENERAL
C     SOLUTION EIGENVECTORS
C
      WRITE(NOUT,99990)((FR(I,J),J=1,NO),I=1,NO)
      WRITE(NOUT,99989)(Q(I),I=1,NO)
      WRITE(NOUT,99988)((B(I,J),J=1,NO),I=1,NO)
      WRITE(NOUT,99987)((Y(I,J),J=1,NO),I=1,NO)
      WRITE(NOUT,99986)(WR(I),WI(I),I=1,NO)
C
C     CALCULATE THE RADIOACTIVE CONTENT AND SPECIFIC ACTIVITIES OF
C     THE COMPARTMENTS
C
      CALL SUBS(Y,WR,NO,Q,NOUT,X(1))
      STOP
99998 FORMAT(25H FINAL SUM OF SQUARES IS ,1PE10.4,15H FINAL FTOL WAS,
     1F5.1)
99997 FORMAT(13H AT THE POINT,1P3E12.4)
99996 FORMAT(13H WITH RESIDS ,1P8E12.4)
99995 FORMAT(22H THIS HAS ERROR NUMBER, I3)
99994 FORMAT(1P5E10.4)
99993 FORMAT(19H1INITIAL FLOW RATES/(1H ,1P5E16.4))
99992 FORMAT(1P5E10.4)
99991 FORMAT(30H0INITIAL AMINO ACID POOL SIZES/(1H ,1P5E16.4/))
99990 FORMAT(17H0FINAL FLOW RATES/(1H ,1P5E16.4))
99989 FORMAT(28H0FINAL AMINO ACID POOL SIZES/(1H ,1P5E16.4))
99988 FORMAT(21H0FINAL RATE CONSTANTS/(1H , 1P5E16.4))
99987 FORMAT(30H0FINAL GENERAL SOLUTION MATRIX/(1H ,1P5E16.4))
99986 FORMAT(18H0FINAL EIGENVALUES/(2H (, 1PE10.4, 1H,,F5.2, 1H)))
      END
```

```
      SUBROUTINE RESID(MD,ND,XC,RC)
C     CALLED BY E04FAF
C     CALCULATES THE VALUES OF THE RESIDUALS RC AT XC
      REAL A(5,5),D(5),Z(5,5),WR(5),WI(5),X02AAF,B(5,5),C(5),Y(5,5)
      INTEGER  X02BAF,NIN,NOUT,I,J,IA,IT,K,L,IZ,IFAIL,M,INTGER(5),N
      REAL FR(5,5), Q(5),XC,RC,T(4),SOL(4),INSOL(4),R(5)
      INTEGER MD,ND,IPT
      DIMENSION XC(ND),RC(MD)
      COMMON FR,Q,Y,WR,WI,B
C
C     SUPPLY THE EXPERIMENTAL DATA POINTS. SCALING OF THE DATA IS
C     REQUIRED. THE EXPERIMENTAL SOLUBLE LYSINE SPECIFIC ACTIVITY
C     DATA AND THE SCALER MULTIPLYER ARE BOTH MULTIPLIED BY 1.0E-06
C     AND THE PROTEIN SPECIFIC ACTIVITY DATA BY 1.0E-02
C
      DATA(T(IPT),IPT=1,4)/5.0,10.0,15.0,20.0/
      DATA(SOL(IPT),IPT=1,4)/3.25,3.86,4.12,4.50/
      DATA(INSOL(IPT),IPT=1,4)/0.47,1.40,2.79,4.36/
C
      NOUT=6
      N=5
C     N SPECIFIES THE MAXIMUM NUMBER OF COMPARTMENTS THAT
C     CAN BE DEALT WITH
C
C     REVISE FLOW RATES ACCORDING TO THE CURRENT ESTIMATE
C     OF THE MINIMUM
      FR(1,2)=XC(2)
      FR(2,1)=FR(1,2)
      FR(2,3)=XC(3)
      FR(4,2)=FR(2,3)
      FR(4,3)=FR(3,4)-FR(2,3)
C
C     CALCULATE RATE CONSTANTS
      DO 14 I=1,N
      DO 15 J=1,N
      B(I,J)=FR(I,J)/Q(I)
   15 CONTINUE
   14 CONTINUE
C     EVALUATE MATRIX OF CONSTANT COEFFICIENTS
      DO 1 I=1,N
      DO2 J=1,N
      IF(I.EQ.J)GO TO 3
      A(J,I)=-B(I,J)
      GO TO 2
    3 A(I,J)=B(I,1)+B(I,2)+B(I,3)+B(I,4)+B(I,5)
    2 CONTINUE
    1 CONTINUE
      IA=5
C     BALANCE MATRIX
      CALL F01ATF(N,X02BAF(IT),A,IA,K,L,D)
C     REDUCE TO HESSENBERG FORM
      CALL F01AKF(N,K,L,A,IA,INTGER)
      IZ=5
C     ACCUMULATE TRANSFORMATIONS
      CALL F01APF(N,K,L,INTGER,A,IA,Z,IZ)
C     CALCULATE EIGENVALUES AND EIGENVECTORS
      IFAIL=1
```



```
      CALL F02AQF(N,K,L,X02AAF(IT),A,IA,Z,IZ,WR,WI,INTGER,IFAIL)
      IF(IFAIL.EQ.0)GO TO 20
      WRITE(NOUT,99996)IFAIL
      STOP
   20 M=N
C     EIGENVECTORS OF ORIGINAL MATRIX FROM THOSE OF BALANCED MATRIX
      CALL F01AUF(N,K,L,M,D,Z,IZ)
C     FIND THE GENERAL SOLUTION THAT SATISFIES THE INITIAL CONDITIONS
      CALL LCPS(Z,N,C,NOUT)
C     EVALUATE ELEMENTS OF THE GENERAL SOLUTION MATRIX
      DO5 I=1,N
      DO6 J=1,N
      Y(I,J)=Z(I,J)*C(J)
    6 CONTINUE
    5 CONTINUE
C
C     CALCULATE THE RADIOACTIVE CONTENT OF COMPARTMENTS 2 AND 4
C     AT T=5, 10, 15 AND 20MIN
C
      DO 9 IPT=1,4
      DO 11 I=2,4
      R(I)=0.0
      DO 10 J=1,N
      X=WR(J)*T(IPT)
C     ENSURE THAT ARGUMENT IS NOT TOO LARGE
      IF(X.GT.600.0)GO TO 10
      R(I)=R(I)+Y(I,J)*EXP(-X)
   10 CONTINUE
   11 CONTINUE
C
C     CALCULATE THE AVERAGE SPECIFIC ACTIVITY OF THE TOTAL SOLUBLE POOL
      SAV=(R(2)+R(4))/(Q(2)+Q(4))
C     CALCULATE RESIDUALS
C     XC(1) IS THE SCALER MULTIPLYER
      RC(IPT)=SAV*XC(1)-SOL(IPT)
C
C     XC(1) HAS BEEN MULTIPLIED BY A SCALING FACTOR OF 1.0E-06 WHILE
C     THE EXPERIMENTAL PROTEIN SPECIFIC ACTIVITY DATA HAS BEEN SCALED
C     BY A FACTOR OF 1.0E-02. THE SIMULATED PROTEIN SPECIFIC ACTIVITY
C     DATA MUST THEREFORE BE MULTIPLIED BY 1.0E+04. NOTE THAT
C     COMPARTMENT 3 IS EQUIVALENT IN SIZE TO 2.2 MG OF PROTEIN

      RC(IPT+4)=R(3)*XC(1)*1.0E+04/2.2-INSOL(IPT)
    9 CONTINUE
      RETURN
99996 FORMAT(25H0ERROR IN F02AQF IFAIL= ,I2)
      END
```

```
      SUBROUTINE LCPS(A,N,C,NOUT)
C     FINDS THE GENERAL SOLUTION SATISFYING THE INITIAL CONDITIONS
      REAL A(5,5),B(5),C(5),AA(5,5),WKS1(5),WKS2(5)
      INTEGER NIN,NOUT,I,N,J,IA,IAA,IFAIL
C
C     SPECIFY THE INITIAL CONDITIONS
      DATA(B(I),I=1,5)/1.0,0.0,0.0,0.0,0.0/
C
      IA=5
      IAA=5
      IFAIL=1
      CALL F04ATF(A,IA,B,N,C,AA,IAA,WKS1,WKS2,IFAIL)
      IF(IFAIL.EQ.0)GO TO 20
      WRITE(NOUT,9999)IFAIL
99990 FORMAT(25H0ERROR IN F04ATF IFAIL= ,I2)
      STOP
   20 RETURN
      END
```



