MULTIPLE FORMS OF ATP CITRATE LYASE FROM RAT LIVER AND BRAIN

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Anne Patricia Wilson

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To

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ABSTRACT

ATP citrate lyase (ATP citrate (pro-3S)-lyase EC 4.1.3.8.), an enzyme which produces cytoplasmic acetyl CoA for biosynthetic pathways, is shown to exist in multiple forms in rat liver and brain. The enzyme in crude supernatants of both tissues has been separated into two fractions by ion-exchange chromatography. The first peak of activity was eluted immediately, without retention, whilst the remaining activity was adsorbed on the column and was eluted by a salt gradient. Whereas only a minor proportion of the total liver activity (15 - 20%) was present in the non-retained, basic peak, it contained 40% of the brain enzyme. Gel filtration of crude liver extracts revealed the presence of a high molecular weight component ($M_r \sim 10^7$) of ATP citrate lyase, comprising 10% of the total activity, in addition to the tetrameric enzyme. This high molecular weight form was absent from brain supernatants. An estimated molecular weight of 410,000 was obtained for the tetramer using a calibrated gel filtration column.

Rechromatography experiments with the non-retained, basic form and the high molecular weight component, from liver, indicated that both are unstable. High-speed centrifugation of liver homogenates showed that the minor peak of activity from ion-exchange is not the same as the high molecular weight activity since removal of the latter did not result in loss of the non-retained peak. No evidence could be found for an association of ATP citrate lyase with the enzymes fatty acid synthetase or acetyl CoA carboxylase, or with mitochondrial membranes.

Inhibition of ATP citrate lyase activity by L-glutamate was investigated. The activity in crude supernatants of brain, and

liver from normal fed rats, was inhibited by 60%; that from liver of starved and refed rats was inhibited by 36%. Experimental evidence suggests that this inhibition is not due to a simple allosteric effect of glutamate.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosine 5'-monophosphate
CM-Sephadex	carboxymethyl Sephadex
CoA	coenzyme A
DCPIP	2,6-dichlorophenol indophenol
DEAE-Sephadex	diethylaminoethyl Sephadex
EDTA	ethylenediaminetetraacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PPO	2,5-diphenyloxazole
RIDCR	rotenone-insensitive NADH cytochrome c reductase
SEM	standard error of the mean
SDS	sodium dodecyl sulphate
Tris	Tris (hydroxymethyl)amino-methane

INTROUCTION

1. General Introduction

ATP citrate lyase (ATP:citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating);EC 4.1.3.8) is a cytoplasmic enzyme which catalyses cleavage of citrate from the mitochondria to produce acetyl CoA and oxaloacetate.

$$\text{citrate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{acetyl CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

Cytoplasmic acetyl CoA is required for biosynthesis of fatty acids, cholesterol, and acetylcholine. Thus, ATP citrate lyase provides a route of transport between the two separate pools of cellular acetyl CoA; one in the mitochondria, the site of production of acetyl CoA from pyruvate, and the second in the cytoplasm, the site of utilisation for biosynthetic pathways.

Citrate exported from the mitochondria and cleaved in the cytoplasm by ATP citrate lyase is now considered the major precursor of cytoplasmic acetyl CoA for fatty acid biosynthesis in liver and adipose tissue. However, the physiological role of ATP citrate lyase in brain is less well understood. It has been shown to be involved in the supply of acetyl CoA for both fatty acid and acetylcholine biosynthesis, and therefore it appears to have a dual role in this tissue. In addition, although ATP citrate lyase is the first enzyme in the pathway of both fatty acid and acetylcholine biosynthesis, no regulatory control mechanism has yet been found, and the role of the enzyme in the rate-limiting control of these pathways is still in dispute.

Hence, an investigation of the properties of ATP citrate lyase from liver and brain was undertaken in an attempt to establish whether the different physiological roles of the enzyme in these two tissues are

reflected in different properties. Moreover, the possibility that the enzyme may be specialised in a particular tissue may be an indication of a regulatory role.

2. Properties of ATP Citrate Lyase

i) General Properties

ATP citrate lyase was first discovered in pigeon liver (Srere and Lipmann, 1953), and has since been found in several tissues, from a number of animals (Hoffmann et al., 1979b; Srere, 1959). The highest activities of the enzyme are found in liver, adipose tissue,lactating mammary gland and brain, and it has been shown to be cytoplasmic in liver (Srere, 1959), adipose tissue (Martin and Denton, 1970; Saggerson, 1974), and brain (Szutowicz and Lysiak, 1980; Tucek, 1967b). The enzyme has been purified from rat liver (Hoffmann et al., 1979b ; Inoue et al., 1966; Linn and Srere, 1979; Redshaw and Loten, 1981; Singh et al., 1976), chicken liver (Srere, 1959), lactating rat mammary gland (Guy et al., 1980, 1981), and rat brain (Szutowicz et al., 1975). ATP citrate lyase from adipose tissue has been partially purified from pig (Mersmann and Houk, 1975); in addition, Ramakrishna and Benjamin (1979) purified a phosphoprotein from rat adipose tissue which they identified as ATP citrate lyase.

The highest specific activity that has been obtained for the pure enzyme was that reported by Linn and Srere (1979); their purification of liver ATP citrate lyase from starved and refed rats yielded a specific activity of 9 units/mg protein when measured by the coupled spectrophotometric assay at 25°C. Other values reported in the literature for the purified liver enzyme (from starved and refed rats) range from 3-8 units/mg protein (Alexander et al., 1979; Hoffmann et al., 1979b; Inoue et al., 1966; Redshaw and Loten, 1981; Singh et al.,

1976; Vogel and Bridger, 1981; Walsh and Spector, 1969). This represents the range obtained for the enzyme purified by different methods, and assayed under different conditions of temperature, pH and protein determination. Hence, although not directly comparable, it gives some indication of the activity of the pure liver enzyme. The specific activity of ATP citrate lyase purified from lactating rat mammary gland was reported to be 3.8 units/mg protein (Guy et al., 1981), which is of the same order as that for the liver enzyme. In contrast, Szutowicz et al. (1975) purified rat brain ATP citrate lyase and determined that the specific activity was only 0.12 units/mg protein. Thus, it appears that the activity of the brain enzyme is somewhat lower than that of liver even taking into account differences in conditions.

Rat liver ATP citrate lyase has a molecular weight of 4×10^5 - 5×10^5 as determined by ultracentrifugation (Inoue et al., 1966) and by sedimentation equilibrium (Singh et al., 1976). SDS polyacrylamide gel electrophoresis of the pure liver enzyme revealed a single band with an estimated molecular weight of 1.2×10^5 (Singh et al., 1976). This indicates that the enzyme consists of four equal sized subunits. Guy et al. (1981) have recently shown that the enzyme purified from lactating mammary gland has a subunit molecular weight similar to that of the liver enzyme. In addition, they observed that the native enzyme protein migrated in the ultracentrifuge in a manner identical to that shown for the liver enzyme (Inoue et al., 1966). No direct determination of the molecular weight of the enzyme from adipose tissue or brain has been reported. However, identification of a fat cell phosphoprotein as ATP citrate lyase (Alexander et al., 1979; Ramakrishna and Benjamin, 1979), was made partly on the basis of the similarity in subunit molecular weight of the two proteins, and the behaviour of the native protein on sucrose

gradient centrifugation. Hence, it would appear that adipose tissue ATP citrate lyase has a similar molecular weight to the liver enzyme.

The amino acid composition has been determined for the liver enzyme (Inoue et al., 1966; Singh et al.,1976; Srere, 1972) and the mammary gland enzyme (Guy et al., 1981). Suzuki et al.(1967) studied the immunochemical properties of the enzyme. Using antibody against rat liver ATP citrate lyase raised in rabbit, they found the enzyme in crude extracts from other tissues was immunochemically indistinguishable from the liver enzyme. In contrast, there appears to be species specificity, since liver ATP citrate lyase from other species showed only partial reaction with antibody to rat liver enzyme.

ATP citrate lyase is unstable both in crude extracts and in the purified state. This instability is partly due to oxidation of essential sulphydryl groups and hence storage in the presence of a sulphydryl reagent, usually 10mM dithiothreitol, is essential (Cottam and Srere, 1969). The enzyme is also susceptible to proteolytic attack (Linn and Srere, 1979), resulting in the formation of a number of small polypeptides as seen by SDS gel electrophoresis of the pure liver enzyme (Guy et al., 1981; Linn and Srere, 1979; Singh et al., 1976). Several different conditions have been used for storage of the enzyme (Cottam and Srere, 1969; Inoue, et al., 1966; Linn and Srere, 1979; Mersmann and Houk, 1975; Redshaw and Loten, 1981; Walsh and Spector, 1969). These include storage in the presence of different sulphydryl reagents, and salts (e.g. KCl,$(NH_4)_2SO_4$,$MgCl_2$), in addition to various pH conditions and temperatures (i.e. 0°C and -20°C).

ii) Reaction Mechanism

The reaction mechanism of the ATP citrate lyase catalysed cleavage of citrate is complex. Kinetic analysis of the enzyme reaction

suggested a sequential mechanism (Farrar and Plowman, 1971a; Plowman and Cleland, 1967). However, isotopic exchange experiments (Farrar and Plowman, 1971b; Plowman and Cleland, 1967) indicated that after phosphorylation of the enzyme by MgATP (Inoue et al.,1968; Plowman and Cleland, 1967), release of MgADP could occur prior to addition of the other two substrates indicating a ping-pong mechanism. Therefore it was proposed that after the initial phosphorylation of the enzyme, the reaction could proceed by different routes depending on the conditions. Schematic representation of the reaction mechanism has been presented by Plowman and Cleland (1967) and Srere (1972). The phosphorylated enzyme has been isolated and identified as a true intermediate (Inoue et al.,1968; Plowman and Cleland, 1967).

ATP citrate lyase shows an absolute specificity for the three substrates, ATP, citrate and CoA (Inoue et al., 1966; Srere, 1961; Mersmann and Houk, 1975). Thus, no appreciable activity was observed when ATP was replaced by another nucleotide triphosphate, or when citrate was replaced by one of several other organic acids tested; pantotheine could not replace CoA. Mg^{2+} is essential for enzyme activity, although it can be replaced with Mn^{2+} or Co^{2+} but with subsequent loss of 20-40% of activity. There is also a requirement for sulphydryl groups on the enzyme, demonstrated by the inhibition of activity by 5,5'-dithiobis(2-nitrobenzoate) (Cottam and Srere, 1969).

The three substrates of ATP citrate lyase are used in equimolar amounts to produce the products of the reaction, acetyl CoA, oxaloacetate, ADP and inorganic phosphate (Srere, 1961). Cleavage of citrate by ATP citrate lyase is stereospecific, such that the carbon-carbon bond broken is the same one that is formed by the condensation of oxaloacetate and acetyl CoA by citrate synthase (Srere and Bhaduri, 1964). Thus, acetyl CoA consists entirely of carbon 1 and 2 of citrate; carbons

4 and 5 are incorporated into the second cleavage product, oxaloacetate.

The pH optimum of liver ATP citrate lyase is between pH 8.4-8.7 for the rat (Inoue et al.,1966) and chicken (Cottam and Srere, 1969) enzyme, whereas a lower optimum of pH 8.0 has been reported for pig adipose tissue enzyme (Mersmann and Houk, 1975). Szutowicz and co-workers (Szutowicz et al., 1971, 1974b, 1975) repeatedly found a pH optimum of pH 7.8 for both liver and brain enzymes.

iii) Kinetic Properties

The equilibrium constant for the ATP citrate lyase catalysed reaction was found to be 1.0-1.5M (Plowman and Cleland, 1967). Although Srere (1961) failed to demonstrate the reverse reaction (i.e. formation of citrate), such reversibility was later shown by others (Inoue et al., 1968; Plowman and Cleland, 1967). However, the reaction was stronger in the direction of citrate cleavage; Plowman and Cleland (1967) found that citrate formation proceeded at only 5% of the rate of citrate cleavage. Therefore, under physiological conditions, the reaction catalysed by ATP citrate lyase is considered irreversible.

ATP citrate lyase from rat liver shows normal Michaelis-Menton kinetics with respect to ATP and CoA. K_m values for these two substrates are in the range of 0.17-0.50mM and 1.6-3.0μM respectively (Hoffmann et al., 1979b; Inoue et al., 1966; Plowman and Cleland, 1967; Ranganathan et al., 1980). Similar K_m values for ATP were reported for the enzyme from rat brain and mammary gland (Guy et al., 1981; Szutowicz et al., 1971, 1974b), and Mersmann and Houk (1975) obtained a K_m of 0.7mM for ATP using partially purified enzyme from pig adipose tissue. K_m values for CoA appear to be more variable; 0.7μM for brain (Szutowicz et al., 1974b) and 12μM for mammary gland (Guy et al., 1981). In contrast however, the kinetics of liver ATP citrate lyase with respect to the third substrate,

citrate, were non-linear, exhibiting an unusual biphasic nature.

Thus, Plowman and Cleland (1967), using purified rat liver ATP citrate lyase, observed non-linear double reciprocal plots of velocity versus citrate concentration over the range of approximately 0.03-2mM citrate. However, in the presence of a high chloride ion concentration (0.25M) such plots became linear, and the K_m for citrate was determined to be 0.071mM. Similar results were later reported by Watson et al. (1969) who calculated two K_m values for citrate; 0.16mM for low citrate concentrations (<1mM) and 5mM for high citrate concentrations. In the presence of high chloride ion concentration, they observed a K_m for citrate of 0.14mM i.e. comparable to that for low citrate concentration. Furthermore, under conditions of high chloride they reported a 50% decrease in V_{max} of the enzyme when measured at low citrate concentrations.

This biphasic nature of the kinetics of the enzyme with respect to citrate has also been observed by Szutowicz and co-workers, for both liver and brain ATP citrate lyase (Szutowicz and Angielski, 1970; Szutowicz et al., 1975). Using a much wider range of citrate concentrations (approximately 0.2-20mM), they obtained biphasic kinetics in the presence of high chloride ion concentration, conditions which had earlier been reported by others to linearise the kinetics. However, in contrast to the work described above, the deviation from linearity was observed at a much higher citrate concentration (3-5mM). It would therefore appear that in the presence of chloride ions a higher concentration of citrate is required for the conversion of the enzyme from the low K_m to the high K_m state. Szutowicz and Angielski (1970) suggested that the enzyme has two independent sites for citrate, a substrate binding site and a regulatory site. Thus, in the presence of a high citrate concentration, citrate binds to the low affinity (high K_m) regulatory site in addition to the high affinity (low K_m) substrate binding site, resulting in activation of the enzyme. No co-operativity of citrate binding was detected

(Szutowicz and Angielski, 1970). The effect of chloride ions under these circumstances would be to act as a competitive inhibitor of citrate at the regulatory site, thus resulting in lower activity of the enzyme as observed by Watson et al. (1969). The inhibition could be overcome by high citrate concentration as indicated by the higher citrate concentration required in the presence of chloride ions to observe the high K_m for citrate.

An alternative explanation put forward by Plowman and Cleland (1967), was that there are two forms of ATP citrate lyase each with a different affinity for citrate. Hence, one form with high affinity for citrate would use Mg-citrate as the substrate, whereas the low affinity form would utilise free citrate. The effect of Cl^- would then be to inhibit the low affinity form. A similar theory was proposed by Szutowicz and Angielski (1970) who suggested the existence of isoenzymes. Hoffmann et al. (1979b) also observed activation of rat liver ATP citrate lyase in the presence of high citrate concentration (20mM). However, they also reported activation of the enzyme in the presence of high chloride ion concentration (0.25M), both at 2mM and 20mM citrate. They explained these results in terms of a general activation of the enzyme by various anions; hence, citrate and chloride ions produced the same effect because of the anionic nature of both. However, they expressed their results only in terms of percentage activation of the enzyme, and therefore comparison with the more detailed results of Watson et al. (1969) is not possible. Nevertheless, the function of a relatively non-specific activation, as suggested by Hoffmann and co-workers, is not readily apparent whereas the more specific activation by the enzyme substrate citrate proposed by others (Plowman and Cleland, 1967; Szutowicz and Angielski, 1970; Watson et al.,1969) is more favourable in terms of regulation of enzyme activity.

Plowman and Cleland (1967) also suggested that the biphasic kinetics could be explained by a random reaction mechanism which would result in presence of more than one enzyme-substrate complex to which citrate could bind. However, the authors pointed out that such a mechanism required that at least two of the substrates should show biphasic kinetics. In this respect it is interesting to note that Mersmann and Houk (1975) observed biphasic kinetics of partially purified ATP citrate lyase from pig adipose tissue with CoA as well as with citrate.

The reports in the literature therefore indicate that the K_m for citrate of ATP citrate lyase is dependent on both the citrate and the chloride ion concentration. In addition, the kinetics of the enzyme with respect to citrate and ATP, depend on the Mg^{2+} concentration indicating the involvement of MgATP and Mg-citrate complexes (Szutowicz and Angielski, 1970). Nevertheless, for given concentrations of Cl^- and Mg^{2+}, there appear to be two K_m values for citrate, one at low citrate concentrations, 0.05-0.16mM, and a higher K_m, 2-5mM, at high citrate concentrations (Plowman and Cleland, 1967; Szutowicz and Angielski, 1970; Szutowicz et al., 1971, 1974b, 1975; Watson et al., 1969). Interestingly however, Inoue et al. (1966) failed to observe any biphasic nature of the kinetics of pure liver ATP citrate lyase with respect to citrate, despite measuring the activity over a range of 1-10mM citrate. This may have been the result of the concentrations of Cl^- and Mg^{2+} in the assay.

There has been very little recent work on the kinetics on ATP citrate lyase and in particular with respect to citrate. However, it seems that this is an important area of research especially in view of the possible regulatory role of citrate. Moreover, citrate is a well-known activator of acetyl CoA carboxylase. It has long been considered the major regulator of the activity of this enzyme, which is generally

accepted as the rate-limiting enzyme of fatty acid biosynthesis. From a practical point of view, assay of ATP citrate lyase at the usual optimal citrate concentration (~20mM) may mask regulatory mechanisms present *in vivo* where conditions of lower citrate concentrations, 0.05-2mM (Greenbaum *et al.*, 1971; Siess *et al.*, 1976, 1978), may affect the K_m of the enzyme for citrate, and the rate of the reaction.

ATP citrate lyase of rat liver and brain is inhibited by two of the reaction products, ADP and inorganic phosphate (Inoue *et al.*, 1966; Szutowicz and Angielski, 1970; Szutowicz *et al.*,1974b, 1975). The inhibition is competitive with respect to ATP and the K_i for ADP is 0.13-0.19mM for both the liver and brain enzyme. The other two products of the reaction, acetyl CoA and oxaloacetate, had no inhibitory effect on liver ATP citrate lyase (Inoue *et al.*,1966).

Szutowicz *et al.*(1974a) reported inhibition of ATP citrate lyase by L-glutamate. The purified enzyme from liver of starved rats, and from adult rat brain was inhibited by 40% and 80% respectively, in the presence of 10mM glutamate. They reported that the inhibition was competitive with respect to ATP, and determined the K_i for the brain enzyme to be 0.3mM. In contrast, glutamate was without effect on the enzyme from newborn rat brain, and liver ATP citrate lyase from starved and refed rats. Szutowicz and co-workers (Szutowicz *et al.*, 1974b, 1975) also showed inhibition of liver and brain ATP citrate lyase by α-keto-glutarate, irrespective of the age or dietary condition of the animals used. 50% inhibition was obtained in the presence of 10mM α-ketoglutarate, and the K_i for the brain enzyme was 9mM.

The physiological significance of the inhibition of ATP citrate lyase by ADP and L-glutamate will be discussed later.

iv) Effects of Diet, Hormones and Development on ATP Citrate Lyase Activity

a) Diet

ATP citrate lyase activity of liver and adipose tissue is dependent on the dietary condition of the animals (Barth et al., 1972; Foster and Srere, 1968; Goodridge, 1968c; Hoffmann et al., 1980c; Kornacker and Ball, 1965; Kornacker and Lowenstein, 1965a,b; Pearce, 1980; Shrago et al.,1971; Szutowicz et al.,1974b; Takeda et al.,1967; Vernon and Walker, 1968b). Rats fed a high carbohydrate diet showed an increased liver ATP citrate lyase activity of 2-13-fold after 3-6 days. The magnitude of the increase was found to be dependent on the composition of the diet; a diet high in fructose produced a greater response than a high glucose diet. Glycerol feeding was also effective in producing an increased ATP citrate lyase activity of the tissue. Moreover, the highest increase in enzyme activity was observed when rats were refed a high carbohydrate diet after an initial 48h starvation. In contrast, refeeding a high fat diet inhibited this increase of activity. Starvation for 48h produced a 3-fold decrease in liver ATP citrate lyase activity.