```
      SUBROUTINE MONIT(M,N,XC,FC,ITERC,SING,LIM)
C     PRINTS OUT VALUES EVERY IPRINT ITERATIONS
C     CALLED BY E04FAF
      LOGICAL SING, LIM
      INTEGER M,N,ITERC
      REAL XC,FC
      DIMENSION XC(N)
      DATA NOUT /6/
      WRITE(NOUT,99999)ITERC,FC
      WRITE(NOUT,99998)(XC(I),I=1,N)
      IF(SING)WRITE(NOUT,99997)
      IF(LIM)WRITE(NOUT,99996)
      RETURN
99999 FORMAT(6H AFTER, I4,14H   ITERATIONS,,22H THE SUM OF SQUARES IS,
     11PE12.4)
99998 FORMAT(13H AT THE POINT,1P3E12.4/)
99997 FORMAT(9H SINGULAR/)
99996 FORMAT(8H LIMITED/)
      END
```



```
      SUBROUTINE SUBS(Y,WR,N,Q,NOUT,SCALER)
C     CALCULATES THE RADIOACTIVE CONTENT AND SPECIFIC ACTIVITIES OF THE
C     COMPARTMENTS
      REAL Y(5,5),R(5),S(5),WR(5),Q(5),FACT
      WRITE(NOUT,99988)
99988 FORMAT(1H0,7X,4HTIME,16X,39H(A) RADIOACTIVE CONTENT OF COMPARTMENT
     1S,/,1H ,27X,37H(B) SPECIFIC ACTIVITY OF COMPARTMENTS)
C
C     SPECIFY RANGE OF TIME
      DO12 L=1,2
      DO8 K=10,100,10
      T=K*(10**L)/100.
C
C     CALCULATE THE RADIOACTIVE CONTENT OF THE COMPARTMENTS
      DO11 I=1,N
   11 R(I)=0.0
      DO9 I=1,N
      DO10 J=1,N
      X=WR(J)*T
C     ENSURE THAT ARGUMENT IS NOT TOO LARGE
      IF(X.GT.600.0)GO TO 10
      R(I)=R(I)+Y(I,J)*EXP(-X)
   10 CONTINUE
      R(I)=R(I)*SCALER
    9 CONTINUE
      WRITE(NOUT,99989)T,(R(I),I=1,N)
99989 FORMAT(1H ,F15.5,10X,3H(A),3X,1P5E13.4)
C
C     CALCULATE SPECIFIC ACTIVITIES
      DO13 I=1,N
   13 S(I)=R(I)/Q(I)
      WRITE(NOUT,99985)(S(I),I=1,N)
99985 FORMAT(1H ,25X,3H(B),2X,1P5E13.4)
    8 CONTINUE
   12 CONTINUE
      RETURN
      END
```

```
INITIAL FLOW RATES
      0.              1.8320E-03      0.              0.              0.
      1.8320E-03      0.              8.0000E-06      0.              0.
      0.              0.              0.              1.4410E-05      0.
      0.              8.0000E-06      6.4100E-06      0.              0.
      0.              0.              0.              0.              0.

INITIAL AMINO ACID POOL SIZES
      2.0000E-01      7.4690E-03      1.0000E+00      4.0220E-03      1.0000E+00

AFTER   0   ITERATIONS, THE SUM OF SQUARES IS  3.0104E-01
AT THE POINT  1.4000E+00  1.8320E-03  8.0000E-06

AFTER   1   ITERATIONS, THE SUM OF SQUARES IS  1.9947E-01
AT THE POINT  1.4035E+00  1.7291E-03  8.5703E-06

AFTER   2   ITERATIONS, THE SUM OF SQUARES IS  1.9860E-01
AT THE POINT  1.4051E+00  1.7372E-03  8.5770E-06

AFTER   3   ITERATIONS, THE SUM OF SQUARES IS  1.9857E-01
AT THE POINT  1.4054E+00  1.7386E-03  8.5779E-06

AFTER   4   ITERATIONS, THE SUM OF SQUARES IS  1.9857E-01
AT THE POINT  1.4055E+00  1.7392E-03  8.5783E-06

AFTER   5   ITERATIONS, THE SUM OF SQUARES IS  1.9873E-01
AT THE POINT  1.4056E+00  1.7439E-03  8.5858E-06

AFTER   6   ITERATIONS, THE SUM OF SQUARES IS  2.0094E-01
AT THE POINT  1.4006E+00  1.7762E-03  8.5977E-06

AFTER   7   ITERATIONS, THE SUM OF SQUARES IS  1.9810E-01
AT THE POINT  1.4097E+00  1.7149E-03  8.5907E-06

AFTER   8   ITERATIONS, THE SUM OF SQUARES IS  1.9809E-01
AT THE POINT  1.4100E+00  1.7112E-03  8.6033E-06

AFTER   9   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4102E+00  1.7113E-03  8.5981E-06

AFTER  10   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4103E+00  1.7113E-03  8.5981E-06

AFTER  11   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4103E+00  1.7114E-03  8.5981E-06

AFTER  12   ITERATIONS, THE SUM OF SQUARES IS  1.9808E 01
AT THE POINT  1.4103E+00  1.7115E-03  8.5979E-06

AFTER  13   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4104E+00  1.7117E-03  8.5976E-06

AFTER  14   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4105E+00  1.7101E-03  8.5980E-06

AFTER  15   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4105E+00  1.7098E-03  8.5981E-06

AFTER  16   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4105E+00  1.7098E-03  8.5983E-06

AFTER  17   ITERATIONS, THE SUM OF SQUARES IS  1.9808E-01
AT THE POINT  1.4105E+00  1.7098E-03  8.5983E-06

FINAL SUM OF SQUARES IS 1.9808E-01 FINAL FTOL WAS-10.0
AT THE POINT  1.4105E+00  1.7098E-03  8.5983E-06
WITH RESIDS  -1.8611E-01  1.3269E-01  1.5382E-01 -1.4153E-01  8.00C01-02  2.3789E-01  9.8612E-02 -1.7151E-01
```

THIS HAS ERROR NUMBER 0

FINAL FLOW RATES

0.	1.7098E-03	0.	0.	0.
1.7098E-03	0.	8.5983E-06	0.	0.
0.	0.	0.	1.4410E-05	0.
0.	8.5983E-06	5.8117E-06	0.	0.
0.	0.	0.	0.	0.

FINAL AMINO ACID POOL SIZES

2.0000E-01	7.4690E-03	1.0000E+00	4.0220E-03	1.0000E+00

FINAL RATE CONSTANTS

0.	8.5488E-03	0.	0.	0.
2.2891E-01	0.	1.1512E-03	0.	0.
0.	0.	0.	1.4410E-05	0.
0.	2.1378E-03	1.4450E-03	0.	0.
0.	0.	0.	0.	0.

FINAL GENERAL SOLUTION MATRIX

3.5666E-02	7.9928E-01	1.6509E-01	-2.7901E-05	0.
-3.5839E-02	2.9675E-02	6.1651E-03	-6.0458E-07	0.
1.7295E-04	-8.2558E-01	8.2543E-01	-1.9019E-05	0.
-1.0605E-08	-3.3674E-03	3.3199E-03	4.7525E-05	0.
0.	0.	0.	0.	0.

FINAL EIGENVALUES
(2.3857E-01, 0.00)
(4.9895E-05, 0.00)
(**********, 0.00)
(3.5886E-03, 0.00)
(0. , 0.00)

TIME		(A) RADIOACTIVE CONTENT OF COMPARTMENTS (B) SPECIFIC ACTIVITY OF COMPARTMENTS				
1.00000	(A)	1.3998E+00	1.0728E-02	6.4202E-06	3.1413E-11	0.
	(B)	6.9990E+00	1.4363E+00	6.4202E-06	7.8103E-09	0.
2.00000	(A)	1.3913E+00	1.9178E-02	2.3827E-05	2.3727E-10	0.
	(B)	6.9567E+00	2.5677E+00	2.3827E-05	5.8992E-08	0.
3.00000	(A)	1.3847E+00	2.5834E-02	4.9888E-05	7.5752E-10	0.
	(B)	6.9233E+00	3.4589E+00	4.9888E-05	1.8834E-07	0.
4.00000	(A)	1.3794E+00	3.1077E-02	8.2765E-05	1.7018E-09	0.
	(B)	6.8969E+00	4.1609E+00	8.2765E-05	4.2312E-07	0.
5.00000	(A)	1.3752E+00	3.5207E-02	1.2101E-04	3.1557E-09	0.
	(B)	6.8761E+00	4.7138E+00	1.2101E-04	7.8462E-07	0.
6.00000	(A)	1.3719E+00	3.8460E-02	1.6349E-04	5.1863E-09	0.
	(B)	6.8596E+00	5.1493E+00	1.6349E-04	1.2895E-06	0.
7.00000	(A)	1.3693E+00	4.1022E-02	2.0929E-04	7.8455E-09	0.
	(B)	6.8466E+00	5.4923E+00	2.0929E-04	1.9506E-06	0.
8.00000	(A)	1.3672E+00	4.3040E-02	2.5772E-04	1.1174E-08	0.
	(B)	6.8362E+00	5.7624E+00	2.5772E-04	2.7781E-06	0.
9.00000	(A)	1.3656E+00	4.4629E-02	3.0822E-04	1.5202E-08	0.
	(B)	6.8280E+00	5.9752E+00	3.0822E-04	3.7797E-06	0.
10.00000	(A)	1.3643E+00	4.5880E-02	3.6034E-04	1.9954E-08	0.
	(B)	6.8215E+00	6.1427E+00	3.6034E-04	4.9613E-06	0.
10.00000	(A)	1.3643E+00	4.5880E-02	3.6034E-04	1.9954E-08	0.
	(B)	6.8215E+00	6.1427E+00	3.6034E-04	4.9613E-06	0.
20.00000	(A)	1.3595E+00	5.0083E-02	9.2147E-04	1.0969E-07	0.
	(B)	6.7977E+00	6.7055E+00	9.2147E-04	2.7272E-05	0.
30.00000	(A)	1.3586E+00	5.0451E-02	1.5008E-03	2.7746E-07	0.
	(B)	6.7930E+00	6.7547E+00	1.5008E-03	6.8986E-05	0.
40.00000	(A)	1.3580E+00	5.0466E-02	2.0815E-03	5.2147E-07	0.
	(B)	6.7900E+00	6.7567E+00	2.0815E-03	1.2966E-04	0.
50.00000	(A)	1.3574E+00	5.0448E-02	2.6620E-03	8.3909E-07	0.
	(B)	6.7872E+00	6.7544E+00	2.6620E-03	2.0863E-04	0.
60.00000	(A)	1.3569E+00	5.0428E-02	3.2423D013	1.2277E-06	0.
	(B)	6.7844E+00	6.7516E+00	3.2423E-03	3.0524E-04	0.
70.00000	(A)	1.3563E+00	5.0407E-02	3.8222E-03	1.6847E-06	0.
	(B)	6.7816E+00	6.7488E+00	3.8222E-03	4.1887E-04	0.
80.00000	(A)	1.3558E+00	5.0386E-02	4.4018E-03	2.2077E-06	0.
	(B)	6.7788E+00	6.7461E+00	4.4018E-03	5.4891E-04	0.
90.00000	(A)	1.3552E+00	5.0365E-02	4.9811E-03	2.7943E-06	0.
	(B)	6.7760E+00	6.7433E+00	4.9811E-03	6.9476E-04	0.
100.00000	(A)	1.3546E+00	5.0345E-02	5.5600E-03	3.4423E-06	0.
	(B)	6.7732E+00	6.7405E+00	5.5600E-03	8.5586E-04	0.

REFERENCES

Adriaens, P., Meesschaert, B. and Vanderhaeghe,H. (1975) Isotope distribution in L-valine-2,3-T. Anal.Biochem., 69, 297-299.

Agrawal, H.C., Bone, A.H. and Davison, A.N. (1970) Effect of phenylalanine on protein synthesis in the developing rat brain. Biochem.J., 117, 325-331.

Airhart, J., Vidrich, A. and Khairallah, E.A. (1974) Compartmentation of free amino acids for protein synthesis in rat liver. Biochem.J., 140, 539-548.

Altman, P.L. and Dittmer, D.S. (Eds), (1964) in "Biology data book", Fed. Amer.Soc.Exp.Biol., Washington.

Andén, N.-E., Dahlström, A., Fuxe, K., Larsson, K., Olson, L. and Ungerstedt, U. (1966) Ascending monoamine neurons to the telencephalon and diencephalon. Acta.Physiol.Scand., 67, 313-326.

Andén, N.-E., Engel, J. and Rubenson, A. (1972) Central decarboxylation and uptake of L-dopa. Naunyn-Schmiedeberg's Arch.Pharmacol., 273, 11-26.

Aoki, K. and Siegel, F.L. (1970) Hyperphenylalaninemia: Disaggregation of brain polyribosomes in young rats. Science, 168, 129-130.

Appeltauer, G.S.L. and Korr, I.M. (1975) Axonal delivery of soluble,insoluble and electrophoretic fractions of neuronal proteins to muscle. Exp.Neurol., 46, 132-146.

Ayres, F. (1974) in "Theory and Problems of Matrices", (Schaum's Outline Series) McGraw-Hill Book Co., New York, London.

Balázs, R. and Cremer, J.E. (Eds), (1973) in "Metabolic compartmentation in the brain". Macmillan Press Ltd., London and Basingstoke.

Baliga, B.S., Pronczuk, A.W. and Munro, H.N. (1968) Regulation of polysome aggregation in a cell-free system through amino acid supply. J.Mol.Biol., 34, 199-218.

Baliga, B.S., Zähringer, J., Trachtenberg, M., Moskowitz, M.A. and Munro, H.N. (1976) Mechanism of D-amphetamine inhibition of protein synthesis. Biochim.Biophys.Acta., 442, 239-250.

Ballard, F.J. (1977) Intracellular protein degradation, in "Essays in Biochemistry" (P.N. Campbell and W.N. Aldridge, eds) Vol.13, pp.1-37. Academic Press, London, New York and San Francisco.

Banker, G. and Cotman, C.W. (1971) Characteristics of different amino acids as protein precursors in mouse brain: Advantages of certain carboxyl-labeled amino acids. Arch.Biochem.Biophys., 142, 565-573.

Bellobono, I.R. (1968) Isotope separation factors of ^{14}C-amino acids in ion-exchange on resins with carboxylic groups. J.Chromatog., 34, 515-519.

Benevenga, N.J., Rogers, Q.R. and Harper, A.E. (1968) Variations in the recovery of carbon-14 in coloured samples treated with peroxide. Anal.Biochem., 24, 393-396.

Berger, B., Tassin, J.P., Blanc, G., Moyne, M.A. and Thierry, A.M. (1974) Histochemical confirmation for dopaminergic innervation of the rat cerebral cortex after distruction of the noradrenergic ascending pathways. Brain Res., 81, 332-337.

Berger, B., Thierry, A.M., Tassin, J.P. and Moyne, M.A. (1976) Dopaminergic innervation of the rat prefrontal cortex: A fluorescence histochemical study. Brain Res., 106, 133-145.

Berkley, K.J., Graham, J. and Jones, E.G. (1977) Differential incorporation of tritiated proline and leucine by neurons of the dorsal column nuclei in the cat. Brain Res., 132, 485-505.

Berl, S., Clarke, D.D. and Schneider, D. (Eds), (1975) in "Metabolic compartmentation and neurotransmission. Relation to brain structure and function". Plenum Press., New York and London.

Berman, M., Shahn, E. and Weiss, M.F. (1962b) The routine fitting of kinetic data to models: A mathematical formalism for digital computers. Biophys.J., 2, 275-287.

Berman, M., Weiss, M.F. and Shahn, E. (1962a) Some formal approaches to the analysis of kinetic data in terms of linear compartmental systems. Biophys.J., 2, 289-316.

Bertler, A., Falck, B., Owman, CH., and Rosengrenn, E. (1966) The localization of monoaminergic blood-brain barrier mechanisms. Pharm.Rev., 18, 369-385.

Bertler, A. and Rosengren, E.(1959) On the distribution in brain of monoamines and of enzymes responsible for their formation. Exp., 15, 382-384.

Blaschko, H. and Chruściel, T.L. (1960) The decarboxylation of amino acids related to tyrosine and their awakening action in reserpine-treated mice. J.Physiol., 151, 272-284.

Bone, A.H. (1975) Investigation of the effect of L-3, 4-dihydroxy-phenylalanine on brain protein synthesis. Biochem.Soc.Trans., 3, 91-93.