Adipose tissue ATP citrate lyase responds to dietary conditions in the same way as liver enzyme, although the magnitude of the changes in activity appear to be lower. In contrast, the activity of the enzyme in brain is not affected by the nutritional status of the animal (Szutowicz et al.,1974b).

The effect of diet on liver and adipose tissue ATP citrate lyase can be correlated with the observed alterations in fatty acid synthesis under the same conditions. Thus, Spencer et al.(1964) measured increased incorporation of citrate into fatty acids of liver in response to starving and refeeding, and a decreased incorporation during starvation. Similar results were obtained in liver and adipose tissue using labelled glucose

as the precursor (Goodridge, 1968c; Saggerson and Greenbaum, 1970b).

b) Hormones

Rat liver ATP citrate lyase activity has been shown to be influenced by hormones (Kornacker and Lowenstein, 1965b; Spence et al., 1979; Szutowicz et al.,1974b). Insulin injection increased ATP citrate lyase activity of liver, and a similar response was observed using cultured hepatocytes incubated with insulin for 24h. In contrast, glucagon produced a 68% decrease in ATP citrate lyase activity of cultured hepatocytes.

ATP citrate lyase activity is decreased in liver of diabetic animals (Kornacker and Lowenstein, 1965b; Szutowicz et al.,1974b; Takeda et al., 1967), in parallel with a decreased rate of fatty acid synthesis. Insulin administration produced an increase in ATP citrate lyase activity which was greater than that seen in normal animals. Furthermore, the enzyme activity of diabetic animals could be increased by feeding a high fructose or high glycerol diet; feeding a high glucose diet had no effect. This can be explained to be the result of impaired glucose metabolism in diabetic animals, whereas utilisation of fructose and glycerol is unaffected. Hence, feeding fructose or glycerol to diabetic animals has the same effect as feeding normal rats any high carbohydrate diet, i.e. increased ATP citrate lyase activity.

No changes have been found in brain ATP citrate lyase in response to insulin or diabetes (Szutowicz et al., 1974b). The effect of hormones on the adipose tissue enzyme does not appear to have been studied in any detail.

c. Development

ATP citrate lyase is also sensitive to developmental changes (Ballard and Hanson, 1967; Goodridge, 1968b,c; Szutowicz et al.,1974b;

Taylor et al.,1967; Vernon and Walker, 1968a,b). In early development, the changes in activity can generally be attributed to the different dietary conditions. Thus, the enzyme activity is high in liver of foetal rats when the diet is high in glucose, but decreases rapidly after birth at the onset of suckling when the diet changes to one high in fat. At weaning there is a large increase in activity corresponding to the change to an essentially high carbohydrate diet. Similar results were obtained by Goodridge (1968b,c) for chicken liver ATP citrate lyase; in this case enzyme activity increased after hatching, with the change from the high fat diet of the yolk to the high carbohydrate diet fed to chicks. As shown for the effects of diet on the enzyme, the changes in ATP citrate lyase activity can be correlated with parallel changes in fatty acid synthesis (Ballard and Hanson, 1967; Goodridge, 1968a,c; Taylor et al.,1967). However, although these dietary changes play a large part in determining the ATP citrate lyase activity of the tissue, Taylor et al. (1967) suggested that the low activity found in suckling rat may be the result of other factors, since weaning rats onto a high fat diet did not completely prevent the normal increase in activity.

These early developmental changes in ATP citrate lyase do not appear to have been studied in detail for the adipose tissue enzyme, although Goodridge (1968b) reported that the enzyme activity showed no change in hatching chicks. He suggested that this was due to the minor role of this tissue in lipogenesis in birds compared to rats.

More recently it has been found that old obese rats have reduced ATP citrate lyase activity in adipose tissue, and a diminished capacity for induction of the liver enzyme, compared to young lean rats (Boll et al., 1982; Hoffmann et al., 1979a). However, this was found to be a result of the obesity of the old rats rather than age (Boll et al., 1982; Hoffmann et al., 1980b).

Brain ATP citrate lyase which, unlike the enzyme from liver and adipose tissue, does not respond to changes in diet or the presence of hormones, is affected by developmental changes. Thus, the enzyme activity was found to decrease in rats from newborn to adult (D'Adamo and D'Adamo, 1968; Szutowicz et al., 1974b). In contrast, Buckley and Williamson (1973) found no such change in activity. Szutowicz et al. (1980) however measured ATP citrate lyase activity in the cerebellum and the cerebrum, rather than the whole brain homogenates used previously. They found that although there was a decrease in activity of the enzyme in the cerebellum, the cerebral activity remained unchanged. They explained these results in terms of a developmental decrease in lipogenesis in both the cerebellum and the cerebrum, but an increase in acetylcholine synthesis in the cholinergic cerebrum. Hence, ATP citrate lyase which is thought to be involved in both lipogenesis and acetylcholine biosynthesis in brain, showed reduced activity in the cerebellum as a result of decreased lipogenesis, but in the cerebrum, the decreased requirement in this pathway was presumably balanced by an increased requirement in acetylcholine biosynthesis.

Another tissue containing a high activity of ATP citrate lyase is mammary gland from lactating rats. Enzyme activity is low in non-lactating tissue, but rises sharply at the start of lactation reaching a maximum by about the fourth day (Baldwin and Milligan, 1966; Howanitz and Levy, 1965; Martyn and Hansen, 1981). A rapid decrease in activity back to normal levels occurs at weaning.

Once again the changes in ATP citrate lyase activity can be correlated with the increased lipogenic capacity of the tissue during lactation (Martyn and Hansen, 1981; Smith and Ryan, 1979).

The changes in the level of ATP citrate lyase in various tissues described here, together with the kinetic properties of the enzyme (see Section 2iii) will now be discussed in terms of the possible regulatory properties of the enzyme.

v) Regulation of ATP Citrate Lyase Activity

Until recently ATP citrate lyase has not been seriously considered as a regulatory enzyme, and hence there has been little investigation of the possible mechanisms involved in the control of enzyme activity. However, as shown in the previous section, ATP citrate lyase activity does change in response to dietary, hormonal and developmental factors. Furthermore, in common with many regulatory enzymes, ATP citrate lyase has complex kinetics; citrate appears to act as an activator as well as a substrate, and the enzyme is inhibited by two of the reaction products, ADP and inorganic phosphate. Therefore, there appear to be two possible levels of control of ATP citrate lyase activity - long-term regulation involved in the overall level of enzyme activity of the tissue, and short-term control, possibly important in the regulation of fatty acid and/or acetylcholine biosynthesis.

The long-term regulation of ATP citrate lyase activity is brought about by changes in the amount of enzyme protein. Thus, the dietary induced increase in liver ATP citrate lyase was shown to be the result of an equivalent increase in the amount of enzyme protein, determined immunochemically (Suzuki et al., 1967). Conversely, starvation resulted in an equivalent decrease in enzyme protein. Several workers demonstrated that the increased ATP citrate lyase activity could be prevented by the use of inhibitors of protein synthesis, indicating that increased amount of enzyme was due to increased synthesis (Gibson et al., 1967; Kornacker and Lowenstein, 1965a; Szutowicz et al.,1974b; Takeda et

al., 1967). More recently Spence et al. (1979) demonstrated that the increase in ATP citrate lyase activity of cultured hepatocytes in response to insulin was accompanied by a similar increase in the amount of enzyme synthesised. However, the decrease in activity produced by glucagon did not result in a significant decrease in the enzyme synthesis. Beins et al.(1980) measured the rate of ATP citrate lyase synthesis in vivo by pulse labelling with tritiated leucine. They observed an increased rate of synthesis after feeding a high carbohydrate diet, and a decreased rate after starvation. However, they did not comment on whether these changes in the rate of synthesis of the enzyme completely accounted for the observed changes in ATP citrate lyase activity. Therefore, although long-term regulation of ATP citrate lyase activity appears to be controlled by the rate of synthesis of the enzyme, the possible involvement of the rate of degradation has not yet been excluded.

Short-term control of enzyme activity may involve allosteric regulation by substrates or other cell metabolites, and/or covalent modification of the enzyme. With reference to ATP citrate lyase, the biphasic kinetics of the reaction with respect to one of the substrates, citrate, and the inhibition of activity by ADP, one of the reaction products, both provide possible mechanisms of allosteric control.

As described earlier ATP citrate lyase has two K_m values for citrate (Section 2iii). One of the possible explanations for this phenomena is that citrate acts at a regulatory site in addition to the substrate binding site, although there appears to be no co-operativity between these sites (Szutowicz and Angielski, 1970).

The concentration of citrate in the cytoplasm varies between 0.05-2mM depending on the conditions of the experiment (Greenbaum et al., 1971; Siess et al.,1976, 1978). There appear to be conflicting reports

however, of the effect of various nutritional conditions on the citrate concentration (Ballard and Hanson, 1969; Greenbaum et al.,1971; Saggerson and Greenbaum, 1970a,b; Siess et al.,1976, 1978; Spencer and Lowenstein, 1967). Nevertheless, the general opinion is that changes in cellular citrate concentration, if any, do not correlate with changes in fatty acid biosynthesis. At first sight, therefore, it would appear that control of ATP citrate lyase by citrate under physiological conditions, is unlikely. However, with reference to short-term regulation of enzyme activity, the immediate changes in citrate concentration need to be considered. Moreover, comparison of the two K_m values of ATP citrate lyase for citrate (0.05-0.16mM and 2-5mM), with the range of citrate concentration measured in the cytoplasm, suggests that depending on the conditions the enzyme may operate at either K_m.

Atkinson and Walton (1967) considered the possibility of control of ATP citrate lyase by product inhibition. ADP is a competitive inhibitor of the enzyme with respect to ATP, and the K_i for ADP is similar to the K_m for ATP, i.e. around 0.2mM (Inoue et al.,1966). Comparison of these values with the cytoplasmic concentrations, ATP ~3mM, ADP ~0.5mM (Tischler et al., 1977), indicates that although the ATP concentration is sufficient to saturate the enzyme, the physiological ADP concentration is high enough to cause some inhibition. Atkinson and Walton (1967) suggested that the enzyme activity could be controlled by the ATP/ADP ratio, and proposed such a mechanism as a model for enzyme regulation by the energy charge of the cell. Thus, whilst absolute concentrations may change little under different physiological conditions, a significant change in the ATP/ADP may be achieved. Seitz et al. (1977) did in fact report a 2-fold increase of this ratio in the cytoplasm, during a 2h period of glucose refeeding. Hence, if ADP does inhibit ATP citrate lyase under physiological conditions, such a change would reduce this inhibition and therefore result in the

expected increase in enzyme activity.

The recent discovery that liver ATP citrate lyase is reversibly phosphorylated at a site(s) distinct from the phosphorylation which occurs as part of the enzyme reaction (Linn and Srere, 1979), has opened up the possibility of regulation of the enzyme by covalent modification.

Phosphorylation of liver ATP citrate lyase has been demonstrated *in vivo*, by injection of [^{32}P] phosphate into rats (Linn and Srere, 1979), and *in vitro*, by incubation of hepatocytes with [^{32}P] phosphate (Janski *et al.*, 1979). This phosphorylation of the enzyme at the structural site(s) could be readily distinguished from the catalytic phosphorylation because of its acid stability, in contrast to the lability of the latter under the same conditions (Linn and Srere, 1979). Incubation of hepatocytes with insulin or glucagon increased ATP citrate lyase phosphorylation (Alexander *et al.*, 1979; Janski *et al.*, 1979), and it was later demonstrated that cAMP-dependent (Alexander *et al.*, 1981; Guy *et al.*, 1980) and cAMP-independent (Ramakrishna and Benjamin, 1981) protein kinases are involved. However, the function of ATP citrate lyase phosphorylation is unclear since it does not produce any detectable change in enzyme activity (Guy *et al.*, 1981; Janski *et al.*, 1979; Linn and Srere, 1979). Moreover, comparison of the properties of the phosphorylated and dephosphorylated liver enzyme indicated that there was no difference in molecular weight, kinetic properties, pH optimum or thermal stability (Ranganathan *et al.*, 1980).

Therefore, although there are several potential mechanisms for short-term regulation of ATP citrate lyase, further investigation is necessary in order to assess the possible physiological significance of such regulation.

3. Physiological Role of ATP Citrate Lyase

i) Role of ATP Citrate Lyase in Fatty Acid Biosynthesis

Biosynthesis of fatty acids from acetyl CoA is catalysed by two enzymes, acetyl CoA carboxylase and fatty acid synthetase complex, and it takes place in the cytoplasm (Abraham et al.,1963; Spencer et al., 1964). Hence, a supply of cytoplasmic acetyl CoA is required, together with a source of reducing equivalents in the form of NADPH. Acetyl CoA is produced in the mitochondria from pyruvate by the enzyme pyruvate dehydrogenase, and therefore a mechanism for transport of the fatty acid precursor into the cytoplasm is required. The inner mitochondrial membrane is considered to be impermeable to acetyl CoA (Spencer and Lowenstein, 1962) thereby excluding the possibility of direct transfer. Thus, the supply of cytoplasmic acetyl units must be derived from mitochondrial acetyl CoA via an indirect route. The possible pathways of this transfer which have been postulated are shown in Fig.1. The three metabolites which have received most attention are citrate, acetate and acetylcarnitine, which require the enzymes ATP citrate lyase, acetyl CoA synthetase (EC 6.2.1.1), and carnitine acetyltransferase (EC 2.3.1.7) respectively to release the acetyl CoA in the cytoplasm. The importance of ATP citrate lyase in providing acetyl CoA for fatty acid biosynthesis will now be considered in more detail, and in relation to the other routes of acetyl group transfer to the cytoplasm.

a) Citrate as the Precursor of Cytoplasmic Acetyl CoA

Citrate was first shown to be an effective precursor of fatty acids in liver. Using crude extracts of pigeon liver, Formica (1962) and Srere and Bhaduri (1962) both demonstrated incorporation of labelled citrate into fatty acids. They observed a reduced incorporation in the presence of unlabelled acetate, and this was interpreted as evidence for

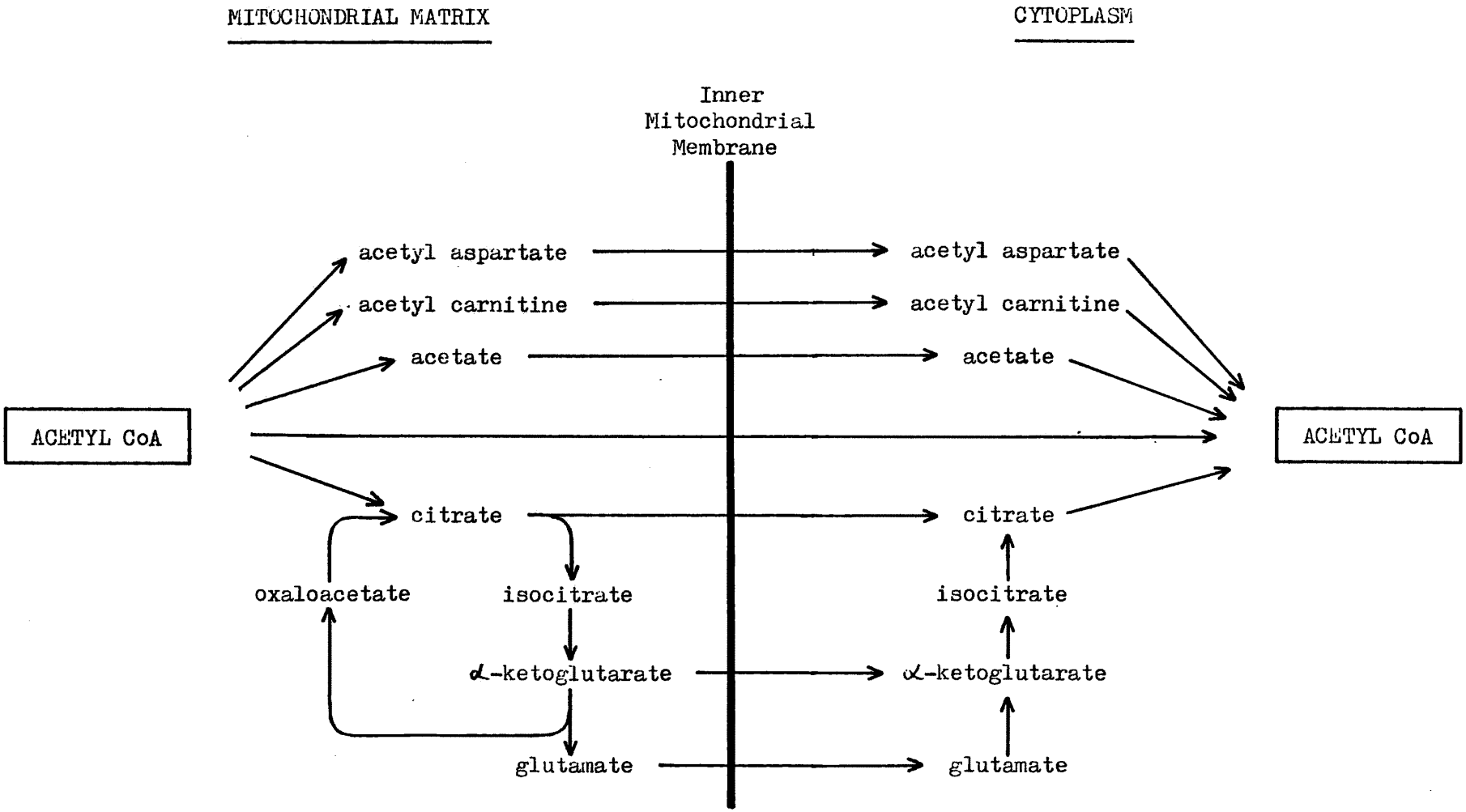


FIG.1

Schematic Representation of the Possible Routes of Acetyl CoA Transport out of the Mitochondria

a common route for both precursors i.e. acetyl CoA. Hence, the involvement of ATP citrate lyase was implicated, since this cytoplasmic enzyme catalyses the cleavage of citrate into acetyl CoA and oxaloacetate. The incorporation of labelled citrate into fatty acids has also been demonstrated in adipose tissue (Shrago et al., 1969), lactating mammary gland (Spencer and Lowenstein, 1962; Spencer et al., 1964), and brain (Dolezal and Tucek, 1981; Tucek et al., 1981).

Utilisation of citrate as a transporter of acetyl units out of the mitochrondria was also shown in experiments using glucose or pyruvate precursors (Bartley et al., 1965; Bressler and Brendel, 1966; Daikuhara et al., 1968). Bartley et al. (1965) measured incorporation of ^{14}C and ^{3}H label into the terminal methyl group of fatty acids from mixed labelled glucose, and found the results were consistent with incorporation via citrate. Similar results were obtained by Rognstad and Katz (1968). Using a reconstructed system consisting of mitochondrial and supernatant fractions, Daikuhara et al. (1968) measured acetyl CoA formation from pyruvate. They demonstrated, by addition of ATP citrate lyase antibody to this system, that more than 80% of the cytoplasmic acetyl CoA from pyruvate was supplied by ATP citrate lyase. This confirmed earlier results from Bressler and Brendel (1966) who concluded from their work that >70% of pyruvate is converted to fatty acids via citrate.

Further investigation of the utilisation of citrate for fatty acid biosynthesis revealed that in addition to a requirement for ATP, Mg^{2+} and CoA, there was no incorporation of carbon 5 of citrate, only carbon 1 (Bhaduri and Srere, 1963; Formica, 1962; Spencer and Lowenstein, 1962). This data is consistent with the known substrate requirements and stereospecificity of ATP citrate lyase, and therefore adds support to the involvement of this enzyme in the pathway from citrate to fatty acids.

The importance of ATP citrate lyase, and hence citrate, in

providing acetyl CoA for fatty acid biosynthesis has also been shown by the use of the specific inhibitor of this enzyme, hydroxycitrate (Szutowicz et al.,1976; Watson et al.,1969). Thus, in the presence of hydroxycitrate, incorporation of labelled citrate and alanine precursors into fatty acids or lipids was inhibited by 60%-80%, both in vitro (Sullivan et al., 1972; Watson and Lowenstein, 1970), and in vivo (Sullivan et al., 1972, 1974). Furthermore, hydroxycitrate also inhibited total liver fatty acid synthesis measured by the incorporation of tritiated water into fatty acids (Lowenstein, 1971). Patel and Clark (1980) observed reduced incorporation of labelled pyruvate into lipids by brain homogenates, in the presence of hydroxycitrate.

b) Other Mechanisms of Acetyl Group Transfer

Despite the fact that there is a large amount of evidence in support of citrate as the major carrier of acetyl units out of the mitochondria, other mechanisms do appear to exist (Fig.1). The most important alternative routes considered are acetate and acetylcarnitine.

Incorporation of acetate into fatty acids has been measured in liver (Bhaduri and Srere, 1963; Formica, 1962; Srere and Bhaduri, 1962; Spencer et al., 1964), mammary gland (Spencer and Lowenstein, 1962) and adipose tissue (Shrago et al.,1969). The enzyme required for acetyl CoA release from acetate, acetyl CoA synthetase, is found in the cytoplasm (Barth et al., 1971; Crabtree et al.,1981; Hoffmann et al.,1978; Szutowicz and Lysiak, 1980), and undergoes adaptive changes similar to those of ATP citrate lyase in response to diet and hormones (Barth et al., 1972; Kornacker and Lowenstein, 1965a,b). However, brain acetyl CoA synthetase increases during development in contrast to the decrease in ATP citrate lyase activity (Buckley and Williamson, 1973; Szutowicz et al., 1980).

The transport of acetyl units in the form of acetylcarnitine has been investigated and although this pathway has been shown to exist in

liver, it is not thought to be of any major importance (Bressler and Brendel, 1966; Daikuhara et al.,1968). The enzyme involved, carnitine acetyltransferase, is mitochondrial (McCaman et al.,1966; Martin and Denton, 1970; Saggerson, 1974; Szutowicz and Lysiak, 1980), but it is thought to be present either on both sides of the inner membrane or localised within the membrane in such a way as to be accesible to substrates on either side (Tucek, 1978). However, Bressler and Brendel (1966) concluded that this enzyme is not involved in fatty acid biosynthesis because changes in activity of carnitine acetyltransferase did not correlate with changes in the rate fatty acid synthesis; for example, starvation produced a 2-fold increase in enzyme activity in contrast to a decreased rate of fatty acid synthesis.

Patel and Clark (1980) reported that part of the cytoplasmic acetyl CoA is provided by N-acetylaspartate in young rat brain. Ketone bodies, an important source of respiratory fuel for the brain in young animals, have also been considered as a source of cytoplasmic acetyl CoA. Thus Patel and Clark (1980) suggested that acetoacetate formed by oxidation of 3-hydroxybutyrate in the mitochondria could be transported into the cytoplasm and converted to acetyl CoA. This conversion of acetoacetate into acetyl CoA in the cytoplasm has been shown to exist in brain, and it provides a cytoplasmic route for utilisation of ketone bodies (Buckley and Williamson, 1973; Patel and Owen, 1976).

A pathway involving transport of acetyl groups out of the mitochondria in the form of α-ketoglutarate and glutamate, with subsequent conversion to citrate, and then cleavage of citrate by ATP citrate lyase, has been proposed. Hence ATP citrate lyase is still involved but a more indirect carrier of acetyl units is utilized. Although such a pathway has been shown to operate in liver (D'Adamo and Haft, 1965), mammary gland (Madsen et al.,1964), and brain (D'Adamo and D'Adamo, 1968), the advantage

over the direct transport in the form of citrate is unclear.

c) Importance of ATP Citrate Lyase in Providing Cytoplasmic Acetyl CoA for Fatty Acid Biosynthesis

Although a number of possible pathways of transport of acetyl units out of the mitochondria have been shown to exist in various tissues, only citrate and acetate appear to be of importance. Attempts to determine the relative contribution of these two metabolites in the formation of cytoplasmic acetyl CoA have included comparison of the rates of incorporation of the labelled precursors into fatty acids (Bhaduri and Srere,1963; Spencer and Lowenstein, 1962; Spencer et al.,1964). Whereas Bhaduri and Srere (1963) found similar rates of incorporation of the ^{14}C-labelled precursors, using pigeon liver supernatant, Spencer and co-workers found greater incorporation of their [^{14}C] citrate precursor compared to the [^{3}H] acetate precursor, using rat mammary gland and liver. Furthermore, they found that the changes in the amount of the precursor incorporation in response to the nutritional and hormonal state of the animals were greater for citrate than acetate, suggesting that the former metabolite is more important. They calculated that the citrate incorporation into fatty acids in liver was greater than the maximal rate of lipogenesis measured in vivo. Hoffmann and Weiss (1978) measured citrate and acetate production from pyruvate by isolated rat liver mitochondria and found citrate production was about 4-fold greater than acetate production.

Comparison of the distribution and activities of the respective enzymes involved in these two pathways, ATP citrate lyase and acetyl CoA synthetase, also favours the citrate pathway. Hence, ATP citrate lyase is exclusively cytoplasmic whereas acetyl CoA synthetase is partially mitochondrial (Barth et al.,1971; Crabtree et al.,1981; Martin and Denton, 1970; Szutowicz and Lysiak, 1980). The highest activities of ATP citrate

lyase are found in tissues active in fatty acid biosynthesis i.e. liver, adipose tissue, lactating mammary gland, and to a lesser extent, brain (Hoffmann et al., 1979b; Srere, 1959). In contrast, acetyl CoA synthetase is present in appreciable amounts in other, non-lipogenic tissues, such as kidney and heart (Barth et al., 1971, 1972). ATP citrate lyase activity was found to be greater than that of acetyl CoA synthetase in several lipogenic tissues (Buckley and Williamson, 1973; Crabtree et al., 1981, Hoffmann et al., 1978; Kornacker and Lowenstein, 1965a,b; Martin and Denton, 1970), a possible indication of the greater importance of the former enzyme.

Kornacker and Lowenstein (1965a) measured the effect of diet on both ATP citrate lyase and acetyl CoA synthetase activities of rat liver supernatants. They concluded that citrate was more important in acetyl group transfer because changes in ATP citrate lyase were greater than those of acetyl CoA synthetase. Similar results were obtained by Hoffmann et al. (1978) using adipose tissue.

Finally, the large inhibition of lipogenesis by hydroxycitrate, the specific inhibitor of ATP citrate lyase, both in vitro and in vivo, provides strong evidence in support of a predominant role for citrate. Thus, cleavage of citrate by ATP citrate lyase is now considered to be the major pathway by which acetyl CoA is transferred from the site of production in the mitochondria, to the cytoplasm, the site of utilisation for fatty acid biosynthesis, at least in liver and adipose tissue (Fig.2). Nevertheless, acetate appears to have an important role in such transport of acetyl units in both liver and adipose tissue of starved animals (Sugden et al., 1982; Triscari and Sullivan, 1977).

In addition to its role in the production of cytoplasmic acetyl CoA, ATP citrate lyase can also participate in the supply of a second requirement of fatty acid biosynthesis, NADPH. Oxaloacetate, the other

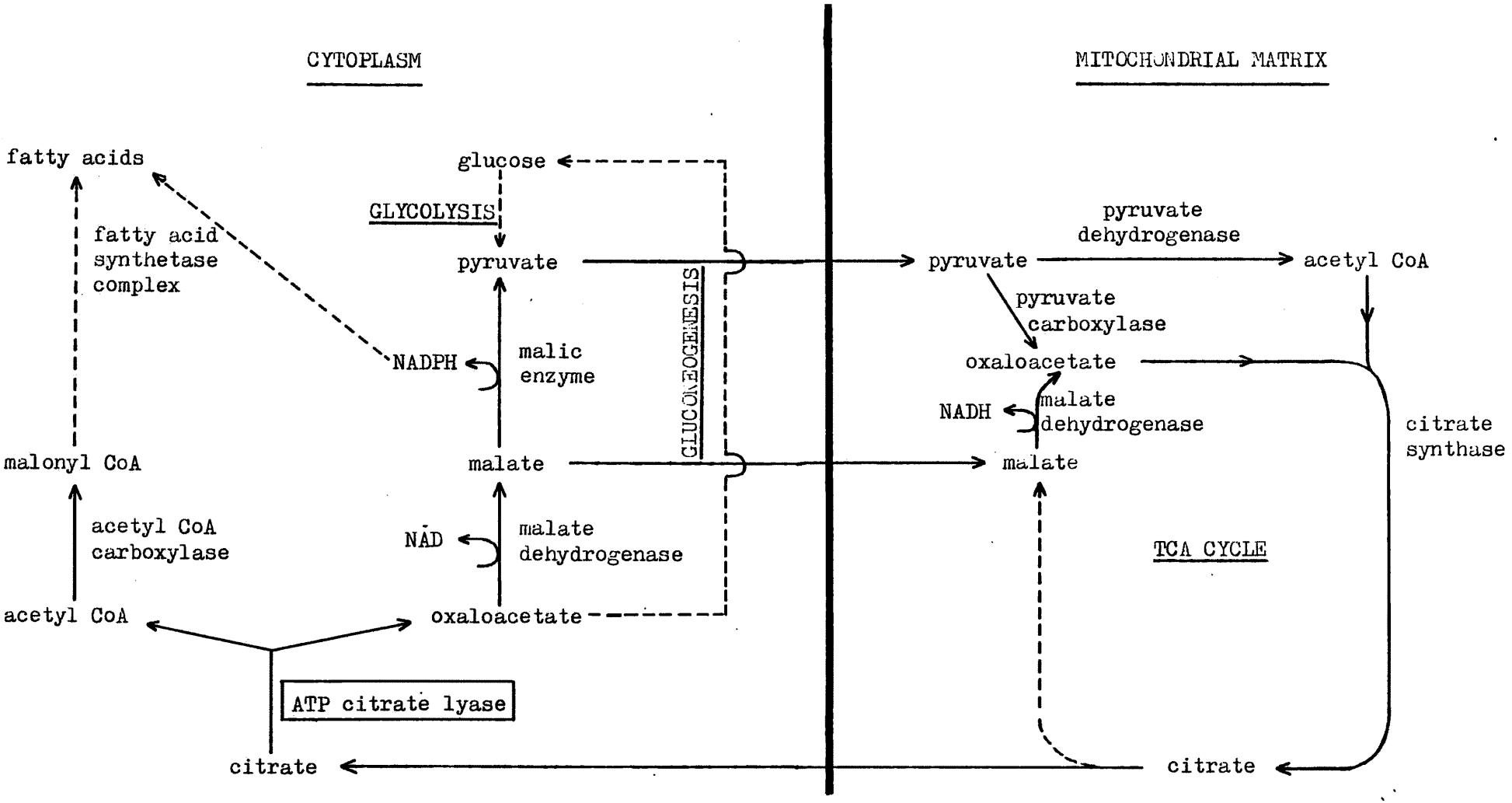


FIG. 2

Scheme Showing the Possible Pathways by which ATP Citrate Lyase can Supply the Precursors of Fatty Acid Biosynthesis

——— single enzyme reactions; ------ metabolic pathways involving more than one enzyme

cleavage product of citrate, although not significantly incorporated into fatty acids (Bhaduri and Srere, 1963), can be utilised to produce cytoplasmic NADPH (Fig.2). Thus, conversion to pyruvate via malate by the successive enzymes malate dehydrogenase (EC 1.1.1.37) and malic enzyme (EC 1.1.1.40), essentially results in formation of NADPH from NADH.

NADPH for fatty acid biosynthesis is produced in the cytoplasm by the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reactions of the pentose phosphate pathway (Flatt and Ball, 1964; Saggerson, 1974). The activities of both these enzymes are influenced by conditions known to influence the rate of fatty acid biosynthesis; the changes observed are consistent with those expected for enzymes which play an important role in fatty acid synthesis (Baldwin and Milligan, 1966; Takeda et al.,1967; Taylor et al., 1967; Vernon and Walker, 1968a,b).

However, it has been estimated that the pentose phosphate pathway can supply only 50-70% of the NADPH required for fatty acid biosynthesis (Flatt and Ball, 1964; Saggerson, 1974). Utilisation of oxaloacetate as described above would therefore provide a source of the remaining NADPH, as suggested by Young et al. (1964) and Kornacker and Ball (1965). The activity of one of the enzymes involved in this pathway, malic enzyme, is high in liver and adipose tissue. Furthermore, the activity of the enzyme has been shown to change in response to dietary and developmental changes in parallel to the changes in ATP citrate lyase activitv, both in liver and in adipose tissue (Ballard and Hanson, 1967; Goodridge, 1968b,c; Kornacker and Ball, 1965; Taylor et al., 1967; Vernon and Walker, 1968a,b; Wise and Ball, 1964). The existence of this pathway therefore supports the view that ATP citrate lyase is important in fatty acid biosynthesis since this enzyme not only produces cytoplasmic acetyl

CoA but can also provide a potential supply of NADPH.

d) Role of ATP Citrate Lyase in the Regulation of Fatty Acid Biosynthesis

Acetyl CoA carboxylase is generally accepted to be the rate-limiting enzyme in fatty acid biosynthesis, and hence the control point of the pathway (for reviews see Lane and Moss, 1971; Volpe and Vagelos, 1976). The enzyme is activated by citrate as a result of a conversion from the inactive protomeric form to the active, polymeric state and control of the polymerisation state of the enzyme in this way is thought to be basis of the regulation of activity. However, the physiological importance of such a mechanism is now considered doubtful (Kim, 1979) since Spencer and Lowenstein (1967) found no correlation between changes in cellular citrate concentration and acetyl CoA carboxylase activity. A second, and at present more favoured, mechanism of regulation of acetyl CoA carboxylase activity is that of covalent modification (Kim, 1979). Thus, reversible phosphorylation of the enzyme by both cAMP-dependent and cAMP-independent protein kinases has been shown to alter enzyme activity (Carlson and Kim, 1973, 1974; Hardie and Cohen, 1978).

ATP citrate lyase can be considered the first enzyme in the pathway of fatty acid biosynthesis, and is therefore potentially a rate-limiting step. Moreover, the fact that the enzyme reaction is physiologically irreversible (Inoue et al., 1968) and consumes ATP makes it a suitable control point. However, there appears to be little positive evidence at present to support a regulatory role of the enzyme in fatty acid biosynthesis, although no thorough investigation of the possible regulatory properties of the enzyme has been made.

A number of workers have measured ATP citrate lyase activity and acetyl CoA carboxylase and/or fatty acid synthetase in the same

sample under optimal assay conditions. Thus, ATP citrate lyase activity was found to be greater than acetyl CoA carboxylase activity in crude supernatants of mammary gland (Howanitz and Levy, 1965), and adipose tissue (Shrago et al., 1969). Shrago et al.(1969) also reported ATP citrate lyase activity to be greater than adipose tissue fatty acid synthetase using crude supernatants, and Finkelstein et al. (1979) observed higher specific activity of purified liver ATP citrate lyase compared to purified fatty acid synthetase from rats under different dietary conditions. These observations therefore suggest that ATP citrate lyase does not have a rate-limiting role in fatty acid biosynthesis. However, in all these reports ATP citrate lyase activity was measured at high citrate concentration (20mM), and as already discussed (Section 2iii)the kinetics of the enzyme reaction are greatly influenced by the citrate concentration. Kornacker and Lowenstein (1963) measured liver ATP citrate lyase under more physiological conditions (0.5mM citrate), and found the activity to be similar to that of fatty acid synthesis. In contrast, Howanitz and Levy (1965) observed a greater reduction of acetyl CoA carboxylase activity than ATP citrate lyase activity in crude supernatants of mammary glands, under similar conditions of reduced citrate concentration. They therefore concluded that acetyl CoA carboxylase was still rate-limiting even at physiological citrate concentrations. Nevertheless, determination of rate-limiting enzymes by measurement of enzyme activities under optimal, and even sub-maximal conditions, cannot alone provide conclusive evidence.

Using a different approach, Foster and Srere (1968) also concluded from their experiments that ATP citrate lyase is not involved in the regulation of fatty acid biosynthesis. They measured the time course of the changes in fatty acid synthesis and ATP citrate lyase activity produced by alterations of the dietary state of the animals.

Although they observed parallel changes, the rate of fatty acid synthesis was found to change before ATP citrate lyase activity. However, the alterations in the enzyme activity observed under these conditions are known to be the result of changes in the amount of enzyme protein, and therefore represents long-term regulation of enzyme activity. Hence, it is probable that such regulation occurs as an adaptation to accommodate the altered requirement for fatty acid synthesising capacity, rather than a mechanism for direct control of the pathway. Short-term regulation of enzyme activity by allosteric modulators and/or covalent modification would not necessarily result in detectable changes in the total enzyme activity measured, but could function to regulate the rate of fatty acid biosynthesis. Such regulation has already been shown for acetyl CoA carboxylase, but the involvement of ATP citrate lyase in this type of control has yet to be demonstrated.

More recently, Rognstad (1979) investigated the possibility of a rate-limiting role for ATP citrate lyase by measuring the flux through the pathway of fatty acid synthesis in hepatocytes, in the presence of increasing amounts of hydroxycitrate, to inhibit ATP citrate lyase activity. From their results they concluded that the rate of enzyme activity was 70% greater than that of fatty acid synthesis, and therefore ATP citrate lyase was not rate-limiting. However, as the authors pointed out, their results were based on the assumption of simple kinetics of competitive inhibition of the enzyme by hydroxycitrate. In view of the known complex kinetics of ATP citrate lyase with respect to citrate this assumption appears to be unrealistic, and therefore their results should be treated with caution.

In conclusion, all these results suggest that ATP citrate lyase is not involved in the regulation of fatty acid biosynthesis. However, since most of the research has been directed towards the role of acetyl

CoA carboxylase as the rate-limiting enzyme, the possibility of a regulatory function for ATP citrate lyase has not been fully explored. Thus, although the enzyme is known to respond to the long-term changes in the rate of fatty acid synthesis, and therefore plays a major role in this pathway, short-term control of the activity as a means of regulation of the pathway requires further investigation particularly as there appear to be several potential mechanisms for such control (Section 2v)

ii) Role of ATP Citrate Lyase in Acetylcholine Biosynthesis in Brain

Acetylcholine is synthesised from choline and acetyl CoA in brain and nervous tissue. The reaction is catalysed by the enzyme choline acetyltransferase (EC 2.3.1.6) which is localised in the cytoplasm of cholinergic nerve endings (Fonnum, 1967; Hebb and Whitacker, 1958; Tucek, 1967a). Hence, acetylcholine biosynthesis requires a source of cytoplasmic choline and acetyl CoA.

Choline is not synthesised in brain tissue and it is now well established that extracellular choline is transported into the nerve endings by a high affinity transport system (for review see Jope, 1979). However, although it is known that the acetyl CoA precursor of acetylcholine originates from glucose, via pyruvate (Browning and Schulman, 1968; Nakamura et al., 1970; Quastel et al., 1936; Tucek and Cheng, 1970) the metabolic route from pyruvate to cytoplasmic acetyl CoA is still disputed (for review see Tucek, 1978).

Acetyl CoA is produced from pyruvate predominantly in the mitochondria by the action of the mitochondrial enzyme pyruvate dehydrogenase (EC 1.2.4.1). Thus, as for fatty acid biosynthesis, a method of transport of acetyl CoA out of the mitochondria is required.