Bradley, P.B. and Hance, A.J. (1957) The effect of chlorpromazine and methopromazine on the electrical activity of the brain in the cat. Electroenceph.Clin.Neurophysiol., 9, 191-215.

Broekkamp, C.L.E., Pijnenburg, A.J.J., Cools, A.R. and Van Rossum, J.M. (1975) The effect of microinjections of amphetamine into the neostriatum and the nucleus accumbens on self-stimulation behaviour. Psychopharmacol., 42, 179-183.

Brostrom, C.O. and Jeffay, H. (1970) Protein catabolism in rat liver homogenates. A re-evaluation of the energy requirement for protein catabolism. J.Biol.Chem., 245, 4001-4008.

Buck, R.C. and Buck, E.F. (1976) in "Introduction to differential equations". Houghton Mifflin Co., Boston, London.

Butcher, L., Engel, J. and Fuxe, K. (1970) L-dopa induced changes in central monoamine neurons after peripheral decarboxylase inhibition. J.Pharm.Pharmac., 22, 313-315.

Butcher, L., Engel, J. and Fuxe, K. (1972) Behavioral, biochemical and histochemical analysis of the central effects of monoamine precursors after peripheral decarboxylase inhibition. Brain Res., 41, 387-411.

Campbell, M.K., Mahler, H.R., Moore, W.J. and Tewari, S. (1966) Protein synthesis systems from rat brain. Biochem., 5, 1174-1184.

Chalmers, J.P. and Wurtman, R.J. (1971) The fate of intracisternally administered norepinephrine-^{3}H in the brain and spinal cord of the rabbit. J.Pharm.Exp.Ther., 178, 8-19.

Chang, Y-F. (1976) Pipecolic acid pathway: The major lysine metabolic route in the rat brain. Biochem.Biophys.Res.Commun., 69, 174-180.

Chase, H.P. and O'Brien, D. (1970) Effect of excess phenylalanine and of other amino acids on brain development in the infant rat. Pediat.Res., 4, 96-102.

Clarke, J.T.R. and Lowden, J.A. (1969) Hyperphenylalaninemia: Effect on the developing rat brain. Can.J.Biochem., 47, 291-295.

Cobelli, C., Polo, A. and Romanin-Jacur, G. (1977) A computer program for the analysis of controllability, observability and structural identifiability of biological compartmental systems. Computer Programs in Biomed., 7, 21-36.

Cohen, S.R., Stampleman, P.F. and Lajtha, A. (1970) The temperature-dependent compartmentation of the 'extracellular space' in mouse brain slices as revealed by the markers: inulin, sucrose, D-mannitol, D-sorbitol and sulfate. Brain Res., 21, 419-434.

Colquhoun, D. (1971) in "Lectures on biostatistics". Clarendon Press, Oxford.

Consroe, P.F. and White, R.P. (1972) Effects of haloperidol and chlorpromazine on central adrenergic and cholinergic mechanisms in rabbits. Arch.Int.Pharmacodyn., 198, 67-75.

Constantinidis, J., Bartholini, G., Tissot, R. and Pletscher, A. (1968) Accumulation of dopamine in the parenchyma after decarboxylase inhibition in the capillaries of brain. Exp., 24, 130-131.

Cools, A.R. and Van Rossum, J.M. (1976) Excitation-mediating and inhibition-mediating dopamine-receptors: A new concept towards a better understanding of electrophysiological, biochemical, pharmacological, functional and clinical data. Psychopharmacol., 45, 243-254.

Costall, B., Marsden, C.D., Naylor, R.J. and Pycock, C.J. (1977a) Stereotyped behaviour patterns and hyperactivity induced by amphetamine and apomorphine after discrete 6-hydroxydopamine lesions of extrapyramidal and mesolimbic nuclei. Brain Res., 123, 89-111.

Costall, B., Naylor, R.J., Cannon, J.G. and Lee , T. (1977b) Differentiation of the dopamine mechanisms mediating stereotyped behaviour and hyperactivity in the nucleus accumbens and caudate-putamen. J.Pharm.Pharmac., 29, 337-342.

Costall, B., Naylor, R.J. and Neumeyer, J.L. (1975a) Differences in the nature of the stereotyped behaviour induced by aporphine derivatives in the rat and in their actions in extrapyramidal and mesolimbic areas. Eur.J.Pharmacol., 31, 1-16.

Costall, B., Naylor, R.J. and Neumeyer, J.L. (1975b) Dissociation by the aporphine derivatives of the stereotypic and hyperactivity responses from injections into the nucleus accumbens septi. J.Pharm.Pharmac., 27, 875-877.

Coulson, W.F. and Hart, B. (1977) Measurement of the rate of incorporation in vivo of amino acid into brain protein. Biochem.Soc.Trans., 5, 1425-1428.

Creese, I., Burt, D.R. and Snyder, S.H. (1975) Dopamine receptor binding: Differentiation of agonist and antagonist states with ^{3}H-dopamine and ^{3}H-haloperidol. Life Sci., 17, 993-1002.

Creese, I., Burt, D.R. and Snyder, S. (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. Science, 192, 481-483.

Creese, I. and Iversen, S.D. (1975) The pharmacological and anatomical substrates of the amphetamine response in the rat. Brain Res., 83, 419-436.

Dahlström, A. and Fuxe, K. (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. 1: Demonstration of monoamines in the cell bodies of brain stem neurons. Acta.Physiol.Scand., 62, supp.232, 1-55.

Davison, A.N. (1961) Metabolically inert proteins of the central and peripheral nervous system, muscle and tendon. Biochem.J., 78, 272-282.

Dean, R.T. and Barrett, A.J. (1976) Lysosomes, in "Essays in Biochemistry" (P.N. Campbell and W.N. Aldridge, eds) Vol.12, pp. 1-40. Academic Press, London, New York and SanFrancisco.

Dewar, A.J. and Winterburn, A.K. (1973) Amphetamine and RNA and protein metabolism in rat brain. Brain Res., 59, 359-370.

Dunlop, D.S., Van Elden, W. and Lajtha, A. (1974) Measurements of rates of protein synthesis in rat brain slices. J.Neurochem., 22, 821-830.

Dunlop, D.S., Van Elden, W. and Lajtha, A. (1975a) A method for measuring brain protein synthesis in young and adult rats. J.Neurochem., 24, 337-344.

Dunlop, D.S. Van Elden, W. and Lajtha, A. (1975b) Optimal conditions for protein synthesis in incubated slices of rat brain. Brain Res., 99, 303-318.

Dunn, A.J. (1975) Intracerebral injections inhibit amino acid incorporation into brain protein. Brain Res., 99, 405-409.

Eccles, J.C. (1973) in "The Understanding of the Brain", pp.167-187. McGraw-Hill Book Co., New York and London.

Elkhawad, A.O. and Woodruff, G.N. (1975) Studies on the behavioural pharmacology of a cyclic analogue of dopamine following its injection into the brains of conscious rats. Br.J.Pharmac., 54, 107-114.

Entingh, D.J. and Damstra , T. (1976) Effects of handling and etherization on incorporation of (^{3}H) lysine into protein of mouse brain and liver. Pharm.Biochem.Behav., 5, 111-116.

Epstein, D., Elias-Bishko, S. and Hershko, A. (1975) Requirement for protein synthesis in the regulation of protein breakdown in cultured hepatoma cells. Biochem. 14, 5199-5204.

Ernst, A.M. (1967) Mode of action of apomorphine and dexamphetamine on gnawing compulsion in rats. Psychopharmacol., 10, 316-323.

Evetts, K.D., Uretsky, N.J., Iversen, L.L. and Iversen, S.D. (1970) Effects of 6-hydroxydopamine on CNS catecholamines, spontaneous motor activity and amphetamine induced hyperactivity in rats. Nature, 225, 961-962.

Fernandez, H.L. and Ramirez, B.U. (1974) Muscle fibrillation induced by blockage of axoplasmic transport in motor neurons. Brain Res., 79, 385-395.

Fibiger, H.C. (1973) Behavioural pharmacology of D-amphetamine: Some metabolic and pharmacological considerations, in "Frontiers in Catecholamine Research" (E. Usdin and S.H. Snyder, eds), pp.933-937. Pergamon Press Inc., New York.

Fisher, R.A. and Yates, F. (1963) in "Statistical Tables for Biological, Agricultural and Medical Research", 6th Edition, Oliver and Boyd, London.

Florio, V. and Longo, V.G. (1971) Neuroleptic drugs and the central dopaminergic system. Antagonism between L-dopa and some butyrophenones on the EEG and motor effects elicited by stimulation of the mesencephalon in the rabbit. Neuropharmacol., 10, 45-54.

Ford, D.H., Rhines, R.K. and Levi, M.A. (1977) Strain differences in the response to morphine on incorporation of ^{3}H-lysine into rat brain protein. Acta.Neurol.Scand., 55, 493-505.

Fouriezos, G. and Wise, R.A. (1976) Pimozide-induced extinction of intracranial self-stimulation: Response patterns rule out motor performance deficits. Brain Res., 103, 377-380.

Fox, B.W. (1976) in "Techniques of sample preparation for liquid scintillation counting", pp.164-166, North-Holland Publishing Co., Amsterdam, Oxford. American Elsevier Publishing Co. Inc., New York.

Franck, G. (1972) Brain Slices, in "The Structure and Function of Nervous Tissue" (G.H. Bourne, ed) Vol.VI, pp.417-465. Academic Press, New York and London.

Fuxe, K., Hökfelt, T., Johansson, O., Jonsson, G., Lidbrink, P. and Ljungdahl, A. (1974) The origin of the dopamine nerve terminals in limbic and frontal cortex. Evidence for meso-cortico dopamine neurons. Brain Res., 82, 349-355.

Fuxe, K. and Ungerstedt, U. (1970) Histochemical, biochemical and functional studies on central monoamine neurons after chronic amphetamine administration, in "Amphetamines and Related Compounds" (E. Costa and S. Garattini, Eds), pp.257-288. Raven Press, New York.

Gainer, H. and Barker, J.L. (1974) Synaptic regulation of specific protein synthesis in an identified neuron. Brain Res., 78, 314-319.

Gaitonde, M.K. and Richter, D. (1956) The metabolic activity of the proteins of the brain. Proc.Roy.Soc., B145, 83-99.

Gan, J.C. and Jeffay, H. (1967) Origins and metabolism of the intracellular amino acid pools in rat liver and muscle. Biochim.Biophys. Acta., 148, 448-459.

Garlick, P.J. and Marshall, I. (1972) A technique for measuring brain protein synthesis. J.Neurochem., 19, 577-583.

Gaull, G.E., Tallan, H.H., Lajtha, A. and Rassin , D.K. (1975) Pathogenesis of brain dysfunction in inborn errors of amino acid metabolism, in "Biology of Brain Dysfunction" (G.E. Gaull, ed) Vol.3, pp.47-143. Plenum Press, New York and London.

Geinismann, Y. (1971) Nucleic acid content of spinal motoneurons and their satellites under orthodromic and antidromic stimulation. A cytospectrophotometric study. Brain Res., 28, 251-262.

German, D.C. and Bowden, D.M. (1974) Catecholamine systems as the neural substrate for intracranial self-stimulation: A hypothesis. Brain Res., 73, 381-419.

Gisiger, V. (1971) Triggering of RNA synthesis by acetylcholine stimulation of the postsynaptic membrane in a mammalian sympathetic ganglion. Brain Res., 33, 139-146.

Glowinski, J. and Iversen, L.L. (1966) Regional studies of catecholamines in the rat brain-I. The disposition of [^{3}H]norepinephrine, [^{3}H] dopamine and [^{3}H]dopa in various regions of the brain. J.Neurochem., 13, 655-669.

Goldstein, M., Fuxe, K., Hökfelt, T. and Joh, T.H. (1971) Immunohistochemical studies on phenylethanolamine-N-methyltransferase, dopa-decarboxylase and dopamine-β-hydroxylase. Exp., 27, 951-952.

Grabowska, M. and Andén, N-E. (1976) Apomorphine in the rat nucleus accumbens: Effects on the synthesis of 5-hydroxytryptamine and noradrenaline, the motor activity and the body temperature. J.Neural.Trans., 38, 1-8.

Guth, L. (1968) "Trophic" influences of nerve on muscle. Physiol.Rev., 48, 645-687.

Gutmann, E. (1973) Critical evaluation and implications of denervation and reinnervation studies of cross-striated muscle, in "Methods of Neurochemistry" (R.Fried, ed) Vol.5, pp.189-254. Marcel Dekker, Inc., New York.

Gutmann, E. (1976) Neurotrophic regulations. Ann.Rev.Physiol., 38, 177-216.

Hamberger, A. and Henn, F.A. (1973) Some aspects of the differential biochemistry and functional relationships between neurons and glia, in "Metabolic Compartmentation in the Brain" (R.Balázs and J.E. Cremer, eds) pp.305-318. Macmillan Press Ltd., London.

Hamilton, P.B. (1965) Amino-acids on hands. Nature, 205, 284-5.

Hemminki,K.(1972) Differential responses of free and bound polysomes to inhibitors and neuroactive substances in vitro. J.Neurochem., 19, 2699-2702

Hemminki, K. (1973) Effects of added substances on RNA and protein synthesis in immature neurons. J.Neurochem., 20, 373-378.

Hider, R.C., Fern, E.B. and London, D.R. (1969) Relationship between intracellular amino acids and protein synthesis in the extensor digitorum longus muscle of rats. Biochem.J., 114, 171-178.

Hider, R.C., Fern, E.B. and London, D.R. (1971) Identification in skeletal muscle of a distinct extracellular pool of amino acids, and its role in protein synthesis. Biochem.J., 121, 817-827.

Hodges, J.R. and Mitchley, S. (1970) The effect of 'training' on the release of corticotrophin in response to minor stressful procedures in the rat. J.Endocr., 47, 253-254.

Holbrook, L. and Brown, I.R. (1976) Disaggregation of brain polysomes after administration of d-lysergic acid diethylamide (LSD) in vivo. J.Neurochem., 27, 77-82.

Holbrook, L. and Brown, I.R. (1977a) Disaggregation of brain polysomes after D-lysergic acid diethylamide administration in vivo: Mechanism and effect of age and environment. J.Neurochem., 29, 461-467.

Holbrook, L. and Brown, I. (1977b) Antipsychotic drugs block LSD-induced disaggregation of brain polysomes. Life.Sci., 21, 1037-1044.

Horn, A.S. (1975) Structure-activity relations for neurotransmitter receptor agonists and antagonists, in "Handb. of Psychopharmacology" (L.L. Iversen, S.D. Iversen and S.H. Snyder, eds) Vol.2, pp.179-243. Plenum Press, New York and London.

Hsia, Y.E. (1976) Disorders of amino acid metabolism, in "Basic Neurochemistry" (G.T. Siegel, R.W. Albers, R. Katzman and B.W. Agranoff, eds) 2nd Edition, pp.500-541. Little, Brown and Co., Boston.

Hubel, D.H. and Wiesel, T.N. (1970) The period of susceptibility to the physiological effects of unilateral eye closure in kittens. J.Physiol., 206, 419-436.

Hughes, J.V. and Johnson, T.C. (1976) The effects of phenylalanine on amino acid metabolism and protein synthesis in brain cells in vitro. J.Neurochem., 26, 1105-1113.

Hughes, J.V. and Johnson, T.C. (1977) The effects of hyperphenylalaninaemia on the concentrations of aminoacyl-transfer ribonucleic acid in viv Biochem.J., 162, 527-537.

Hughes, J.V. and Johnson, T.C. (1978) Experimentally induced and natural recovery from the effects of phenylalanine on brain protein synthesis. Biochim.Biophys.Acta., 517, 473-485.

Ilan, J. and Singer, M. (1975) Sampling of the leucine pool from the growing peptide chain: Difference in leucine specific activity of peptidyl-transfer RNA from free and membrane bound polysomes. J.Mol.Biol., 91, 39-51.

Iversen, S.D., Kelly, P.H., Miller, R.J. and Seviour, P. (1975) Amphetamine and apomorphine responses in the rat after lesion of mesolimbic or striatal dopamine neurons. Brit.J.Pharmac., 54, 244P.

Jackson, D.M., Andén, N.-E. and Dahlström, A. (1975) A functional effect of dopamine in the nucleus accumbens and in some other dopamine-rich parts of the rat brain. Psychopharmacol., 45, 139-149.

Jakoubek, B. (1974) in "Brain Function and Macromolecular Synthesis" Pion Ltd., London.

Jakoubek, B. and Semiginovský, B. (1970) The effect of increased functional activity on the protein metabolism of the nervous system. Int.Rev.Neurobiol., 13, 255-288.

Johnson, D., White, J., Lim, L. and Davison, A.N. (1975) The effect of experimental hyperphenylalaninaemia on polyribosomes of the developing rat brain. Biochem.Soc.Trans., 3, 93-94.

Jones, B.E., Bobillier, P., Pin, C. and Jouvet, M. (1973) The effect of lesions of catecholamine-containing neurons upon monoamine content of the brain and EEG and behavioral waking in the cat. Brain Res., 58, 157-177.