ATP citrate lyase is present in brain (Srere, 1959), and has been shown to be cytoplasmic (Szutowicz and Lysiak, 1980; Tucek, 1967b). Therefore, transport of acetyl units out of the mitochondria in the form of citrate, with subsequent cleavage of citrate by ATP citrate lyase, may provide the cytoplasmic acetyl CoA for acetylcholine synthesis. Since such a pathway exists in liver and adipose tissue to produce cytoplasmic acetyl CoA for fatty acid biosynthesis (Bressler and Brendel, 1966; Daikuhara et al., 1968; Martin and Denton, 1970), and also appears to be involved in fatty acid synthesis in brain (Dolezal and Tucek, 1981; Patel and Owen, 1980) it is not unreasonable to suppose that it may operate in a similar manner in acetylcholine synthesis. Furthermore, the recent interest in ATP citrate lyase with respect to a regulatory role of the enzyme in fatty acid biosynthesis suggests that it could also be involved in regulation of acetylcholine biosynthesis. This is of particular importance as one of the possible mechanisms involved in regulation of acetylcholine synthesis is thought to be control of the supply of precursors, acetyl CoA and choline.

a) Source of Cytoplasmic Acetyl CoA

The fact that pyruvate was found to be the most effective precursor of acetyl CoA for acetylcholine synthesis indicated that the supply of acetyl CoA is via the mitochondria, the site of formation from pyruvate. This was confirmed by the findings that acetylcholine synthesis was closely linked to oxidative metabolism (Gibson and Blass, 1976; Gibson et al., 1975; Ksiezak and Gibson, 1981). In contrast to these results however, Lefresne et al. (1977) found no such correlation between these two parameters. In view of the fact that pyruvate has been found to be a better precursor than other metabolites such as citrate, acetate and acetylcarnitine, they proposed that cytoplasmic acetyl CoA could be derived from

pyruvate outside the mitochondria, by a cytoplasmic pyruvate dehydrogenase-like enzyme. They later reported indirect evidence in support of such a mechanism (Lefresne et al.,1978). However, no cytoplasmic pyruvate dehydrogenase activity has been detected in brain (Szutowicz and Lysiak, 1980; Szutowicz et al., 1981b). Furthermore, Jope and Jenden (1980) concluded from their results with specific inhibitor of mitochondrial pyruvate transport, α-cyano-4-hydroxycinnamic acid, that utilisation of pyruvate via the mitochondria was necessary for acetylcholine biosynthesis. There is therefore no direct evidence in support of the proposed cytoplasmic pyruvate dehydrogenase. The fact that exogenous pyruvate appears to be a better precursor of acetylcholine synthesis than other metabolites may simply reflect more rapid uptake of pyruvate. The rate of uptake of the various precursors, and the possibility of preferential metabolism in the glial cells rather than the neurones in experiments with brain slices need to be considered. Moreover, it has been suggested that exogenous citrate enters a different compartment to endogenous citrate released from the mitochondria (Sterling and O'Neill,1978; Szutowicz et al., 1981a), and is not utilised for acetylcholine biosynthesis. Hence, utilisation of exogenous substrates does not necessarily reflect the situation in vivo, particularly in a heterogenous tissue such as brain.

Having established that cytoplasmic acetyl CoA is derived from the mitochondria, it was necessary to determine the mechanism by which acetyl CoA is transported into the cytoplasm. Since citrate is important in liver for providing cytoplasmic acetyl CoA for fatty acid biosynthesis, and may also be involved in fatty acid biosynthesis in brain, a similar role in providing acetyl CoA for acetylcholine biosynthesis was considered. However, labelled citrate was found to be a poor precursor of acetylcholine, both in vitro (Dolezal and Tucek, 1981; Lefresne et al., 1977; Nakamura

et al., 1970), and in vivo (Tucek and Cheng, 1970, 1974). Nevertheless, indirect evidence, using mixed-labelled glucose, suggested that citrate is involved in acetylcholine synthesis (Sollenberg and Sorbo, 1970; Sterling and O'Neill,1978).

More recently, use of hydroxycitrate, the specific inhibitor of ATP citrate lyase (Szutowicz et al., 1976; Watson et al., 1969), has provided good evidence for the involvement of this enzyme, and hence citrate, in acetylcholine biosynthesis. Thus, when cytoplasmic cleavage of citrate was blocked by inhibition of ATP citrate lyase in the presence of hydroxycitrate, acetylcholine synthesis from glucose was reduced by 25-30% in rat brain slices (Gibson and Shimada, 1980; Sterling and O'Neill, 1978; Tucek et al., 1981), and in rat synaptosomes (Gibson and Shimada, 1980; Szutowicz et al., 1981a). Although no attempt was made to measure the inhibition of ATP citrate lyase by hydroxycitrate under these conditions, the concentration of the inhibitor used (1.0-7.5mM) was far greater than the K_i for the brain enzyme (0.8μM) (Szutowicz et al.,1976). Furthermore, Tucek et al.(1981) demonstrated that inhibition of acetylcholine synthesis was >80% when citrate was the sole precursor. The fact that this inhibition was reduced to only 30% in the presence of glucose precursor, suggested that citrate is not the only source of cytoplasmic acetyl CoA. However, Sterling and O'Neill(1978) who first reported this effect of hydroxycitrate, suggested that other pathways of acetyl CoA transport only became operational when the citrate route was blocked.

Therefore, despite strong indirect evidence in support of citrate as an acetyl group transporter, direct incorporation of label from citrate into acetylcholine appeared to argue against the involvement of citrate. However, the apparent lack of incorporation of these earlier experiments could be explained by the low uptake of citrate. Although several groups have demonstrated that citrate does enter synaptosomes and is metabolised

(Gibson and Shimada, 1980; Lefresne et al.,1977; Szutowicz et al., 1981a, 1982a), Dolezal and Tucek (1981) found that acetylcholine synthesis from citrate in rat brain slices was doubled in the absence of calcium. They suggested that formation of a citrate-calcium complex prevented entry of citrate into the cells. Nakamura et al.(1970) and Lefresne et al. (1977) both used incubations containing calcium and found poor utilisation of citrate for acetylcholine biosynthesis. Furthermore, high concentrations of citrate (>2.5mM) have been shown to inhibit total acetylcholine synthesis from glucose (Gibson and Shimada, 1980; Tucek et al., 1981).

Studies of the regional and subcellular distribution of ATP citrate lyase in brain provided further evidence in support of a role for citrate as the transporter of acetyl groups for acetylcholine biosynthesis. The activity of the enzyme was found to be higher in regions containing a high proportion of cholinergic neurones (Hayashi and Kato, 1978; Szutowicz and Lysiak, 1980; Szutowicz et al., 1980, 1982a, 1982c). Furthermore, subcellular fractionation indicated that the enzyme is localised in synaptosomes (Szutowicz et al.,1977) and preferentially in cholinergic nerve endings (Szutowicz and Lysiak, 1980; Szutowicz et al., 1982c). Szutowicz et al.(1982c) demonstrated good correlation between choline acetyltransferase and ATP citrate lyase activity of various fractions from different regions of rat brain.

In addition to subcellular fractionation of brain tissue, the localisation of brain ATP citrate lyase in cholinergic neurones has also been investigated by the use of lesions to specifically remove cholinergic terminals. Thus, Szutowicz et al.(1982b) measured a 33% decrease in ATP citrate lyase activity compared to 70-80% decrease of choline acetyltransferase, as a result of such experiments. This was interpreted as evidence for the presence of the enzyme in cholinergic neurones. No change

in activity was observed for either acetyl CoA synthetase or carnitine acetyltransferase, two other enzymes which can produce cytoplasmic acetyl CoA.

In contrast to this work by Szutowicz and co-workers, others concluded from the results of their experiments on the enzyme activity in different brain regions (Harvey et al.,1982), and the effects of lesion of cholinergic terminals on the enzyme activity (Sterri and Fonnum, 1980), that there is no correlation between ATP citrate lyase activity and cholinergic function. However, in both cases the role of ATP citrate lyase in fatty acid biosynthesis in non-cholinergic nerve endings and in glial cells, did not appear to have been considered. Harvey et al.(1982) measured the relative activities of choline acetyltransferase and ATP citrate lyase in brain regions containing high cholinergic activity. They did not however, compare these results with an area of low cholinergic activity, where the ATP citrate lyase activity would presumably represent that involved only in fatty acid biosynthesis. In addition, comparison of their results with those of Szutowicz and Lysiak (1980), showed that although the specific activities of choline acetyltransferase in different brain regions were similar, the specific activity of ATP citrate lyase obtained by Harvey et al.(1982) was 10-fold lower than that found by Szutowicz and co-workers. The authors themselves commented on this difference but could offer no explanation. Sterri and Fonnum (1980) measured the effect of lesion of cholinergic neurones on choline acetyltransferase and acetyl CoA producing enzymes. They concluded that the small decrease in ATP citrate lyase activity (15%) compared to choline acetyltransferase (90%) was evidence against the presence of the former enzyme in cholinergic neurones. However, they did not measure the effect of lesion on a marker cytoplasmic enzyme such as lactate dehydrogenase. The decrease in activity of other

acetyl CoA producing enzymes (pyruvate dehydrogenase and carnitine acetyltransferase), which they took as evidence for localisation in cholinergic neurones, was no greater than that of other mitochondrial enzymes not involved in acetyl CoA metabolism. Their evidence is therefore not very convincing.

Hence, the results from the different experimental approaches described here provide strong evidence in support of a role for ATP citrate lyase in the supply of cytoplasmic acetyl CoA for acetylcholine biosynthesis. In addition, the developmental changes in brain ATP citrate lyase described earlier (Section 2iv c) can be explained in terms of the changes in fatty acid and acetylcholine synthesis in different areas of brain. Nevertheless, experiments with hydroxycitrate to inhibit ATP citrate lyase indicated that this enzyme is responsible for the supply of only one third of the cytoplasmic acetyl CoA (Ricny and Tucek, 1982; Tucek et al., 1981).

Two other methods of acetyl group transport out of the mitochondria which have received attention are acetate and acetylcarnitine. Incorporation of radioactivity into acetylcholine has been demonstrated using labelled acetate (Dolezal and Tucek, 1981; Lefresne et al., 1977; Nakamura et al., 1970; Sollenberg and Sorbo, 1970) and labelled acetylcarnitine (Dolezal and Tucek, 1981; Tucek et al., 1981). However, as with experiments using labelled citrate as a precursor, the incorporation was poor compared to that from glucose or pyruvate.

Unlike ATP citrate lyase, the enzymes involved in the formation of acetyl CoA from acetate and acetylcarnitine, acetyl CoA synthetase and carnitine acetyltransferase respectively, are not found exclusively in the cytoplasm. In fact, acetyl CoA synthetase is located mainly in the mitochondria (Szutowicz and Lysiak, 1980; Tucek, 1967b), and Szutowicz and Lysiak (1980) suggested that most of the brain activity was associated

with the glial cells rather than neurones. However, there does appear to be a minor proportion of activity in the cytoplasm, and it is possible that this may be specifically localised in a compartment associated with acetylcholine synthesis. Carnitine acetyltransferase is also a mitochondrial enzyme (McCaman et al.,1966; Szutowicz and Lysiak, 1980). Although it can catalyse transfer of acetyl CoA across the inner mitochondrial membrane, the mechanism involved is not known. It has been suggested that the enzyme is present either in the inner mitochondrial membrane, such that it is accessible to substrates on both sides, or as a soluble enzyme present on both sides of the membrane (Tucek, 1978). However, there is no evidence to suggest that carnitine acetyltransferase is specifically localised in cholinergic neurones (Szutowicz and Lysiak, 1980).

Therefore, the subcellular localisation of these two enzymes acetyl CoA synthetase and carnitine acetyltransferase, does not appear to be consistent with a major role for either in acetylcholine biosynthesis. In addition, the activities of both enzymes increased during development in the cerebellum and the cerebrum, with no significant differences between these two regions (Szutowicz et al., 1980). Hence, there is no evidence for a specialised role for either enzyme in acetylcholine synthesis since there was no greater increase in activity in the cholinergic cerebrum compared to the cerebellum, despite an increase in acetylcholine biosynthesis in this region.

b) Role of ATP Citrate Lyase in the Regulation of Acetylcholine Biosynthesis

The mechanism involved in the regulation of acetylcholine biosynthesis is at present unknown. However, there are currently four alternative possibilities under consideration: regulation by control of the high affinity choline transport system; product inhibition of

choline acetyltransferase; control of the supply of precursors, acetyl CoA and choline; control by changes in the concentrations of substrates and products, governed by the Law of Mass Action. Jope (1979) published a model of control of acetylcholine synthesis based on the data available at that time.

Clearly, knowledge of the immediate source of cytoplasmic acetyl CoA for acetylcholine synthesis is important in order to establish the possible sites of control. The evidence to date suggests that ATP citrate lyase is important in providing a supply of cytoplasmic acetyl CoA via citrate, but it does not appear to be the only route, as already discussed. The fact that ATP citrate lyase activity is much greater than that of choline acetyltransferase activity in synaptosomes and brain homogenates led Szutowicz and co-workers to conclude that the enzyme does not have a rate-limiting role (Szutowicz and Lysiak, 1980; Szutowicz et al., 1977, 1980, 1982a). However, choline acetyltransferase is specifically located in cholinergic neurones which represent only 10% of synaptosomes. Therefore comparison of total activities may not reflect the relative activities of the two enzymes in cholinergic neurones.

Szutowicz et al. (1981a) suggested that citrate transport was possibly the rate-limiting factor since they calculated that the rate of endogenous citrate cleavage by ATP citrate lyase was greater than the rate of the endogenous citrate flux to acetylcholine via ATP citrate lyase. However, this may also reflect rate-limiting control by choline acetyltransferase.

Ricny and Tucek (1980, 1981) measured the content of acetyl CoA and acetylcholine in rat brain slices under different conditions. They found a linear relationship between these two parameters and concluded that synthesis of acetylcholine is controlled by the Law of Mass Action. Further investigation by these workers (Ricny and Tucek, 1982) indicated that an increased supply of citrate may increase the synthesis of

acetylcholine. This implies that since ATP citrate lyase is required for the utilisation of citrate for acetylcholine synthesis it may be involved in the regulation of the pathway.

In conclusion, although there is good evidence for the involvement of ATP citrate lyase is acetylcholine biosynthesis, there is as yet, no positive evidence to suggest a regulatory role for the enzyme in this pathway. The high activity of the enzyme compared to choline acetyltransferase has suggested that it is not rate-limiting in acetylcholine biosynthesis. However, ATP citrate lyase is also involved in fatty acid biosynthesis in brain, and is present in non-cholinergic synaptosomes and glial cells, in addition to cholinergic synaptosomes. In contrast, choline acetyltransferase is found exclusively in cholinergic synaptosomes where it functions only in acetylcholine biosynthesis. Therefore, comparison of these two enzymes with respect to their roles in acetylcholine biosynthesis must take into account these differences, and clearly, a rate-limiting role for ATP citrate lyase cannot yet be excluded.

4. Aims of this Study

ATP citrate lyase is known to be involved in the supply of acetyl CoA for both fatty acid and acetylcholine biosynthesis. However, the role of the enzyme in the regulation of these pathways, if any, has not been established.

In view of the dual role of ATP citrate lyase in brain, a comparison of the enzyme from rat liver and brain was considered important since any difference in properties may reflect the involvement of the enzyme in the two separate biochemical pathways. Moreover, such an investigation may reveal evidence in support of a regulatory role of the enzyme. Hence, two aspects of the enzyme were considered in this

study: 1. Does the enzyme exist in different forms, possibly isoenzymes, which would correspond to the two functions i.e. supply of acetyl CoA for fatty acid and acetylcholine biosynthesis? 2. Is there any evidence for short-term control of the enzyme in either liver or brain, which may be involved in regulation of either fatty acid or acetylcholine biosynthesis? The latter point is particularly important in view of the recent discovery of reversible phosphorylation of the enzyme, the significance of which is unknown.

A full understanding of the physiological role of ATP citrate lyase is important for several reasons. The enzyme from liver, and to a lesser extent from adipose tissue, undergoes long-term changes in activity in response to diabetes and obesity. Although this is thought to represent adaptation of the enzyme as a result of decreased fatty acid biosynthesis, knowledge of any regulatory properties of the enzyme would be of benefit when considering the treatment of these two conditions. Moreover, the specific inhibitor of ATP citrate lyase, hydroxycitrate, has been considered as a possible anti-obesity drug (Cawthorne and Arch, 1982; Sullivan and Triscari, 1977), and for use in the treatment of hypertriglyceridemia (Sullivan et al., 1977), as a result of its inhibition of ATP citrate lyase and hence fatty acid biosynthesis. Obviously, an understanding of the regulation of fatty acid biosynthesis, and the effects of any other sites of action of hydroxycitrate, for example inhibition of ATP citrate lyase involved in acetylcholine biosynthesis in brain, would be necessary before such treatment could be considered for clinical use. The role of brain ATP citrate lyase in acetylcholine biosynthesis is particularly important since the supply of precursors is thought to be involved in the regulation of this pathway. Clearly, understanding the control of this pathway would be of value in establishing the causes, and possible

treatment of diseases in which acetylcholine biosynthesis is impaired such as Alzehiemer's disease (Marchbanks, 1982).

ATP citrate lyase activity has been determined in human liver and adipose tissue (Hoffmann et al., 1980a; Shrago et al., 1969, 1971; Suzuki and Okuda, 1981). However, as a result of the low activity of the enzyme compared to that of corresponding rat tissues, and the apparent lack of response to nutritional status, it was suggested that the enzyme has no significant role in human tissues. Nevertheless, if ATP citrate lyase is found to be an important regulatory enzyme in rat, its physiological function in human tissues would have to be re-evaluated.

MATERIALS AND METHODS

1. Materials

The reagents for the ATP citrate lyase assay, NADH (disodium salt), malate dehydrogenase (pig heart, mitochondrial, $(NH_4)_2SO_4$ suspension), 2-mercaptoethanol, and the substrates, ATP, coenzyme A (disodium salts), and citrate (tripotassium salt) were obtained from Sigma (St. Louis, USA). Substrates and cofactors for other enzymes, commercial enzyme preparations, L-glutamate (monopotassium salt), glycylglycine, potassium cyanide, aminooxyacetate hemihydrochloride, dithiothreitol, oxaloacetate (free acid), rotenone, phenylmethylsulphonyl fluoride, bovine serum albumin and molecular weight standard proteins were also from Sigma. DEAE-Sephadex A-25, CM-Sephadex A-25, Sepharose 6B, Sepharose 2B, Sephadex G100 (superfine grade), Sephadex G25 (medium grade) and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden); phosphocellulose was from Carl Schleicher and Schüll (Dassel, West Germany). Radiochemicals [1,5-^{14}C] citric acid monohydrate and sodium [^{14}C] bicarbonate were purchased from Amersham International (Amersham UK). All other chemicals were from BDH (Poole, UK).

2. Animals

Adult female Wistar rats (200-250g) were obtained from the departmental animal house, maintained at 21°C on a 12h light/dark cycle. Newborn rats were taken from two litters at 1-2 days old irrespective of their sex. All animals were killed about 2h after the start of the light cycle. Three different dietary conditions were used: i) normal fed, ad libitum, a standard laboratory PRD diet (see Appendix for diet composition), ii) starved for 48h, but allowed water only, iii) starved for 48h with water only, and refed ad libitum, a high carbohydrate diet

consisting of PRD diet, plus 5% sucrose (w/v) added to the drinking water, for 72h.

3. ATP Citrate Lyase Assays

i) Spectrophotometric Assay

The enzyme was assayed by the coupled spectrophotometric assay of Srere (1959) following a method similar to that of Szutowicz et al. (1974a). The assay mixture contained 50mM Tris/HCl buffer pH 7.8, 10mM $MgCl_2$, 100mM KCl, 5mM ATP, 0.2mM CoA, 20mM citrate, 0.15mM NADH, 10mM mercaptoethanol, malate dehydrogenase, 2iu/ml, and enzyme sample in a final volume of 1ml. A stock solution of buffer containing $MgCl_2$ and KCl was stored at 4°C. The pH was checked before use and adjusted if necessary with concentrated HCl. Stock solutions of the other reagents were made in the assay buffer. ATP, NADH, CoA and malate dehydrogenase were all stored at -20°C and used within 5 days, citrate was kept at 4°C, and mercaptoethanol was freshly prepared each day.