Jones, D.A. and McIlwain, H. (1971) Amino acid distribution and incorporation into protein in isolated, electrically-stimulated cerebral tissues. J.Neurochem., 18, 41-58.

Kandera, J., Levi, G. and Lajtha, A. (1968) Control of cerebral metabolite levels. II. Amino acid uptake and levels in various areas of the rat brain. Arch.Biochem.Biophys., 126, 249-260.

Kärjä, J., Kärki, N.T. and Tala, E. (1961) Inhibition by methysergid of 5-hydroxytryptophan toxicity to mice. Acta.Pharmacol.Toxicol., 18, 255-262.

Kelly, P.H. (1975) Unilateral 6-hydroxydopamine lesions of nigrostriatal or mesolimbic dopamine-containing terminals and the drug-induced rotation of rats. Brain Res., 100, 163-169.

Kelly, P.H., Miller , R.J. and Neumeyer, J.L. (1975a) Effect of aporphine alkaloids on central dopamine receptors. Brit.J. Pharmac., 54, 271P.

Kelly, P.H., Miller, R.J. and Neumeyer, J.L. (1976) Aporphines 16. Action of aporphine alkaloids on locomotor activity in rats with 6-hydroxydopamine lesions of the nucleus accumbens. Eur.J.Pharmacol., 35, 85-92.

Kelly, P.H., Seviour, P.W. and Iversen, S.D. (1975b) Amphetamine and apomorphine responses in the rat following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum. Brain Res., 94, 507-522.

Khairallah, E.A. and Mortimore, G.E. (1976) Assessment of protein turnover in perfused rat liver. Evidence for amino acid compartmentation from differential labelling of free and tRNA-bound valine. J.Biol.Chem., 251, 1375-1384.

Kuntzman, R., Shore, P.A., Bogdanski, D. and Brodie, B.B. (1961) Microanalytical procedures for fluorometric assay of brain dopa-5HTP decarboxylase, norepinephrine and serotonin, and a detailed mapping of decarboxylase activity in brain. J.Neurochem., 6, 226-232.

Kuromi, H. and Hasegawa, S. (1975) Neurotrophic effect of spinal cord extract on membrane potentials of organ-cultured mouse skeletal muscle. Brain Res., 100, 178-181.

Lajtha, A. (1964) Protein metabolism of the nervous system. Int.Rev.Neurobiol., 6, 1-98.

Lajtha, A., Furst, S., Gerstein, A. and Waelsch, H. (1957) Amino acid and protein metabolism of the brain-I. Turnover of free and protein bound lysine in brain and other organs. J.Neurochem., 1, 289-300.

Lajtha, A.,Latzkovits, L. and Toth, J. (1976) Comparison of turnover rates of proteins of the brain, liver and kidney in mouse in vivo following long term labeling. Biochim.Biophys.Acta., 425, 511-520.

Lajtha, A. and Marks, N. (1969) Dynamics and significance of cerebral protein metabolism. Dis.Nerv.Syst., 30, (Suppl.) 36-43.

Lajtha, A. and Toth, J. (1966) Instability of cerebral proteins. Biochem.Biophys.Res.Commun., 23, 294-298.

Lajtha, A. and Toth, J. (1974) Postmortem changes in the cerebral free amino acid pool. Brain Res., 76, 546-551.

Lal, S. and Feldmüller, F. (1975) Effect of amphetamine and apomorphine on brain monoamines and behaviour in the immature and young adult rat . Arch.Int.Pharmacodyn., 218, 239-251.

Lal, S. and Sourkes, T.L. (1973) Ontogeny of stereotyped behaviour induced by apomorphine and amphetamine in the rat. Arch.Int.Pharmacodyn., 202, 171-182.

Lang, J.F. (1971) Chemical vs. color quenching in automatic external standard calibration. Application of empirical observations in a computer program, in "Organic Scintillators and Liquid Scintillation Counting" (D.L. Horrocks and C-T. Peng, eds), pp.823-833. Academic Press, New York and London.

Langer, S.Z. (1975) Denervation supersensitivity, in "Handb. of Psychopharmacol"(L.L. Iversen, S.D. Iversen and S.H. Snyder, eds) Vol.2, pp.245-280. Plenum Press, New York and London.

Layne, E. (1957) Spectrophotometric and turbidimetric methods for measuring proteins, in "Methods in Enzymology" (S.P.Colowick and N.O. Kaplin, eds) Vol.III, pp.447-454. Academic Press, New York.

Lentz, T.L. (1974a) Effect of brain extracts on cholinesterase activity of cultured skeletal muscle. Exp.Neurol., 45, 520-526.

Lentz, T.L. (1974b) Neurotrophic regulation at the neuromuscular junction. Ann.N.Y.Acad.Sci., 228, 323-337.

Levi, G., Kandera, J. and Lajtha, A. (1967) Control of cerebral metabolite levels. 1.Amino acid uptake and levels in various species. Arch.Biochem.Biophys., 119, 303-311.

Lindroos, O.F.C. and Oja, S.S. (1971) Hyperphenylalanaemia and the exchange of tyrosine in adult rat brain. Exp.Brain Res., 14, 48-60.

Lindvall, O. and Björklund, A. (1974) The organization of the ascending catecholamine neuron systems in the rat brain. Acta.Physiol.Scand.Suppl., 412, 1-48.

Lindvall, O., Björklund, A., Moore, R.Y. and Stenevi, U. (1974) Mesencephalic dopamine neurons projecting to neocortex. Brain Res., 81, 325-331.

Liu, Y.P., Ambani, L.M. and Van Woert, M.H. (1972) L-Dihydroxyphenylalanine: Effect on levels of amino acids in rat brain. J.Neurochem., 19, 2237-2239.

Loh, H., Hitzemann, R. and Stolman, S. (1973) Effect of d-amphetamine on brain protein synthesis. Toxicol.Appl.Pharmacol., 26, 476-481.

Loizou, L.A. (1972) The postnatal ontogeny of monoamine-containing neurones in the central nervous system of the albino rat. Brain Res., 40, 395-418.

Long, C. (ed), (1961) in "Biochemists' Handbook", E. & F.N.Spon Ltd., London.

Lund-Andersen, H. and Møller, M. (1977) Uptake of inulin by cells in rat brain cortex. Exp.Brain Res., 28, 37-50.

Lytle, L.D., Hurko, O., Romero, J.A., Cottman, K., Leehey, D. and Wurtman, R.J. (1972) The effects of 6-hydroxydopamine pretreatment on the accumulation of dopa and dopamine in brain and peripheral organs following L-dopa administration. J.Neural.Trans., 33, 63-71.

McIlwain, H. (1970) Metabolic adaptation in the brain. Nature, 226, 803-806.

McIlwain, H. and Rodnight, R. (1962) in "Practical Neurochemistry" pp.109-133. J. and A. Churchill Ltd., London.

MacInnes, J.W. and Schlesinger, K. (1971) Effects of excess phenylalanine on *in vitro* and *in vivo* RNA and protein synthesis and polyribosome levels in brains of mice. Brain Res., 29, 101-110.

Magour, S., Coper, H. and Fähndrich C.H.R. (1976) The effect of chronic self-administration of d-amphetamine on food intake, locomotor activity, and C^{14}-leucine incorporation into cerebral cortex protein. Psychopharmacol., 45, 267-270.

Maickel, R.P., Cox, R.H., Miller, F.P., Segal, D.S. and Russell, R.W. (1969) Correlation of brain levels of drugs with behavioural effects. J.Pharmacol.Exp.Ther., 165, 216-224.

Maj, J., Grabowska, M. and Gajda, L. (1972) Effect of apomorphine on motility in rats. Eur.J.Pharmacol., 17, 208-214.

Maker, H.S., Clarke, D.D. and Lajtha, A.L. (1976) Intermediary metabolism of carbohydrates and amino acids, in "Basic Neurochemistry" (G.J. Siegel, R.W. Albers, R.Katzman, B.W. Agranoff, eds) 2nd Edition, pp.279-307. Little, Brown and Co.,Boston.

Mans, R.J. and Novelli, G.D. (1961) Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. Arch.Biochem.Biophys., 94, 48-53.

Matsen, F.A. and Franklin, J.L. (1950) A general theory of coupled sets of first order reactions. J.Am.Chem.Soc., 72, 3337-3341.

Miller, R.J. and Iversen L.L. (1974) Effect of psychoactive drugs on dopamine (3,4-dihydroxyphenethylamine)-sensitive adenylate cyclase activity in corpus striatum of rat brain. Biochem.Soc.Trans., 2, 256-259.

Moldave, K., Castelfranco, P. and Meister, A. (1959) The synthesis and some properties of amino acyl adenylates. J.Biol.Chem., 234, 841-848.

Morell, P., Bornstein, M.B. and Raine, C.S. (1976) Diseases involving myelin, in "Basic Neurochemistry" (G.J. Siegel, R.W. Albers, R. Katzman, B.W. Agranoff, eds) 2nd Edition, pp.581-604. Little, Brown and Co., Boston.

Mortimore, G.E., Woodside, K.H. and Henry, J.E. (1972) Compartmentation of free valine and its relation to protein turnover in perfused rat liver. J.Biol.Chem., 247, 2776-2784.