Assays were preincubated in 1cm path length plastic cuvettes for 7 min at 37°C to enable determination of endogenous NADH oxidation, and then the reaction was started by the addition of CoA. The oxidation of NADH was followed continuously at 340nm in a Beckmann spectrophotometer at 37°C. One unit of enzyme activity is defined as that amount of enzyme required to transform one µmole NADH per min at 37°C (ε_{NADH} = 6.22 x 10^3 $cm^{-1}M^{-1}$).

ii) Radiochemical Assay

This assay was performed by a modification of the method of Suzuki et al. (1967), using Warburg flasks (3.5cm base diameter) equipped with a side arm and a centre well. The assay components were the same as for the spectrophotometric assay except that NADH and malate dehydrogenase

were omitted, and citrate was replaced by 20mM [1,5-^{14}C] citrate (0.01 µCi/µmole). A total volume of 1ml was used.

The flasks containing the assay mixture were preincubated for 7 min in a shaking water bath at 37°C, and the reaction was started by the addition of ATP or CoA. After a suitable incubation at 37°C, usually 10 min, the reaction was stopped by addition of 0.2ml 2M HCl from the side arm of the flask. Small glass tubes (0.5 x 1.5cm) containing 0.15ml ethanolamine/ethylene glycol monomethyl ether (1:2 v/v) to absorb [^{14}C] carbon dioxide (Jeffay and Alvarez, 1961), were placed in the centre wells and the flasks were sealed with subaseal stoppers. Oxaloacetate produced by the ATP citrate lyase reaction was decarboxylated by injection of 0.5ml 0.75M phthalate buffer pH 4.5 (15.3g potassium hydrogen phthalate + 1.8g sodium hydroxide in 100ml water), followed by 0.5ml $Al_2(SO_4)_3$ solution (33.3% w/v) as described by Krebs and Eggleston (1945).

After a further incubation period of 60 min at 25°C in a shaking water bath, the glass tubes and contents were removed from the flasks and placed in scintillation vials containing 10ml toluene PPO (5g/l) / ethylene glycol monomethyl ether (2:1 v/v). Radioactivity was measured in a Packard scintillation counter. One unit of enzyme activity is defined as that amount of enzyme required to produce one µmole $^{14}CO_2$ per min at 37°C.

The efficiency of carbon dioxide absorption by ethanolamine was determined by acidification of a standard 5mM $NaH^{14}CO_3$ solution (0.004 µCi/µmole). Varying amounts of $NaH^{14}CO_3$ in 50mM Tris/HCl buffer pH 9.0 (1ml final volume) were acidified by addition of 0.2ml 2M HCl from the side arm. After incubation for 25 min at 37°C, radioactive carbon dioxide trapped by the ethanolamine/ethylene glycol monomethyl ether in the centre well was measured.

The efficiency of oxaloacetate decarboxylation was measured using a standard manometric technique (Umbreit et al., 1972). Oxaloacetate in a final volume of 1.2ml containing 50mM Tris/HCl buffer pH 7.8, and 0.2ml 2M HCl, was decarboxylated as described above. The reaction was performed in double-armed Warburg flasks, containing phthalate buffer in one arm and the $Al_2(SO_4)_3$ solution in the other. After 10 min equilibration at 25°C, the contents of the side arms were tipped into the main compartment and readings were taken at intervals until carbon dioxide evolution was complete.

4. Determination of Inhibition of ATP Citrate Lyase Activity by L-Glutamate

Assay mixtures containing all the components of the spectrophotometric assay except CoA, were incubated for 30 min at 37°C, in the presence or absence of 10mM L-glutamate. The reaction was started by the addition of CoA, and ATP citrate lyase activity was determined by measurement of NADH oxidation at 340nm.

In addition, a modified assay used by Szutowicz et al. (1974a) was also employed, in which the concentrations of ATP, citrate, and $MgCl_2$ were changed from 5mM, 20mM and 10mM to 1mM, 2mM, and 13mM respectively.

5. Preparation of Crude Tissue Extracts

Initially, crude supernatants of rat liver and brain were obtained by homogenisation of the tissue in 4 vol. and 2 vol. (w/v) respectively, of 5mM Tris/HCl buffer pH 7.4, containing 0.2M KCl and 1mM dithiothreitol. Homogenisation was performed using a power driven Potter-Elvehjem glass-Teflon homogeniser, and the resulting homogenates were centrifuged at 30,000g for 30 min at 4°C (Szutowicz et al., 1974a).

For partial purification of the enzyme by ion-exchange chromatography, supernatants were prepared by a procedure similar to that used by Hoffmann et al. (1979b). Tissues (liver 1:4 (w/v), brain 1:2 (w/v)) were homogenised in 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol, and 0.25M sucrose, and homogenates were centrifuged at 45,000g for 30 min at 4°C. All subsequent crude tissue extracts were obtained by this method. In addition, for some liver supernatant preparations, a prespin at 2,000g for 10 min at 4°C, was performed prior to centrifugation at 45,000g.

6. Ion-Exchange Chromatography

i) DEAE-Sephadex

ATP citrate lyase from liver and brain was partially purified on DEAE-Sephadex A-25 following the method of Hoffmann et al. (1979b). Crude supernatants (prepared as above) were dialysed against 30-50 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol, and 0.25M sucrose, for about 2h at 4°C, prior to application to the column.

Three different column sizes were routinely used: 2.26 x 22cm (bed volume 88ml) and 1.6 x 11cm (bed volume 22ml), using glass columns (Wright Ltd.), and 0.9 x 5cm (bed volume 3ml) columns made in 2ml plastic syringes. All columns were packed and equilibrated with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, and 1mM dithiothreitol, at a flow rate of 30ml/h.

After sample application, the 2.26 x 22cm columns were washed with 150ml (~1.5 bed volumes) of the above buffer, followed by a linear KCl gradient (0-0.4M) in 400ml of elution buffer. A flow rate of 15ml/h was routinely used, although this was sometimes reduced when running gradients to enable the run to be continued overnight. 8ml fractions

were collected into tubes containing 1ml (0.125 vol.) of 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. For the smaller columns these volumes were reduced in proportion to the bed volume, except that for the 3ml columns, 1ml fractions were collected into 0.125ml 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. Step gradients were routinely used for this size column.

ii) CM-Sephadex

3ml bed volume columns (0.9 x 5cm) of CM-Sephadex A-25 were packed and equilibrated with 20mM sodium phosphate buffer pH 7.0 or pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. Crude liver supernatants prepared in 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, were dialysed against 100 vol. of the above phosphate buffer containing 0.25M sucrose, at either pH 7.0 or pH 8.0, for 12h at 4°C.

After application of the sample (1ml), columns were washed with 10ml phosphate buffer (without sucrose), followed by two 10ml step gradients of 0.1M and 1M NaCl. 1ml fractions were collected.

iii) Phosphocellulose

Ion-exchange chromatography of rat liver and brain supernatants on phosphocellulose was performed in a similar way to DEAE-Sephadex chromatography. Before preparing the columns, the phosphocellulose was precycled with NaOH and HCl. After allowing the gel to swell in distilled water overnight at room temperature, the water was replaced with excess 0.5M NaOH, and stirred gently for 30 min. The gel was then washed with distilled water on a Buchner funnel until neutral before being gently stirred for a further 30 min in the presence of excess 0.1M HCl. Following repeated washing with distilled water to remove the acid, the gel was equilibrated first with 0.4M Tris/HCl buffer pH 7.0 containing 20mM EDTA,

and finally with column elution buffer, 20mM Tris/HCl buffer pH 7.0, containing 1mM EDTA and 1mM dithiothreitol. 1.6 x 6cm columns (bed volume 12ml) were packed and equilibrated with this buffer at a flow rate of 30ml/h.

Crude supernatants of liver and brain were prepared as described for DEAE-Sephadex ion-exchange chromatography and were then dialysed at 4°C against 50 vol. of the homogenisation buffer at pH 7.0 instead of pH 8.0, for 2h. After sample application, the columns were washed with 25ml 20mM Tris/HCl buffer pH 7.0, containing 1mM EDTA and 1mM dithiothreitol at a flow rate of 15ml/h. A linear gradient of NaCl (0-1M) in 100ml of the same buffer was then applied, followed by 25ml 2M NaCl in the elution buffer. The linear gradients were run at a flow rate of 15ml/h or 6ml/h for experiments with liver or brain supernatants, respectively. 1ml fractions were collected into tubes containing 0.125ml 1M Tris/HCl buffer pH 7.0, containing 10mM dithiothreitol.

7. Gel Filtration Chromatography

i) Sepharose 6B and Sepharose 2B

Gel filtration of crude tissue supernatants was performed using Sepharose 6B and Sepharose 2B columns (2.26 x 27.5cm, bed volume 110ml). 5ml samples of liver supernatants were applied, without dialysis, to columns equilibrated with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. For gel filtration of brain supernatants, the sample volume was increased (10-13ml) because of the lower activity of brain ATP citrate lyase. ATP citrate lyase activity was eluted with the same buffer at a flow rate of 8ml/h. 2.5ml fractions were collected into tubes containing 0.3ml 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The void volumes of both columns were determined from the elution volume of Blue Dextran 2000.

The Sepharose 6B column was calibrated according to the method of Andrews (1965), using the molecular weight standard proteins, catalase, 210,000 (bovine liver), pyruvate kinase, 237,000 (rabbit muscle), ferritin, 440,000 (horse spleen), and thyroglobulin, 669,000 (porcine, Type II), in 5ml of elution buffer. Elution of these marker proteins was determined by absorbance at 280nm except for pyruvate kinase which was measured enzymatically (see Section 8v). In addition, elution of lactate dehydrogenase activity present in liver supernatants was also used for column calibration.

ii) Sephadex G25 and Sephadex G100

Sephadex G25 (1.6 x 9cm, bed volume 18ml) and Sephadex G100 (1.6 x 6.5cm, bed volume 13ml) columns were packed and equilibrated with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, at a flow rate of 35-40ml/h. Crude supernatants of liver or brain (1-4ml) were eluted with 1.5 bed volumes of the above buffer. 1ml fractions were collected.

All chromatographic procedures were performed at 4°C. Livers were from normal fed, starved, or starved and refed rats, as described in Results; brains from normal fed rats were routinely used. Linear ionic gradients used in ion-exchange chromatography, were measured using a portable conductivity meter. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

8. Marker Enzyme Assays

All enzyme assays were performed at 37°C except pyruvate kinase activity which was measured at 30°C. A final volume of 1ml was used for all assays and the reactions were followed continuously in a Beckmann

spectrophotometer. One unit of enzyme activity is defined as that amount of enzyme required to produce one μmole of product, or transform one μmole of substrate per min. Molar extinction coefficients used were ε_{NADH} 6.22 x 10^3, $\varepsilon_{cyt\ c\ red.}$ 18.5 x 10^3, and ε_{DCPIP} 21 x 10^3 $cm^{-1}M^{-1}$.

i) Citrate Synthase EC 4.1.3.7.

The assay mixture contained 50mM Tris/HCl buffer pH 8.0, 6.7mM L-malate, 0.2mM NAD, 0.13mM acetyl CoA, malate dehydrogenase, 125iu/ml, and enzyme sample. The reaction was started by addition of acetyl CoA and the reduction of NAD was followed at 340nm (Ochoa, 1955).

ii) Fatty Acid Synthetase System

The assay mixture contained 100mM potassium phosphate buffer pH 6.5, 2.5mM EDTA, 10mM cysteine, 0.06mM acetyl CoA, 0.07mM malonyl CoA, 0.15mM NADPH, BSA, 0.3mg/ml, and enzyme sample. The reaction was started by addition of malonyl CoA and oxidation of NADPH was followed at 340nm (Lynen, 1969).

iii) Lactate Dehydrogenase EC 1.1.1.27

The assay mixture contained 0.1M potassium phosphate buffer pH 7.0, 2mM pyruvate, 0.15mM NADH, and enzyme sample. The reaction was started by addition of pyruvate and oxidation of NADH was followed at 340nm (Stolzenbach, 1966).

iv) NADPH Cytochrome c Reductase EC 1.6.2.4

The assay mixture contained 33mM potassium phosphate buffer pH 7.3, cytochrome c (Type III), 1mg/ml, 0.1mM NADPH, 1mM KCN, and enzyme sample. The reaction was started by addition of NADPH and reduction of cytochrome c was followed at 550nm (modified from Masters et al., 1967).

v) Pyruvate Kinase EC 2.7.1.40

The assay mixture contained 40mM triethanolamine buffer pH 7.4, 8mM Mg_2SO_4, 70mM KCl, 0.15mM NADH, 1mM ADP, 0.67mM phosphoenolpyruvate, lactate dehydrogenase in 1% BSA, 2iu/ml, and enzyme sample. The reaction was started by addition of phosphoenolpyruvate and oxidation of NADH was followed at 340nm (Bücher and Pfleiderer, 1955).

vi) Rotenone-Insensitive NADH Cytochrome c Reductase EC 1.6.99.3

The assay mixture contained 12mM glycylglycine buffer pH 7.5, cytochrome c (Type III), 1mg/ml, rotenone, 2 µg/ml, NADH, 1mg/ml, 1mM KCN and enzyme sample. Rotenone was added to the assay as a solution in methoxyethanol (0.2mg/ml). The reaction was started by addition of enzyme sample and reduction of cytochrome c was followed at 550nm (Smoly et al., 1971).

vii) Succinate Dehydrogenase EC 1.3.99.1

The assay mixture contained 50mM potassium phosphate buffer pH 7.6, 1mM KCN, 0.04mM DCPIP, 20mM succinate and enzyme sample. The reaction was started by addition of succinate and the reduction of DCPIP was followed at 600nm (Earl and Korner, 1965).

9. Subcellular Fractionation

Crude mitochondrial fractions were prepared by a simple two-step procedure. Rat liver was homogenised in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, using a power driven Potter-Elvehjem glass-Teflon homogeniser with 0.25mm clearance. The homogenate was centrifuged at 600g for 10 min and the supernatant obtained was then centrifuged at 15,000g for 15 min (Johnson and Lardy, 1967). The pellet obtained from this centrifugation was resuspended in homogenising buffer, and centrifuged at 15,000g for 15 min.

This was repeated, and the final pellet, the crude mitochondrial fraction after two washes, was resuspended in a suitable volume of buffer. All steps were performed at 4°C.

10. Statistical Analysis

Standard error of the mean (SEM) was calculated from the sample standard deviation i.e. $\sigma_{n-1}/\sqrt{n}$ where n = number of observations. Significance of the results was tested at the 5% level by Student's t test. However, because the data was obtained from independent experiments, and the assumptions of the parametric t test may not be valid, results were also analysed by non-parametric statistics, at the same significance level. The Mann-Whitney U test was used for independent samples and the Wilcoxin matched-pairs signed-ranks test for paired data (Siegel, 1956).

RESULTS

1. ATP Citrate Lyase Activity of Rat Liver and Brain

i) Spectrophotometric Assay

ATP citrate lyase activity was measured by a coupled spectrophotometric assay as described in Methods. Oxaloacetate produced by the enzyme reaction is converted to malate by exogenous malate dehydrogenase, and the oxidation of NADH is followed spectrophotometrically at 340nm. CoA was routinely used to start the reaction after an initial preincubation period at 37°C, but one of the other substrates, ATP or citrate, could also be used without affecting the result. ATP citrate lyase activity was determined by subtracting the rate of endogenous NADH oxidation, measured during the preincubation period in the absence of one of the substrates, from that obtained with the complete assay mixture. All three substrates ATP, CoA and citrate, were required for enzyme activity, and using this assay the minimum change in optical density which could be measured corresponded to oxidation of 3×10^{-4} µmoles NADH per min. Enzyme activity was directly proportional to the amount of crude tissue supernatant (0-50 µl liver; 0-100 µl brain), equivalent to approximately 0-1 mg total protein (Fig. 3).

The activity of ATP citrate lyase in crude supernatants from liver and brain is shown in Table 1. The specific activity of the liver enzyme from normal fed rats, 0.016 units/mg protein, corresponds to literature values, for similar crude extracts, ranging from 0.004 - 0.013 units/mg protein (Pearce, 1980; Vernon and Walker, 1968b; Shargo *et al.*, 1971; Ramakrishna and Benjamin, 1979; Kornacker and Lowenstein, 1965a; Hoffmann *et al.*, 1979b). When rats were starved for 48h, a 50% decrease in the liver enzyme activity was obtained,

FIG. 3

ATP Citrate Lyase Activity of Liver and Brain Supernatants: Activity v Amount of Sample

Crude supernatants of a) liver and b) brain from normal fed rats were prepared by homogenisation of the tissue in 4 vol. and 2 vol. respectively of 5mM Tris/HCl buffer pH 7.4, containing 0.2M KCl and 1mM dithiothreitol, followed by centrifugation of homogenates at 30,000g for 30 min. Brain supernatant was dialysed against ~500 vol. homogenising buffer at 4°C overnight. ATP citrate lyase activity was measured at 37°C by the coupled spectrophotometric assay using different amounts of the supernatant sample. The reaction was started by addition of CoA after a 7 min preincubation at 37°C. Results are from two separate experiments, a) and b), and were obtained from duplicate assays.

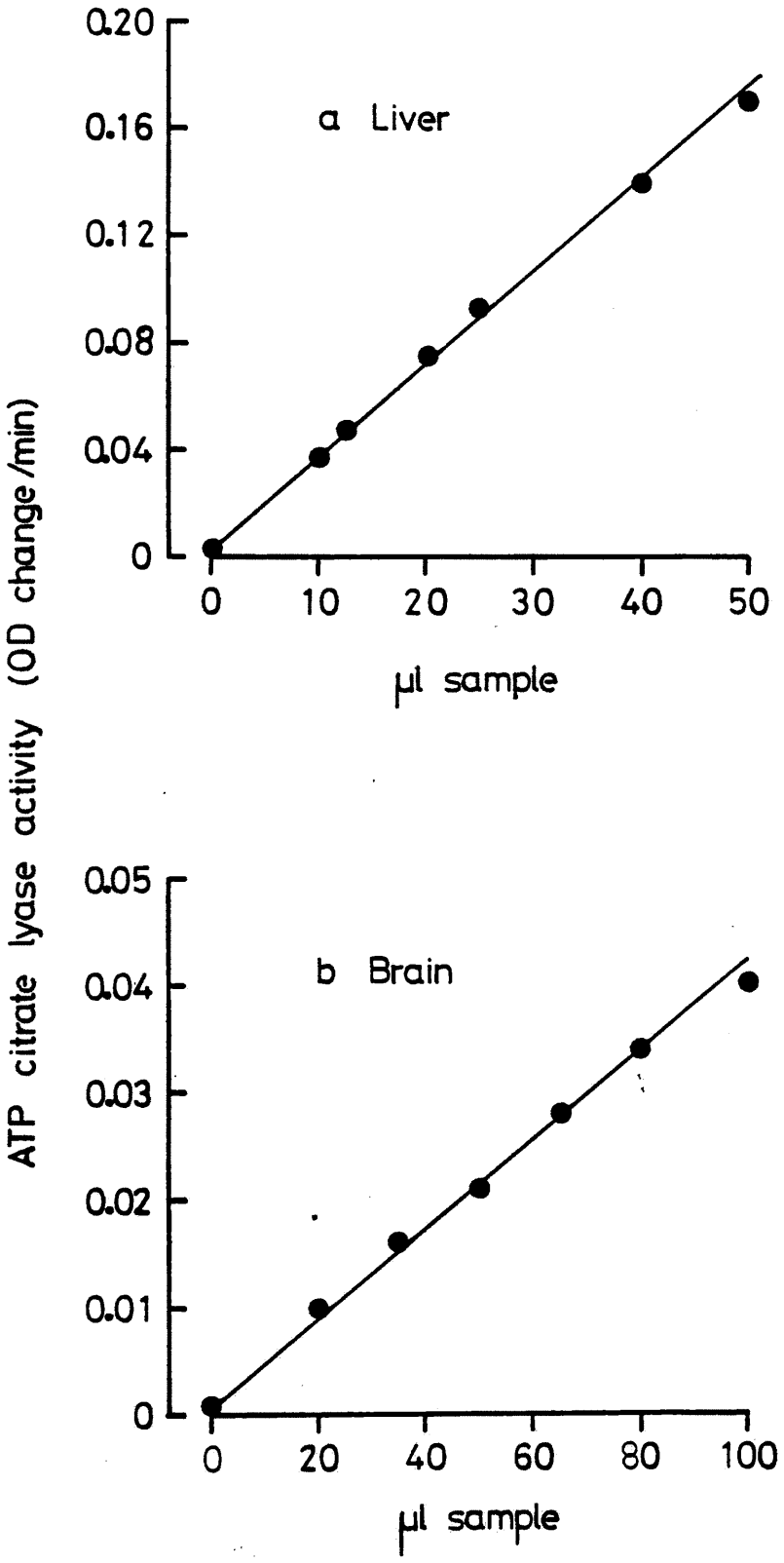

ATP citrate lyase activity (OD change/min)
0.20
0.16
0.12
0.08
0.04
0
a Liver
0
10
20
30
40
50
µl sample
0.05
0.04
0.03
0.02
0.01
0
b Brain
0
20
40
60
80
100
µl sample