Moskowitz, M.A., Rubin, D., Liebschutz, J., Munro, H.N., Nowak, T.S. and Wurtman, R.J. (1977) The permissive role of hyperthermia in the disaggregation of brain polysomes by L-dopa or D-amphetamine. J.Neurochem., 28, 779-782.

Moskowitz, M.A., Weiss, B.F., Lytle, L.D., Munro, H.N. and Wurtman, R.J. (1975) d-Amphetamine disaggregates brain polysomes via a dopaminergic mechanism. Proc.Nat.Acad.Sci., USA., 72, 834-836.

Neidle, A., Kandera, J. and Lajtha, A. (1973) The uptake of amino acids by the intact olfactory bulb of the mouse: A comparison with tissue slice preparations. J.Neurochem., 20, 1181-1193.

Neidle, A., Kandera, J. and Lajtha, A. (1975) Compartmentation and exchangeability of brain amino acids: Evidence from studies of transport into tissue slices. Arch.Biochem.Biophys., 169, 397-405.

Ng, K.Y., Chase, T.N., Colburn, R.W. and Kopin, I,J. (1970) L-dopa induced release of cerebral monoamines. Science, 170, 76-77.

Ng, K.Y., Chase, T.N., Colburn, R.W. and Kopin, I.J. (1971) Dopamine: Stimulation induced release from central neurons. Science, 172, 487-489.

Noujaim, A., Ediss, C. and Wiebe, L. (1971) Precision of some quench correction methods in liquid scintillation counting, in "Organic Scintillators and Liquid Scintillation Counting" (D.L. Horrocks and C.T. Peng, eds) pp.705-717. Academic Press, New York and London.

Nowak, T.S. and Munro, H.N. (1977) Inhibition of cell-free protein synthesis initiation by amphetamine: Association with reduction in tRNA aminoacylation. Biochem.Biophys.Res.Comm., 77, 1280-1285.

Oh, T.H. (1975) Neurotrophic effects: Characterization of the nerve extract that stimulates muscle development in culture. Exp.Neurol., 46, 432-438.

Oh, T.H. (1976) Neurotrophic effects of sciatic nerve extracts on muscle development in culture. Exp.Neurol., 50, 376-386.

Oja, S.S. (1967) Studies on protein metabolism in developing rat brain. Ann.Acad.Sci.Fenn.A.V., 131, 1-81.

OjA, S.S. (1973) Comments on the measurement of protein synthesis in the brain. Int.J.Neuroscience, 5, 31-33.

Oldendorf, W.H. (1971) Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. Amer.J.Physiol., 221, 1629-1639.

Palladin, A.V., Belik, Ya.A. and Polyakova, N.M. (1977) in "Protein Metabolism of the Brain" (A.Lajtha, ed of English translation) pp.169-202. Plenum Press, New York.

Patterson, M.S. and Greene, R.C. (1965) Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal.Chem., 37, 854-857.

Peavy, D.E. and Hansen, R.J. (1976) Lack of effect of amino acid concentration on protein synthesis in the perfused rat liver. Biochem.J., 160, 797-801.

Phillips, A.G., Carter, D.A. and Fibiger, H.C. (1976) Dopaminergic substrates of intracranial self-stimulation in the caudate-putamen. Brain Res., 104, 221-232.

Pieri, L., Pieri, M. and Haefely, W. (1974) LSD as an agonist of dopamine receptors in the striatum. Nature, 252, 586-588.

Pijnenburg, A.J.J., Honig, W.M.M. and Van Rossum, J.M. (1975) Effects of antagonists upon locomotor stimulation induced by injections of dopamine and noradrenaline into the nucleus accumbens of nialimide-pretreated rats. Psychopharmacol., 41, 175-180.

Pijnenburg, A.J.J., Honig, W.M.M., Van Der Heyden, J.A.M. and Van Rossum, J.M. (1976) Effects of chemical stimulation of the mesolimbic dopamine system upon locomotor activity. Eur.J.Pharmacol., 35, 45-58.

Pijnenburg, A.J.J. and Van Rossum, J.M. (1973) Stimulation of locomotor activity following injection of dopamine into the nucleus accumbens. J.Pharm.Pharmac., 25, 1003-1005.

Pijnenburg, A.J.J., Woodruff, G.N. and Van Rossum, J.M. (1973) Ergometrine induced locomotor activity following intracerebral injection into the nucleus accumbens. Brain Res., 59, 289-302.

Pletscher, A. and Gey, K.F. (1962) Topographical differences in the cerebral metabolism of DL-2-^{14}C-3,4-dihydroxyphenylalanine. Exp., 18, 512-513.

Portugal, F.H., Elwyn, D.H. and Jeffay, H. (1970) Free lysine compartments in rat liver cells. Biochim.Biophys.Acta., 215, 339-347.

Randrup, A. and Munkvad, I. (1966a) Role of catecholamines in the amphetamine excitatory response. Nature, 211, 540.

Randrup, A. and Munkvad, I.(1966b) Dopa and other naturally occuring substances as causes of stereotypy and rage in rats. Acta.Psychiat. Scand., Suppl. 191 (Vol 42), 193-199.

Randrup, A. and Munkvad, I. (1970) Biochemical, anatomical and psychological investigations of stereotyped behavior induced by amphetamines, in "Amphetamines and related compounds" (E.Costa and S. Garattini, eds) pp.695-713. Raven Press, New York.

Randrup, A., Munkvad, I. and Udsen, P. (1963) Adrenergic mechanisms and amphetamine induced abnormal behaviour. Acta.Pharmacol.et Toxicol., 20, 145-157.

Rees, H.D., Brogan, L.L., Entingh, D.J., Dunn, A.J., Shinkman, P.G., Damstra-Entingh, T., Wilson, J.E. and Glassman, E. (1974) Effect of sensory stimulation on the uptake and incorporation of radioactive lysine into protein of mouse brain and liver. Brain Res., 68, 143-156.

Richardson, K. and Rose, S.P.R. (1971) A diurnal rhythmicity in incorporation of lysine into rat brain regions. Nature, 233, 182-184.

Roberts, S. (1974) Effects of amino acid imbalance on amino acid utilization, protein synthesis and polyribosome function in cerebral cortex, in "Aromatic Amino Acids in the Brain" Ciba Foundation Symposium 22 (new series) pp.299-324. Elsevier, Amsterdam.

Roberts, S. and Morelos, B.S. (1965) Regulation of cerebral metabolism of amino acids - IV. Influence of amino acid levels on leucine uptake, utilization and incorporation into protein in vivo. J.Neurochem., 12, 373-387.

Roberts, S. and Morelos, B.S. (1976) Role of ribonuclease action in phenylalanine-induced disaggregation of rat cerebral polyribosomes. J.Neurochem., 26, 387-400.

Roel, L.E., Schwartz, S.A., Weiss, B.F., Munro, H.N. and Wurtman, R.J. (1974) In vivo inhibition of rat brain protein synthesis by L-dopa. J.Neurochem., 23, 233-239.

Rolls, E.T. (1971) Contrasting effects of hypothalamic and nucleus accumbens septi self-stimulation on brain stem single unit activity and cortical arousal. Brain Res., 31, 275-285.

Rolls, E.T., Kelly, P.H. and Shaw, S.G. (1974) Noradrenaline, dopamine, and brain-stimulation reward. Pharm.Biochem.Behav., 2, 735-740.

Rose, S.P.R. (1973) Cellular compartmentation of metabolism in the brain, in "Metabolic Compartmentation in the Brain " (R. Balázs and J.E. Cremer, eds) pp.287-304. Macmillan Press Ltd., London.

Rose, S.P.R. and Sinha, A.K. (1974) Incorporation of amino acids into proteins in neuronal and neuropil fractions of rat cerebral cortex: Presence of a rapidly labelling neuronal fraction. J.Neurochem., 23, 1065-1076.

Sabri, M.I., Bone, A.H. and Davison, A.N. (1974) Turnover of myelin and other structural proteins in the developing rat brain. Biochem.J., 142, 499-507.

Sandoval, M.E., Palacios, R. and Tapia, R. (1976) Studies on the relationship between GABA synthesis and protein synthesis in brain. J.Neurochem., 27, 667-672.

Sandoval, M.E. and Tapia, R. (1975) GABA metabolism and cerebral protein synthesis. Brain Res., 96, 279-286.

Scales, B. (1972) Questions regarding the occurrence of unwanted luminescence in liquid scintillation samples, in "Liquid Scintillation Counting" (M.A. Crook, P. Johnson and B. Scales, eds) Vol.2, pp.101-107. Heyden and Son Ltd., London.

Schotman, P., Lipon, L and Gispen, W.H. (1974) Conversion of [4,5-^{3}H] leucine into ^3H_2O and tritiated metabolites in rat brain tissue. Comparison of a peripheral and intracranial route of administration. Brain Res., 70, 377-380.

Schotman, P., Reith, M.E.A. and Gispen, W.H. (1977) Effects of stressful procedures as ether anesthesia and intracranial injections on amino acid incorporation into brain protein. Brain Res.Bull., 2, 83-87.