TABLE 1

ATP Citrate Lyase Activity of Crude Supernatants from Rat Liver and Brain

		ATP Citrate Lyase Activity			Total Protein		Specific Activity	
		Units/ml		Units/g wet wt.	mg/ml		Units/mg	
Liver								
Normal fed	a	0.36 ± 0.03	(14)	1.8	21.7 ± 0.6	(8)	0.016 ± 0.002	(8)
	b	0.49 ± 0.08	(9)	2.5	-		-	
Starved	a	0.17	(2)	0.85	24.4	(2)	0.0074	(2)
Starved/refed	a	1.4 ± 0.1	(22)	7.0	22.4 ± 2.8	(9)	0.084 ± 0.01	(9)
Brain								
Adult normal fed	a	0.056 ± 0.004	(8)	0.17	10.3 ± 0.5	(4)	0.0058 ± 0.0007	(4)
	b	0.058 ± 0.007	(7)	0.17				
Adult starved/refed	b	0.036	(2)	0.11	8.0	(2)	0.0045	(2)
Newborn normal fed	a	0.041	(2)	0.21	6.6	(1)	0.0056	(1)

TABLE 1 - Continued

ATP citrate lyase activity was measured by the coupled spectrophotometric assay described in Methods. Supernatants were prepared by one of two methods: a) tissue homogenised in 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose. Liver and newborn brain were homogenised in 4 vol. buffer; adult brain was homogenised in 2 vol. buffer. Homogenates were centrifuged at 45,000g for 30 min at 4°C; b) tissue homogenised in 5mM Tris/HCl buffer pH 7.4, containing 0.2M KCl and 1mM dithiothreitol. Liver was homogenised in 4 vol. buffer, brain in 2 vol. Homogenates were centrifuged at 30,000g for 30 min at 4°C. Rats were starved for 48h with water only; starved/refed rats were starved for 48h with water only and then refed normal diet + 5% sugar solution ad libitum for 72h. Livers were from adult rats, brains were from adult or newborn rats as indicated. For some liver supernatants two livers were used, and as many as 14 adult and 28 newborn brains were used for each brain supernatant preparation. Results are means $\pm$ SEM with number of determinations in brackets.

whereas refeeding a high carbohydrate diet for 72h after an initial 48h starvation produced an average 5-fold increase in activity. Such diet-induced changes in ATP citrate lyase activity originally reported by Kornacker and Lowenstein (1965a), were subsequently shown to be the result of changes in the amount of enzyme (Suzuki et al., 1967; Takeda et al., 1967). Literature values for the specific activity of liver ATP citrate lyase in crude extracts from 48h starved rats average 0.003 units/mg protein (Kornacker and Lowenstein, 1965a; Hoffmann et al., 1980c), representing a 2-9-fold decrease. Induction of the enzyme by starving and refeeding produces increases in activity of 2-13-fold with specific activities of the enzyme in crude extracts ranging from 0.012 - 0.16 units/mg protein (Plowman and Cleland, 1967; Hoffmann et al., 1979b; Inoue et al., 1966; Kornacker and Lowenstein, 1965a; Linn and Srere, 1979; Redshaw and Loten, 1981).

ATP citrate lyase activity of crude brain supernatants from adult, normal fed rats, was $<10\%$ that of liver extracts from similar animals, when compared on a tissue weight basis. The specific activity, 0.0058 units/mg protein, was 36% of the value for the liver enzyme (Table 1). Literature values for brain ATP citrate lyase specific activity in crude extracts vary from 0.0016 - 0.0070 units/mg protein (D'Adamo and D'Adamo, 1968; Hoffmann et al., 1979b; Szutowicz et al., 1975; Simpson, 1981; Szutowicz and Lysiak, 1980). In contrast to the liver enzyme, no change in the activity of brain ATP citrate lyase was obtained when rats were starved for 48h and refed a high carbohydrate diet for 72h (Table 1), an observation previously reported by Szutowicz et al. (1974b). Furthermore, the enzyme from newborn rat brain had the same specific activity as that from adult brain (Table 1). This result is in agreement with that of Buckley and Williamson (1973), but

a decrease in brain ATP citrate lyase activity from newborn to adult has been observed by others (D'Adamo and D'Adamo, 1968; Szutowicz et al., 1974b). However, Szutowicz et al. (1980) recently presented evidence which indicated that apparent developmental changes in enzyme activity differ in different brain areas. Thus, in the essentially non-cholinergic cerebellum, ATP citrate lyase activity was found to decrease during development, in parallel with reduced lipogenesis. In the cholinergic cerebrum however, no change in the enzyme activity was observed and this was explained to be due to an increase in activity to accommodate the increased acetylcholine synthesis, together with a decrease in activity of the enzyme involved in lipogenesis. Therefore, developmental changes of the enzyme cannot be determined using whole brain extracts since such regional changes would not be taken into consideration.

Crude extracts of liver and brain were prepared by homogenisation of the tissue in one of two isolation buffers, a (Hoffmann et al., 1979b) and b (Szutowicz et al., 1974a). Tris/HCl was used as the buffer in both media but at different concentrations and pH, a, 20mM, pH 8.0; b, 5mM pH 7.4 . In addition, one contained non-ionic sucrose (0.25M) as the osmotic support (a), whereas the other (b), was made isotonic by addition of 0.2M KCl. However, as shown in Table 1, neither liver nor brain ATP citrate lyase activity was significantly affected by the extraction method used. The method of Hoffmann et al. (1979 b) was routinely employed.

The endogenous NADH oxidation present in crude supernatants of liver and brain was measured, and expressed as a percentage of the total NADH oxidation obtained for the complete assay mixture (Table 2). For liver supernatants from normal fed rats, this background activity represented 17% of the total activity whereas after induction of ATP

TABLE 2

% Total NADH Oxidation due to Endogenous Activity

Crude Supernatant	% Total Rate due to Endogenous NADH Oxidation
Liver	
Normal fed	17 ± 2 (11)
Starved/refed	7.5 ± 0.5 (22)
Brain	
Normal fed	33 ± 4 (6)

ATP citrate lyase activity was measured at 37°C by the coupled spectrophotometric assay, as described in Methods. Cuvettes containing all the assay components except CoA were incubated at 37°C for 7 min prior to starting the enzyme reaction. During this time NADH oxidation was measured, and the amount of this endogenous activity is expressed as a percentage of the total NADH oxidation obtained after addition of CoA. Supernatants were prepared by homogenisation of tissue (liver 1:4 vol. w/v; brain 1:2 vol. w/v) in 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol, and 0.25M sucrose, and centrifugation of homogenates at 45,000g for 30 min at 4°C. Results are means ± SEM with number of determinations in brackets.

citrate lyase by starving and refeeding rats, it was reduced to 8%. Crude supernatants of brain however, contained a high background activity comprising 33% of the total NADH oxidation. In addition, this endogenous activity was non-linear, thereby making determination of brain ATP citrate lyase more difficult.

Attempts to reduce the background activity by dialysis, particularly for brain supernatants, were not effective (Table 3). In fact, dialysis of supernatants against 30-50 vol. homogenising buffer for 2h at 4°C appeared to increase the endogenous activity of liver supernatants from normal fed rats. The background activity of liver supernatants from starved and refed rats, and of brain supernatants, was not affected by dialysis. No change in ATP citrate lyase activity was observed after dialysis of either liver or brain supernatants (Table 3).

Crude supernatants were routinely stored at 4°C and maintained maximum activity for 3-4 days. However, activity could be restored after this period by incubation with 5mM dithiothreitol for several hours at 0-4°C. This phenomenon was described by Cottam and Srere (1969) and occurs due to reduction of essential sulphydryl groups on the enzyme. The degree of activation obtained varied from sample to sample such that the enzyme activity was increased to 30-70% of the original activity. Typical activation curves are shown in Fig. 4. In addition, this activation was also observed in the cuvette when using aged samples, and is probably due to the presence of mercaptoethanol and CoA, two sulphydryl reagents present in the assay.

ii) Radiochemical Assay

This assay was developed as an alternative to the coupled spectrophotometric assay. [4-^{14}C]oxaloacetate produced from the cleavage

TABLE 3

Effect of Dialysis on ATP Citrate Lyase Activity and Endogenous NADH Oxidation of Crude Liver and Brain Supernatants

	Crude Supernatant		Dialysed Supernatant	
	ATP Citrate Lyase Activity	Endogenous NADH Oxidation	ATP Citrate Lyase Activity	Endogenous NADH Oxidation
	Units/ml	% total	Units/ml	% total
Liver				
Normal fed	0.37 ± 0.06	16 ± 2	0.36 ± 0.06	23 ± 3*
Starved/refed	1.2 ± 0.1	6.6 ± 0.2	1.1 ± 0.1	7.8 ± 0.6
Brain				
Normal fed	0.058 ± 0.004	25 ± 6	0.053 ± 0.006	30 ± 7

* Significantly different from crude supernatant $p = 0.01$ (Wilcoxin test); $p = 0.002$ (Student's paired t test).

Crude supernatants of liver and brain were prepared by homogenisation of the tissue (liver 1:4 vol. w/v, brain 1:2 vol. w/v), in 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, followed by centrifugation of homogenates at 45,000g for 30 min at 4°C. Dialysed supernatants were obtained by dialysis of the crude extracts against 30-50 vol. homogenising buffer for 2h at 4°C. ATP citrate lyase activity was measured by the coupled spectrophotometric assay described in Methods. Endogenous NADH oxidation in the absence of CoA, is expressed as a percentage of the total rate obtained for the complete assay mixture. Results are means ± SEM for 9 experiments with liver supernatants from normal fed rats, and starved/refed rats, and 6 experiments with brain supernatants.

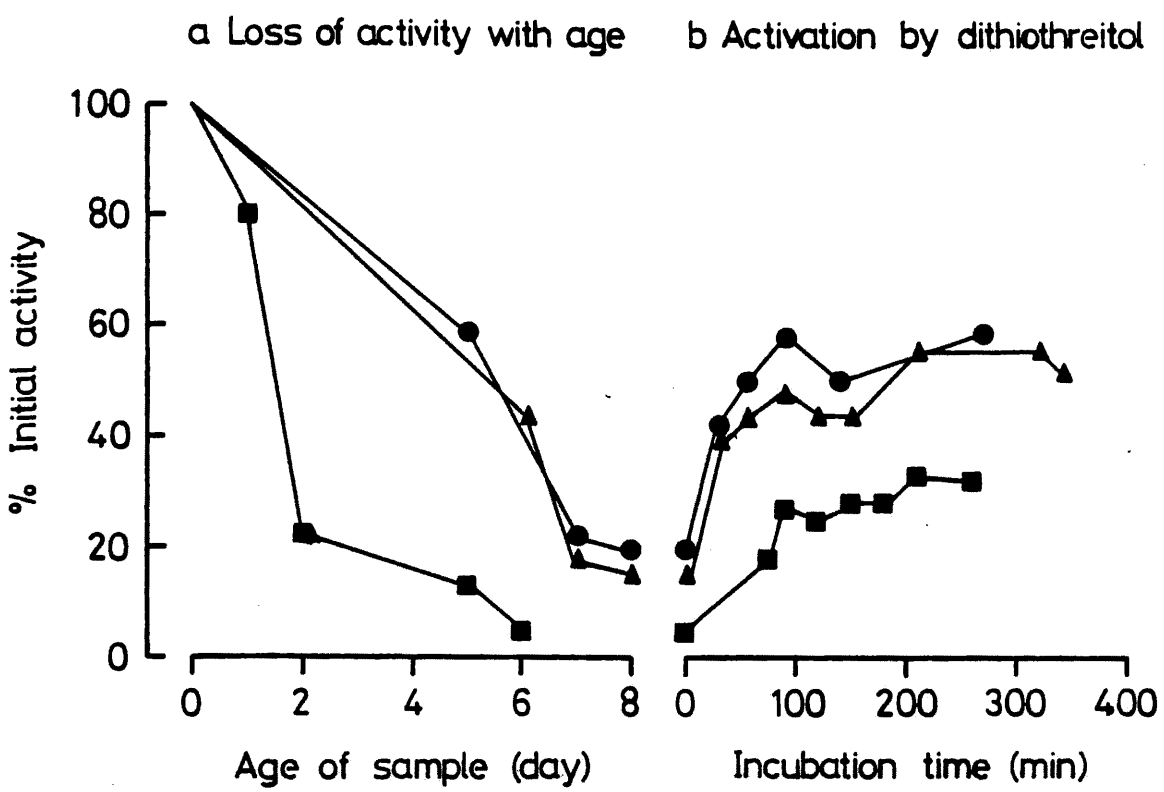
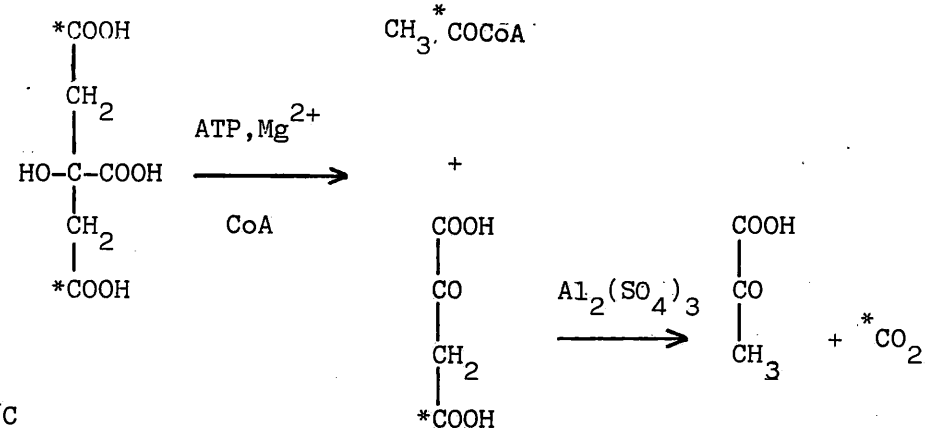


FIG. 4

Activation of Liver ATP Citrate Lyase by 5mM Dithiothreitol

The graph shows a) loss of activity of liver ATP citrate lyase of samples stored at 4°C, and b) reactivation of the same samples by incubation with dithiothreitol at 4°C. Results are from three experiments and are expressed as the % initial activity (part a, day 0). Each sample was incubated with 5mM dithiothreitol in buffer on the last day shown in part a, and aliquots (50 µl) were removed for enzyme assay at the times indicated. All samples were from DEAE-Sephadex ion-exchange chromatography of liver supernatants from normal fed rats (see Methods); ● column fraction from first-eluted enzyme peak; ▲ column fraction from second-eluted enzyme peak; ■ 3 fractions containing maximum activity of second-eluted enzyme peak, pooled and concentrated to 0.5 original volume with polyethylene glycol. ATP citrate lyase activity was measured by the spectrophotometric assay at 37°C; results are averages of duplicate assays except ■ part b.

of [1,5-^{14}C] citrate by ATP citrate lyase is decarboxylated to release [^{14}C] carbon dioxide, which is trapped and the radioactivity determined. The enzyme shows stereospecificity for the substrate citrate (Srere and Bhaduri, 1964), such that only carbon 5 of citrate is incorporated into oxaloacetate.



*C = ^{14}C

The assay procedure was modified from that of Suzuki et al. (1967) using the components of the spectrophotometric assay but without the coupling system, malate dehydrogenase and NADH. The reaction mixture was incubated in Warburg flasks, and the oxaloacetate produced was decarboxylated using the method of Krebs and Eggleston (1945) employed by Suzuki et al. (1967). The amount of [^{14}C] carbon dioxide released was determined using the ethanolamine/ethylene glycol monomethyl ether/toluene PPO trapping and counting system described by Jeffay and Alvarez (1961). This method has several advantages compared to the methanolic hyamine employed by Suzuki et al. (1967), such as less severe quenching and higher carbon dioxide trapping capacity (1ml ethanolamine can react with 8 mmoles CO_2). In addition, ethanolamine is less expensive and more readily available than hyamine. A further advantage of absorbing the carbon dioxide directly is that the homogenous scintillation mixture obtained enables greater counting efficiency than methods involving gas absorption on paper.

Ethanolamine, which traps carbon dioxide by formation of a

carbonate, is a quenching agent and therefore it is necessary to use the minimum volume required to trap all the carbon dioxide produced. Moreover, as the carbonate formed is insoluble in the toluene PPO scintillation mixture, ethylene glycol monomethyl ether is required to act as a solubilising agent. Thus, [^{14}C] carbon dioxide was trapped in the 1:2 (v/v) ethanolamine/ethylene glycol monomethyl ether solution suggested by Jeffay and Alvarez (1961). A total volume of 0.15ml was used which contained excess ethanolamine to trap [^{14}C] carbon dioxide but had no quenching effect. This trapping solution was placed in small glass tubes held in the centre wells of the Warburg flasks. At the end of the assay, these tubes were transferred to scintillation vials containing the toluene PPO/ethylene glycol monomethyl ether (2:1 v/v) scintillant. Thus, all the [^{14}C] carbon dioxide trapped by the ethanolamine was transferred quickly and without loss. The presence of the glass tubes in the scintillation vials had no effect on the measurement of radioactivity.

The efficiency of ^{14}C determination was 78-82%, and correction was made using a quench curve, which was obtained with ^{14}C-hexadecane standard (0.39 μCi/ml), and chloroform as a quenching agent. The scintillation mixture was the same as that used for the assay, toluene PPO/ethylene glycol momomethyl ether (2:1 v/v). Recovery of [^{14}C] carbon dioxide released by acidification of standard Na$H^{14}CO_3$ was 100% when determined by the method described above, and the radioactivity measured was directly propertional to the amount of bicarbonate (0-0.02 μCi) (Fig.5).

Release of [^{14}C] carbon dioxide from the assay mixture was obtained by decarboxylation of the oxaloacetate produced during the enzyme reaction. This involved injection of phthalate buffer, followed by $Al_2(SO_4)_3$, into sealed Warburg flasks containing the enzyme assay

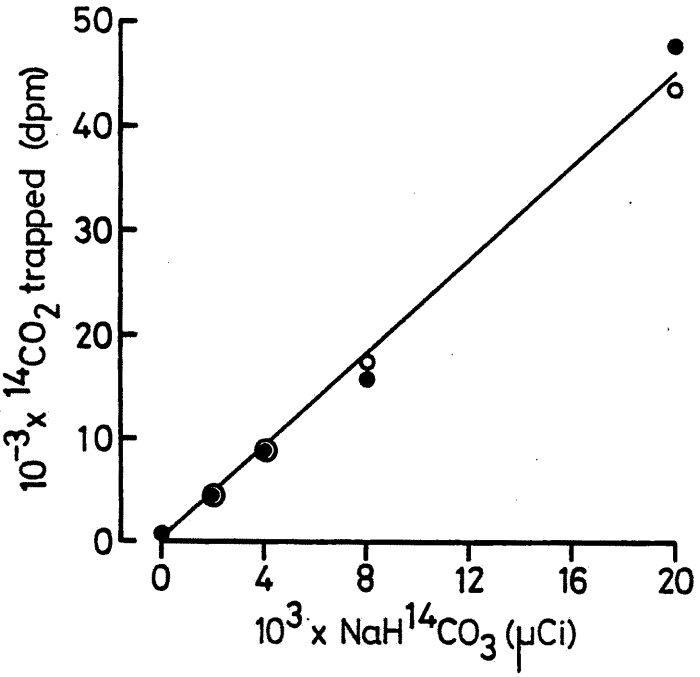


FIG. 5

Amount of $^{14}CO_2$ Trapped by Ethanolamine v Amount of $NaH^{14}CO_3$ Acidified

Varying amounts of 5mM $NaH^{14}CO_3$ (0.004μCi/μmole) and 50mM Tris/HCl buffer pH 9.0 (1ml final volume) were incubated at 37°C in the main compartment of a sealed Warburg flask, containing 0.2ml 2M HCl in the side arm. After 5 min equilibration, the acid was tipped into the main compartment and incubation was continued for a further 25 min. The $^{14}CO_2$ released was trapped by 0.15ml ethanolamine/ethylene glycol monomethyl ether (1:2 v/v), present in a glass tube held in the centre well of the flask. ^{14}C radioactivity (•) was determined as described in Methods. Results are from one experiment and are typical of three. o indicates the expected ^{14}C radioactivity assuming 100% efficiency of trapping.

mixture and the carbon dioxide trapping solution (Krebs and Eggleston, 1945). Decarboxylation of standard oxaloacetate solutions was measured manometrically because [4-^{14}C] oxaloacetate is not readily available. The amount of carbon dioxide evolved was directly proportional to the amount of oxaloacetate (0-5 μmoles) (Fig. 6). Recovery of carbon dioxide was 82% and appropriate correction was made in the enzyme assays.

Since the procedures involved in the measurement of oxaloacetate were found to operate with high efficiency, this assay system appeared to be suitable for the determination of ATP citrate lyase activity. However, when crude supernatants of liver or brain, from normal fed rats, were used in the assay, the ^{14}C radioactivity measured was lower (>50%) than that expected from spectrophotometric assays of the same samples; duplicate assays were inconsistent and there was no increase in the amount of [^{14}C] carbon dioxide detected with increased amounts of the sample. Control assays performed in the absence of enzyme sample revealed a high and variable background (150 - 350 dpm) compared to vials containing scintillation mixture only (50 - 60 dpm). Further investigation indicated that this was due to decarboxylation of [1,5-^{14}C] citrate, since it could be produced by incubation of [1,5-^{14}C] citrate and buffer alone. Although this [^{14}C] carbon dioxide released represented <0.1% of the total citrate it was sufficient to produce as much as a 7-fold increase in the background radioactivity.

However, despite this high background radioactivity, the activity of partially purified liver ATP citrate lyase from DEAE-Sephadex ion-exchange chromatography (activity eluted by the gradient) could be measured with this assay. Thus, the amount of [^{14}C] carbon dioxide was shown to be directly proportional to the amount of sample

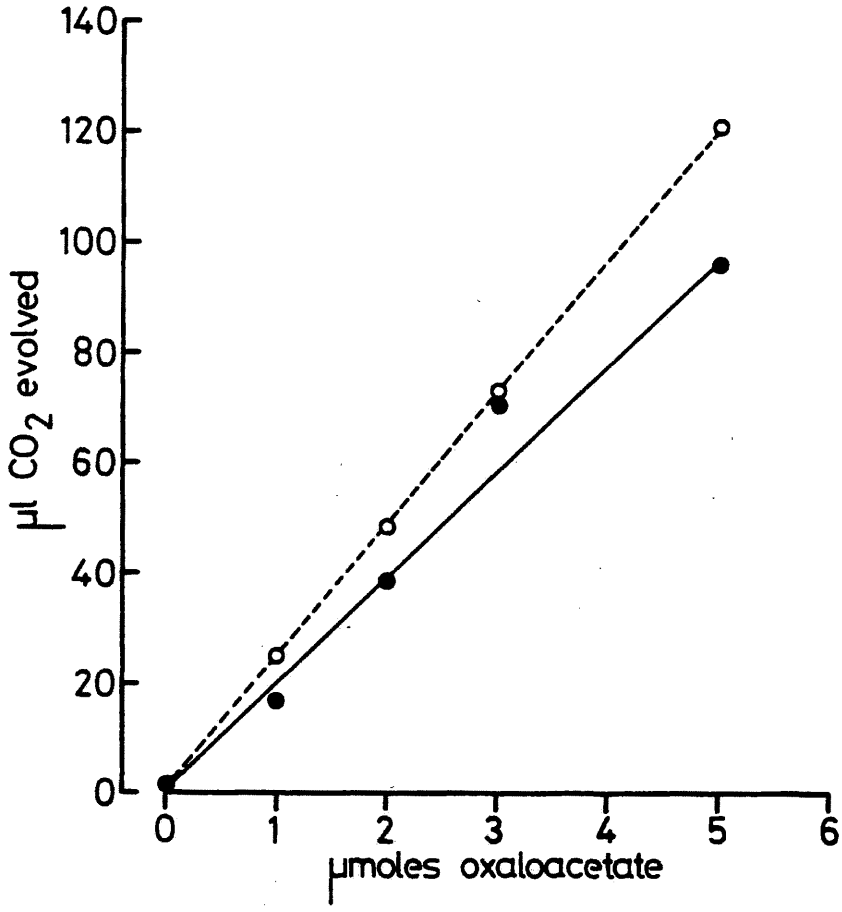


FIG. 6

Amount of CO_2 Released v Amount of Oxaloacetate Decarboxylated

Oxaloacetate in 50mM Tris/HCl buffer pH 7.8 (1ml final volume), and 0.2ml 2M HCl, were equilibrated at 25°C in double-armed Warburg flasks. After 10 min, oxaloacetate was decarboxylated by addition of 0.5ml 0.75M phthalate buffer pH 4.5, followed by 0.5ml $Al_2(SO_4)_3$ solution (33.3% w/v) from the side arms. CO_2 evolution (•) was measured manometrically at 25°C. Results are from one experiment and are typical of two. o indicates the expected amount of CO_2 evolved assuming 100% efficiency of decarboxylation.

(0 - 75µl) and to the incubation time at 37°C (0-40 min) (Figs. 7 and 8). The enzyme activity measured was in good agreement with results from the coupled spectrophotometric assays of the same samples (Table 4), and reactions started with either ATP or CoA produced the same result.

Although this assay was suitable to use with partially purified samples of liver, it could not be used to measure ATP citrate lyase activity of crude supernatants, possibly due to utilisation of the [^{14}C] oxaloacetate produced by other enzymes. Therefore, because of its limited use, and the fact that it was more time consuming than the spectrophotometric assay, it was not used in any further experiments.

2. Ion-Exchange Chromatography of Crude Supernatants

i) DEAE-Sephadex

a) Liver

DEAE-Sephadex anion-exchange chromatography of liver supernatants from normal fed rats revealed two peaks of ATP citrate lyase activity. The first peak was eluted immediately, within a single bed volume, followed by the major proportion of the recovered activity, which was eluted with the gradient (Fig. 9). A similar two-peak profile, with the same distribution of activity, was observed for the enzyme from liver of starved and refed rats. In both cases about 16% of the total recovered activity was present in the non-retained, basic peak (Table 5 and Fig. 10).

These results are in contrast to those of Hoffmann et al. (1979b), who used this method in the purification of liver ATP citrate lyase from starved and refed rats, and observed only a single peak of activity which was eluted by the gradient. Comparison with their results

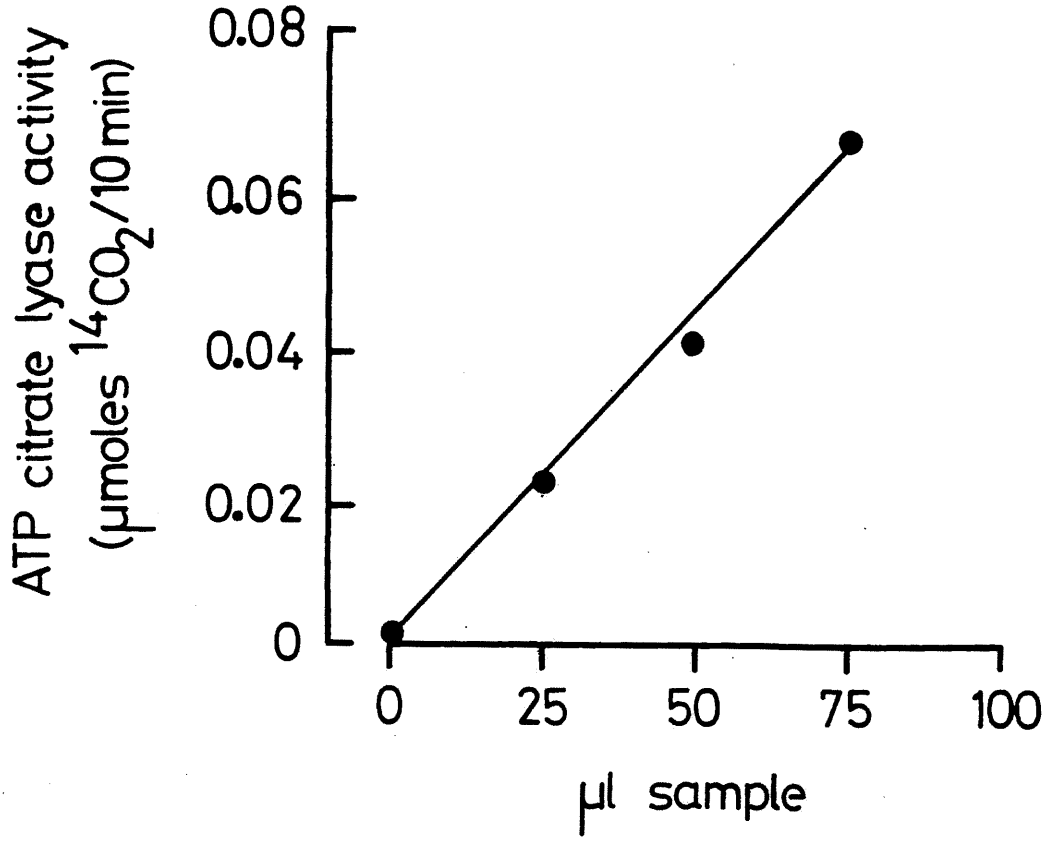


FIG. 7

Radiochemical Assay of Liver ATP Citrate Lyase: $^{14}CO_2$ Released v Amount of Sample

ATP citrate lyase activity was measured by the radiochemical assay as described in Methods. Enzyme samples were preincubated in the assay mixture for 7 min at 37°C before the reaction was started by the addition of ATP, followed by a further 10 min incubation at 37°C. $^{14}CO_2$ released by decarboxylation of the [4-^{14}C] oxaloacetate produced by the enzyme reaction, was trapped by ethanolamine/ethylene glycol monomethyl ether, and the radioactivity determined. Assays without enzyme sample were used as blanks. The ATP citrate lyase activity of the sample measured by the spectrophotometric assay, using a 50 μl sample, was 0.093 units/ml. The enzyme sample used was a column fraction containing ATP citrate lyase activity eluted from a DEAE-Sephadex column by the salt gradient. Results are from one experiment and are typical of four. Values have been corrected for the 82% efficiency of oxaloacetate decarboxylation.

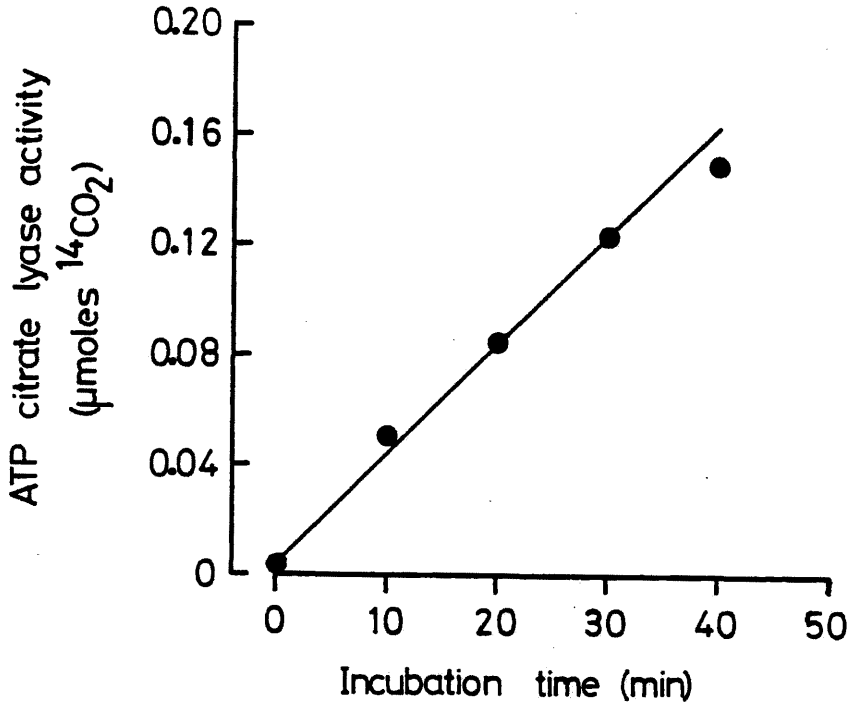


FIG. 8

Radiochemical Assay of Liver ATP Citrate Lyase: $^{14}CO_2$ Released v Incubation Time

ATP citrate lyase activity was measured by the radiochemical assay as described in Methods. Enzyme samples (100μl) were preincubated in the assay mixture for 7 min at 37°C before the reaction was started by the addition of ATP, followed by further incubation for 10-40 min as indicated. $^{14}CO_2$ released by decarboxylation of the [4-^{14}C] oxaloacetate produced by the enzyme reaction, was trapped by ethanolamine/ethylene glycol monomethyl ether, and the radioactivity determined. Assays in which the enzyme reaction was stopped immediately after addition of ATP were used as blanks. The enzyme sample used was a column fraction containing ATP citrate lyase activity eluted from a DEAE-Sephadex column by the salt gradient and concentrated to half the original volume with polyethylene glycol. Results are from a single experiment, and have been corrected for the 82% efficiency of oxaloacetate decarboxylation.

TABLE 4

Comparison of the Radiochemical and Spectrophotometric Assays for the Determination of Liver ATP Citrate Lyase Activity

Radiochemical Assay		Spectrophotometric Assay
μmoles CO_2/10min	Units/ml	Units/ml
0.028	0.056	0.044
0.041	0.082	0.092
0.098	0.20	0.19

Results are from three experiments using three different samples of partially purified liver ATP citrate lyase, and are averages of duplicate assays. The first two values were obtained using individual fractions eluted from DEAE-Sephadex ion-exchange columns by a KCl gradient; for the third value, the enzyme sample used consisted of several fractions eluted from a DEAE-Sephadex column, which had been pooled and concentrated with polyethylene glycol. Both assays were performed using 50μl samples, as described in Methods. Results from the radiochemical assay were obtained after a 10 min incubation at 37°C, and the reaction was started with ATP. Values have been corrected for the 82% efficiency of oxaloacetate decarboxylation. Spectrophotometric assays were started with CoA.

FIG. 9

DEAE-Sephadex Ion-Exchange Chromatography of ATP Citrate Lyase Activity of Crude Liver Supernatant

Dialysed supernatant (34ml) prepared from liver of a normal fed rat was applied to a 2.26 x 22cm (88ml bed volume) DEAE-Sephadex column. ATP citrate lyase activity (●) was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, followed by a linear gradient of 0-0.4M KCl (o). 8ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The elution profile is from a single experiment and is typical of three using this size column.

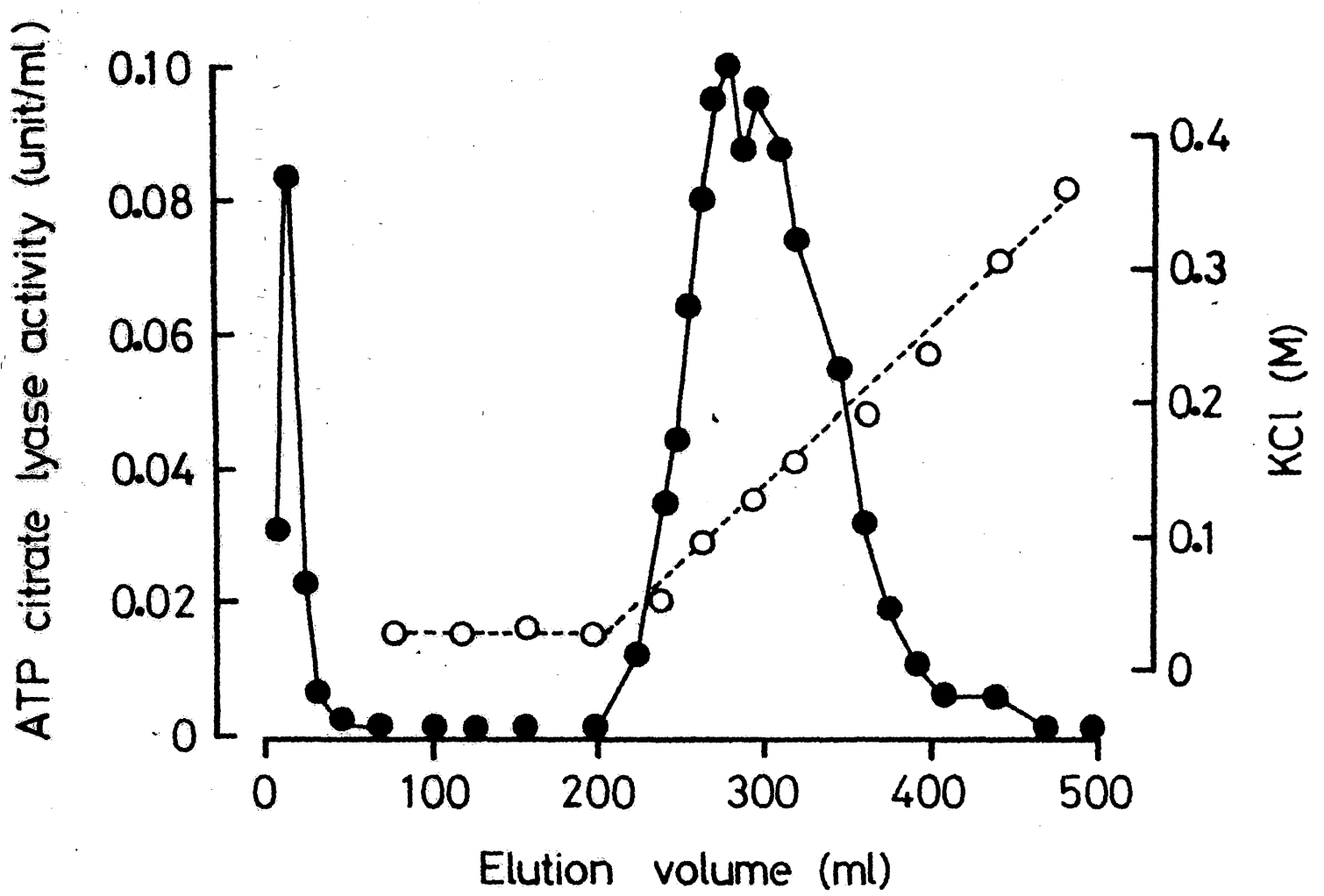

ATP citrate lyase activity (unit/ml)
0.10
0.08
0.06
0.04
0.02
0
0.4
0.3
0.2
0.1
0
KCl (M)
0
100
200
300
400
500
Elution volume (ml)

TABLE 5

% Total Recovered Activity found in the First-Eluted Peak from DEAE-Sephadex Ion-Exchange Chromatography of Liver and Brain Supernatants

Dialysed Supernatant	% Total Recovered Activity
Liver	
Normal fed	14.2 ± 1.6 (5)
Starved/refed	17.7 ± 3.6 (7)
Brain	39.9 ± 7.4 (3)*

* Significantly different from liver, normal fed and starved/refed, $p < 0.02$ (Mann-Whitney U test).

DEAE-Sephadex ion-exchange chromatography of dialysed liver and brain supernatants was performed as described in Methods. The total enzyme activity (units) in the non-retained peak was determined and is expressed as a percentage of the total recovered activity from the two peaks. Results were obtained using different column sizes (see Methods) and are means ± SEM for the number of experiments in brackets. Brains were from normal fed rats.