Segal, H.L. (1976) Mechanism and regulation of protein turnover in animal cells, in "Current Topics in Cellular Regulation" (B.L. Horecker and E.R. Stadtman, eds) Vol.11, pp.183-201. Academic Press, New York.

Sellinger, O.Z. and Nordrum, L.M. (1969) A regional study of some osmotic, ionic and age factors affecting the stability of cerebral lysosomes. J.Neurochem., 16, 1219-1229.

Seta, K., Sansur, M. and Lajtha, A. (1973) The rate of incorporation of amino acids into brain proteins during infusion in the rat. Biochim.Biophys.Acta., 294, 472-480.

Sidransky, H., Bongiorno, M., Sarma, D.S.R. and Verney, E. (1967) The influence of tryptophan on hepatic polyribosomes and protein synthesis in fasted mice. Biochem.Biophys.Res.Comm., 27, 242-248.

Siegel, F.L., Aoki, K. and Colwell, R.E. (1971) Polyribosome disaggregation and cell-free protein synthesis in preparations from cerebral cortex of hyperphenylalaninemic rats. J.Neurochem., 18, 537-547.

Simpson, M.V. (1953) The release of labeled amino acids from the proteins of rat liver slices. J.Biol.Chem., 201, 143-154.

Smythies, J.R. (1970) The chemical nature of the receptor site. A study in the stereochemistry of synaptic mechanisms. Int.Rev.Neurobiol., 13, 181-222.

Snyder, S.H. (1975) The glycine synaptic receptor in the mammalian central nervous system. Br.J.Pharmac., 53, 473-484.

Sourkes, T.L. (1971) Possible new metabolites mediating actions of L-dopa. Nature, 229, 413-414.

Spano, P.F., Kumakura, K., Tonon, G.C., Govoni, S. and Trabucchi, M. (1975) LSD and dopamine-sensitive adenylate-cyclase in various rat brain areas. Brain Res., 93, 164-167.

Spiegel, M.R. (1971) in "Theory and Problems of Advanced Mathematics for Engineers and Scientists" (Schaum's Outline Series) McGraw-Hill Book Co., New York, London.

Steinberg, D. and Vaughan, M. (1956) Observations on intracellular protein catabolism studied in vitro. Arch.Biochem.Biophys., 65, 93-105.

Stephenson, G. (1966) in "Mathematical Methods for Science Students" Fourth Impression, Longmans, London.

Stinus, L., Thierry, A.M. and Cardo, B. (1976) Effects of various inhibitors of tyrosine hydroxylase and dopamine beta-hydroxylase on rat self-stimulation after reserpine treatment. Psychopharmacol., 45, 287-294.

Swaiman, K.F., Hosfield, W.B. and Lemieux, B. (1968) Elevated plasma phenylalanine concentration and lysine incorporation into ribosomal protein of developing brain. J.Neurochem., 15, 687-690.

Swick, R.W. (1958) Measurement of protein turnover in rat liver. J.Biol.Chem., 231, 751-764.

Taub, F. and Johnson, T.C. (1975) The mechanism of polyribosome disaggregation in brain tissue by phenylalanine. Biochem.J., 151, 173-180.

Tiplady, B. (1972) Brain protein metabolism and environmental stimulation, effects of forced exercise. Brain Res., 43, 215-225.

Toth, J. and Lajtha, A. (1977) Rates of exchange of free amino acids between plasma and brain in mice. Neurochemical Res., 2, 149-160.

Ungerstedt, U. (1971a) Stereotaxic mapping of the monoamine pathways in the rat brain. Acta.Physiol.Scand., Suppl. 367, 1-48.

Ungerstedt, U. (1971b) Postsynaptic supersensitivity after 6-hydroxy-dopamine induced degeneration of the nigro-striatal dopamine system. Acta.Physiol.Scand., Suppl.367, 69-93.

Van Riezen, H. (1972) Different central effects of the 5-HT antagonists mianserine and cyproheptadine. Arch.Int.Pharmacodyn., 198, 256-269.

Van Venrooij, W.J., Poort, C., Kramer, M.F. and Jansen, M.T. (1972) Relationship between extracellular amino acids and protein synthesis in vitro in the rat pancreas. Eur.J.Biochem., 30, 427-433.

Verity, M.A. and Brown, W.J. (1968) Structure-linked activity of lysosomal enzymes in the developing mouse brain. J.Neurochem., 15, 69-80.

Vidrich, A., Airhart, J., Bruno, M.K. and Khairallah, E.A. (1977) Compartmentation of free amino acids for protein biosynthesis. Influence of diurnal changes in hepatic amino acid concentrations on the composition of the precursor pool charging aminoacyl-transfer ribonucleic acid. Biochem.J., 162, 257-266.

Von Hungen, K., Roberts, S. and Hill, D.F. (1974) LSD as an agonist and antagonist at central dopamine receptors. Nature, 252, 588-589.

Von Hungen, K., Roberts, S. and Hill, D.F. (1975) Interactions between lysergic acid diethylamide and dopamine-sensitive adenylate cyclase systems in the rat brain. Brain Res., 94, 57-66.

Votava, Z. and Dyntarová, H. (1970) in "The Neuroleptics" (D.P. Bobon, J.A.P. Janssen and J. Bobon, eds) pp.102-103, Karger, Basel.

Waisman, H.A., Wang, H.L., Palmer, G. and Harlow, H.F. (1960) Phenylketonurea in infant monkeys. Nature, 188, 1124-1125.

Walter, H. (1960) Protein catabolism. Nature, 188, 643-647.

Weiss, B.F., Liebschutz, J.L., Wurtman, R.J. and Munro, H.N. (1975) Participation of dopamine- and serotonin-receptors in the disaggregation of brain polysomes by L-dopa and L-5-HTP. J.Neurochem., 24, 1191-1195.

Weiss, B.F., Munro, H.N., Ordonez, L.A. and Wurtman, R.J. (1972) Dopamine: Mediator of brain polysome disaggregation after L-dopa. Science, 177, 613-616.

Weiss, B.F., Munro, H.N. and Wurtman, R.J. (1971) L-dopa: Disaggregation of brain polysomes and elevation of brain tryptophan. Science. 173, 833-835.

Weiss, B.F., Roel, L.E., Munro, H.N. and Wurtman, R.J. (1974a) L-dopa, polysome aggregation and cerebral synthesis of protein, in "Aromatic Amino Acids in the Brain" Ciba Foundation Symposium 22 (new series) pp.325-332. Elsevier, Amsterdam.

Weiss, B.F., Roel, L.E., Munro, H.N. and Wurtman, R.J. (1974b) The effect of L-dopa on brain polysomes and protein synthesis: Probable mediation by intracellular dopamine. Adv.Neurol., 5, 87-96.

Weiss, B.F., Wurtman, R.J. and Munro, H.N. (1973) Disaggregation of brain polysomes by L-5-hydroxytryptophan. Life Sci., 13, 411-416.

Weissbach, H.and Ochoa, S. (1976) Soluble factors required for eukaryotic protein synthesis. Ann.Rev.Biochem., 45, 191-216.

Widelitz, M.M., Coryell, M.R. and Avadhani, N.G. (1977) Reversal of amphetamine induced polysome dissociation by neuroleptic agents in rat brain. Biochem.Biophys.Res.Comm., 76, 1223-1229.

Widelitz, M.M., Coryell, M.R., Widelitz, H. and Avadhani, N.G. (1975) Dissociation of rat brain polyribosomes in vivo by amphetamines. Brain Res., 100, 215-220.

Widelitz, M.M., Coryell, M.R., Widelitz, H., Avadhani, N.G. (1976) Effects of amphetamine administration in vivo on in vitro protein synthesizing system from rat brain. J.Neurochem., 27, 471-475.

Wilkinson, J.H. (1966) Calculation of eigensystems of matrices, in "Numerical Analysis. An Introduction" (J. Walsh, ed). Academic Press, London and New York.

Winkelman, J. and Slater, G. (1967) Chemiluminescence of liquid scintillation mixture components. Anal.Biochem., 20, 365-368.

Wise, R.A. (1978) Neuroleptic attenuation of intracranial self-stimulation: reward or performance deficits? Life Sci., 22, 535-542.

Wong, P.W.K., Fresco, R. and Jastice, P. (1972) The effect of maternal amino acid imbalance on fetal cerebral polyribosomes. Metab.Clin.Exp., 21, 875-881.

Woodside, K.H. and Mortimore, G.E. (1972) Suppression of protein turnover by amino acids in the perfused rat liver. J.Biol.Chem., 247, 6474-6481.

Woolf, L.I. (1970) Myelin deficiencies related to inborn errors of human metabolism, in "Myelination" (A.N. Davison and A. Peters, main authors) pp.183-198. Charles C. Thomas, Illinois.

Zilversmit, D.B., Entenman, C. and Fishler, M.C. (1943) On the calculation of "turnover time" and "turnover rate" from experiments involving the use of labeling agents. J.Gen.Physiol., 26, 325-331.