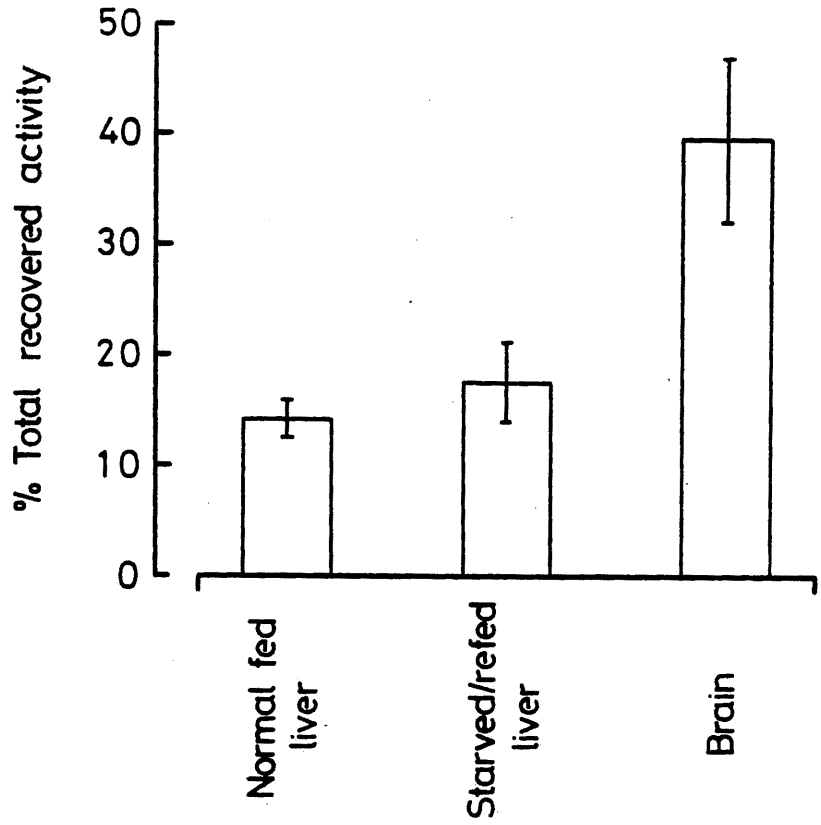


FIG. 10

% Total Recovered ATP Citrate Lyase Activity found in the First-Eluted Peak from DEAE-Sephadex Ion-Exchange Chromatography of Liver and Brain Supernatants

As for Table 5.

shows that the second peak in Fig. 9 was eluted with maximum activity at the same KCl concentration (0.1M). Also, similar increases in specific activity of the enzyme, approximately 10-fold, were obtained (Table 6). Hence, the major proportion of ATP citrate lyase activity was eluted as described by Hoffmann et al. (1979b). However, an additional peak containing a minor component of activity was also observed.

In order to determine whether this first-eluted peak was simply due to column overloading, the sample volume was reduced by 75%. However, the minor component of activity was still present, and there was no reduction in the proportion of the total activity it contained (Table 7, Exp.1). Furthermore, induction of ATP citrate lyase by refeeding rats a high carbohydrate diet, after a period of starvation, produces a large increase in the enzyme activity without changing the protein concentration of the supernatant (see Table 1). When such 'induced supernatant' samples, containing the same total ATP citrate lyase activity as normal fed samples, but with only 25% of the total protein (Table 7, Exp.3), were applied to ion-exchange columns two peaks were still obtained. Thus the presence of the basic peak, containing the same proportion of the total activity as in experiments with supernatants from normal fed rats, indicates that non-adsorption of this activity is not due to competition with other proteins. The possibility of column overloading can therefore be excluded.

ATP citrate lyase has been shown to be susceptible to nicking by proteolytic enzymes (Singh et al., 1976; Linn and Srere, 1979). This therefore presents another explanation for the apparent heterogeneity of the enzyme observed by ion-exchange chromatography. Supernatants were routinely applied to columns immediately after dialysis. However, when crude supernatants were either aged for 24h at 4°C before dialysis, or

TABLE 6

Comparison of the Increase in Specific Activity of Liver ATP Citrate Lyase, obtained by DEAE-Sephadex Ion-Exchange Chromatography, with that observed by Hoffmann et al.(1979b)

Source of ATP Citrate Lyase	Volume ml	Activity Units	Total Protein mg	Specific Activity Units/mg	Total Yield %	Increase in Specific Activity
1. Normal fed						
Total Applied -- supernatant	34	11	620	0.018		
Recovered - gradient fractions	12	2.4	19	0.13	105	x 7.0
2. Starved/refed						
Total Applied -- supernatant	34	61	670	0.091		
Recovered - gradient fractions	9	4.0	5.4	0.74	107	x 8.0
Hoffmann et al. (1979b)						
Total Applied - supernatant	42	36	617	0.060		
Recovered - gradient fractions	136	34	54	0.60		x 10

TABLE 6 - continued

Livers from normal fed, or starved and refed rats were homogenised in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose. Homogenates were centrifuged at 45,000g for 30 min at 4°C, and the supernatants were applied, after dialysis, to DEAE-Sephadex column, (2.26 x 22cm). 34ml samples were eluted with the above buffer without sucrose, followed by a linear KCl gradient. 8ml fractions were collected into 1ml Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The specific activity of ATP citrate lyase eluted by the gradient was determined using fractions containing maximum enzyme activity. For the experiment with starved/refed rat liver results are from a single fraction; for the experiment with normal fed rat liver, three maximum activity fractions were pooled and concentrated to half the original volume by polyethylene glycol. Results are from single experiments and are typical of 4 using normal fed rats, and 2 using starved/refed rats. 'Total Yield' represents the total recovery of activity from the column i.e. from both the non-retained and retained peaks.

TABLE 7

Effect of Changing Total ATP Citrate Lyase Activity and/or Total Protein Applied to DEAE-Sephadex Ion-Exchange Columns, on % Total Recovered Activity in the Non-Retained Peak

Experiment	Dietary State of Animal	Sample Volume ml	Total Activity Applied Units	% Total Recovered Activity in Non-Retained Peak
1. Decreased Enzyme Activity and Total Protein	a. normal fed	34	13	13.0
	b. normal fed	8	3.8	13.6
2. Increased Enzyme Activity	a. normal fed	34	13	13.0
	b. starved/refed	34	52	18.6
3. Decreased Total Protein	a. normal fed	8.5	2.4	18.2
	b. starved/refed	2.0	2.7	22.5

TABLE 7 - Continued

DEAE-Sephadex ion-exchange chromatography of liver supernatants was performed as described in Methods. Results represent three sets of experiments: 1. Supernatants from liver of normal fed rats were applied to DEAE-Sephadex columns, 2.26 x 22cm (88ml bed volume). Results are a) average of three experiments, b) from one experiment; 2. Supernatants from liver of a) normal fed, and b) starved and refed rats, were applied to DEAE-Sephadex columns, 2.26 x 22cm (88ml bed volume). Results are a) from same experiments as is 1a, b) average of two; 3. Supernatants from liver of a) normal fed, and b) starved and refed rats, were applied to DEAE-Sephadex columns 1.6 x 11cm (22ml bed volume). Results are a) from one experiment, and b) average of two. Changes in sample volume represent different amounts of total protein applied to the columns as well as different amounts of ATP citrate lyase activity. Average protein concentrations of supernatants are 21.7mg/ml and 22.4mg/ml for normal fed and starved/refed respectively (see Table 1). Total recovery of activity from both peaks averaged 107 $\pm$ 10% for the nine separate experiments; differences in recovery between experiments did not affect results.

dialysed for 24h, prior to column chromatography, two peaks of ATP citrate lyase were still obtained. More direct evidence against the involvement of proteolysis was obtained by ion-exchange chromatography in the presence of the proteolytic inhibitor phenylmethylsulphonyl fluoride (0.2mM). Thus, liver supernatant prepared in buffer containing phenylmethylsulphonyl fluoride had the same proportion of the total ATP citrate lyase activity in the non-retained, basic form as the control supernatant, prepared from the same tissue but homogenised in the absence of the inhibitor (Fig. 11). The concentration of phenylmethylsulphonyl fluoride used here has recently been shown to protect acetyl CoA carboxylase, resulting in changes in the properties of the enzyme (Song and Kim, 1981).

The use of Sephadex G25 gel filtration of supernatants in place of dialysis had no effect on the elution profile of ATP citrate lyase from DEAE-Sephadex ion-exchange columns. Thus, the enzyme heterogeneity is not due to an artefact of dialysis.

The possibility that citrate synthase acting in the reverse direction is responsible for the presence of the minor peak was considered. However, citrate synthase activity in crude liver supernatants was negligible. Furthermore, when column fractions containing either first peak or second peak activity were assayed using the ATP citrate lyase assay, but in the absence of ATP which inhibits citrate synthase, no activity was detected. This confirms the absence of citrate synthase in these fractions.

b) Brain

ATP citrate lyase in crude supernatants of adult rat brain also eluted as two peaks of activity from DEAE-Sephadex ion-exchange columns (Fig. 12). However, in contrast to the liver enzyme, a large proportion of the recovered activity, 40%, was eluted in the non-retained

FIG. 11

DEAE-Sephadex Ion-Exchange Chromatography of Crude Liver Supernatants in the Presence of Phenylmethylsulphonyl Fluoride

Livers from two starved and refed rats were minced separately and each divided in half. Each one of the four liver portions was homogenised separately in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol, 0.25M sucrose, and either 0.2mM phenylmethylsulphonyl fluoride (PMSF) in propan-2-ol (one portion from each liver), or an equal amount of propan-2-ol only (one portion from each liver). PMSF was dissolved in propan-2-ol and diluted 1:2 (v/v) with homogenising buffer. The required volume was then added to homogenisation and column buffers to give 0.2mM PMSF. Control buffers (i.e. -PMSF) contained the same volume of a 1:2 (v/v) solution of propan-2-ol in buffer. Each homogenate was centrifuged at 45,000g for 30 min at 4°C; the two supernatants containing PMSF (i.e. one from each liver) were combined, as were the two supernatants without PMSF. After dialysis of each sample (i.e. + and - PMSF) against 50 vol. homogenising buffer for 2h at 4°C, 2ml samples were applied to DEAE-Sephadex columns (1.6 x 11cm). ATP citrate lyase activity (●) was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, and 1mM dithiothreitol, $\pm$ PMSF, followed by a step gradient of 0.4M KCl (o). 2ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, contiaining 10mM dithiothreitol. Results are from a single experiment; a second profile obtained for each sample (i.e. + and - PMSF) after 24h dialysis, produced the same results.

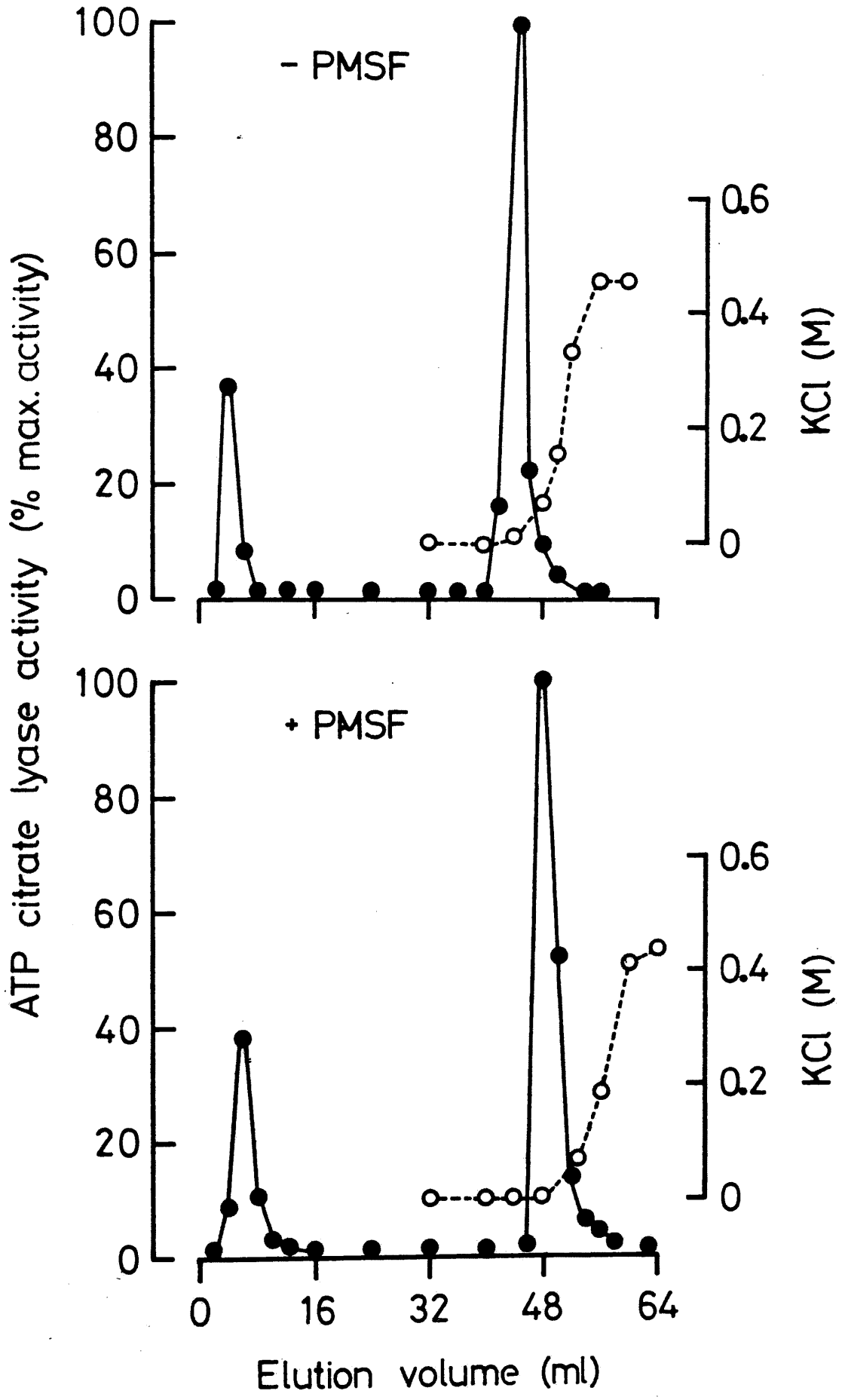
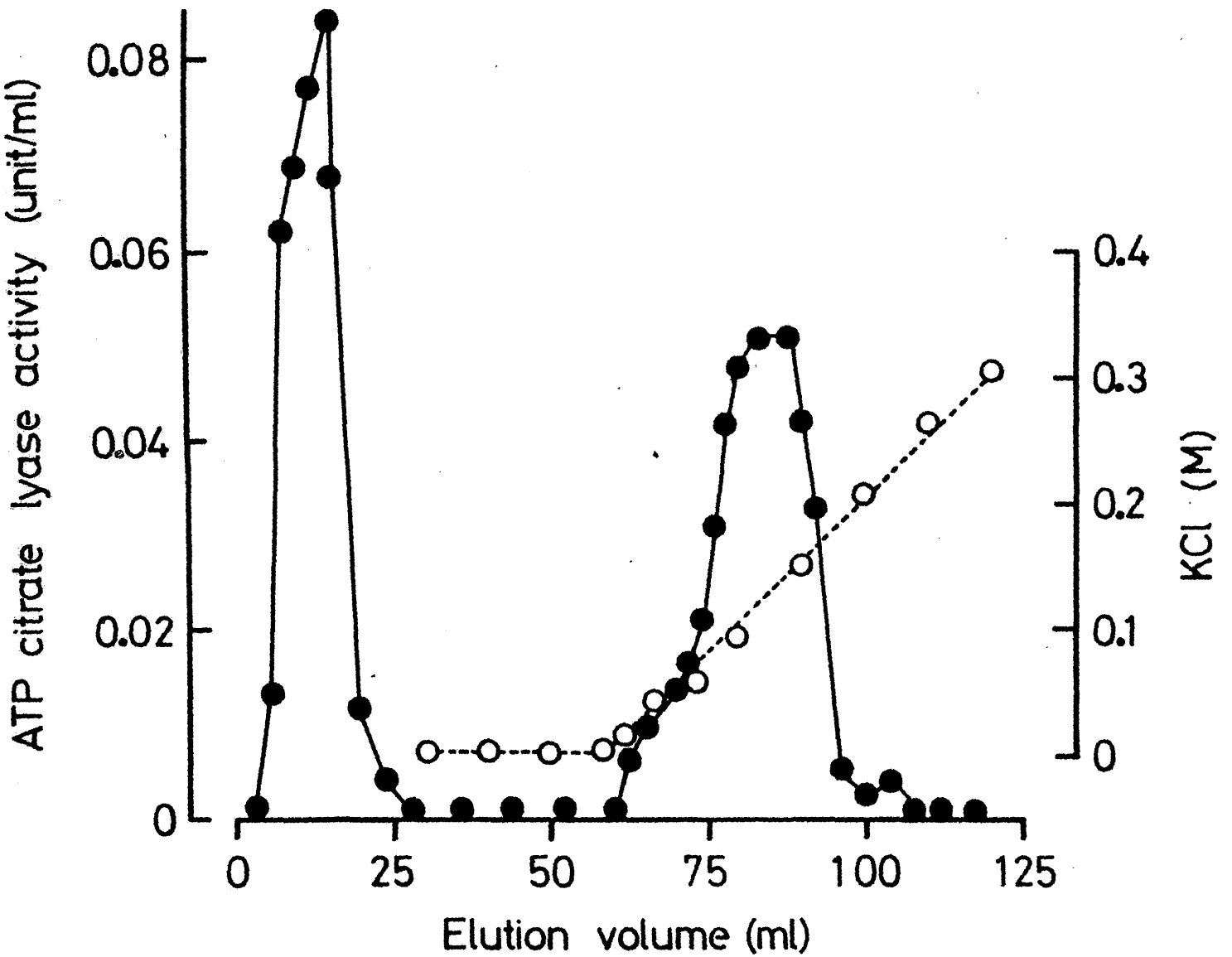

- PMSF
+ PMSF
ATP citrate lyase activity (% max. activity)
KCl (M)
Elution volume (ml)
100
80
60
40
20
0
0.6
0.4
0.2
0
16
32
48
64

FIG. 12

DEAE-Sephadex Ion-Exchange Chromatography of ATP Citrate Lyase Activity of Crude Brain Supernatant

Dialysed supernatant (20ml) prepared from brains of 8 normal fed rats was applied to a 1.6 x 11cm (22ml bed volume) DEAE-Sephadex column. ATP citrate lyase activity (●) was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, followed by a linear gradient of 0-0.4M KCl (o). 2ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The elution profile is from a single experiment and is typical of three using the same size column.


ATP citrate lyase activity (unit/ml)
0.08
0.06
0.04
0.02
0
0.4
0.3
0.2
0.1
0
KCl (M)
0
25
50
75
100
125
Elution volume (ml)

peak (Table 5 and Fig. 10). The remaining activity was eluted by the ionic gradient at a similar KCl concentration to the liver enzyme, around 0.1M.

Initially all ion-exchange columns were run over two days and fractions were assayed for ATP citrate lyase activity the second day after preparation of the supernatant. However, use of smaller columns (22ml instead of 88ml bed volume) enabled chromatography to be performed overnight so that fractions were assayed the day after supernatant preparation. Average recovery of activity (total from both peaks) from all columns, both liver and brain, was 109 $\pm$ 6% from 36 experiments. Some experiments however, resulted in unexpectedly high recoveries of over 170%. Possible explanations for this are discussed later.

ii) CM-Sephadex

Since the minor component of ATP citrate lyase activity was not adsorbed on the DEAE-Sephadex anion-exchange column, ion-exchange chromatography was performed using a cation-exchange column. Hence, the ATP citrate lyase not retained by the positively charged DEAE-Sephadex, may be adsorbed by a negatively charged cation-exchanger.

Therefore, cation-exchange chromatography was performed on CM-Sephadex using supernatants prepared in the same way as for DEAE-Sephadex anion-exchange chromatography, except that samples were dialysed against phosphate buffers. However, all the ATP citrate lyase activity was eluted without retention, at both pH's used, pH 7.0 and pH 8.0, resulting in 100% recovery of activity before application of the NaCl gradients. Neither the phosphate buffer, nor the lower pH (pH 7.0), had any effect on the ATP citrate lyase activity of the supernatants compared to control samples in Tris/HCl buffer at pH 8.0.

iii) Phosphocellulose

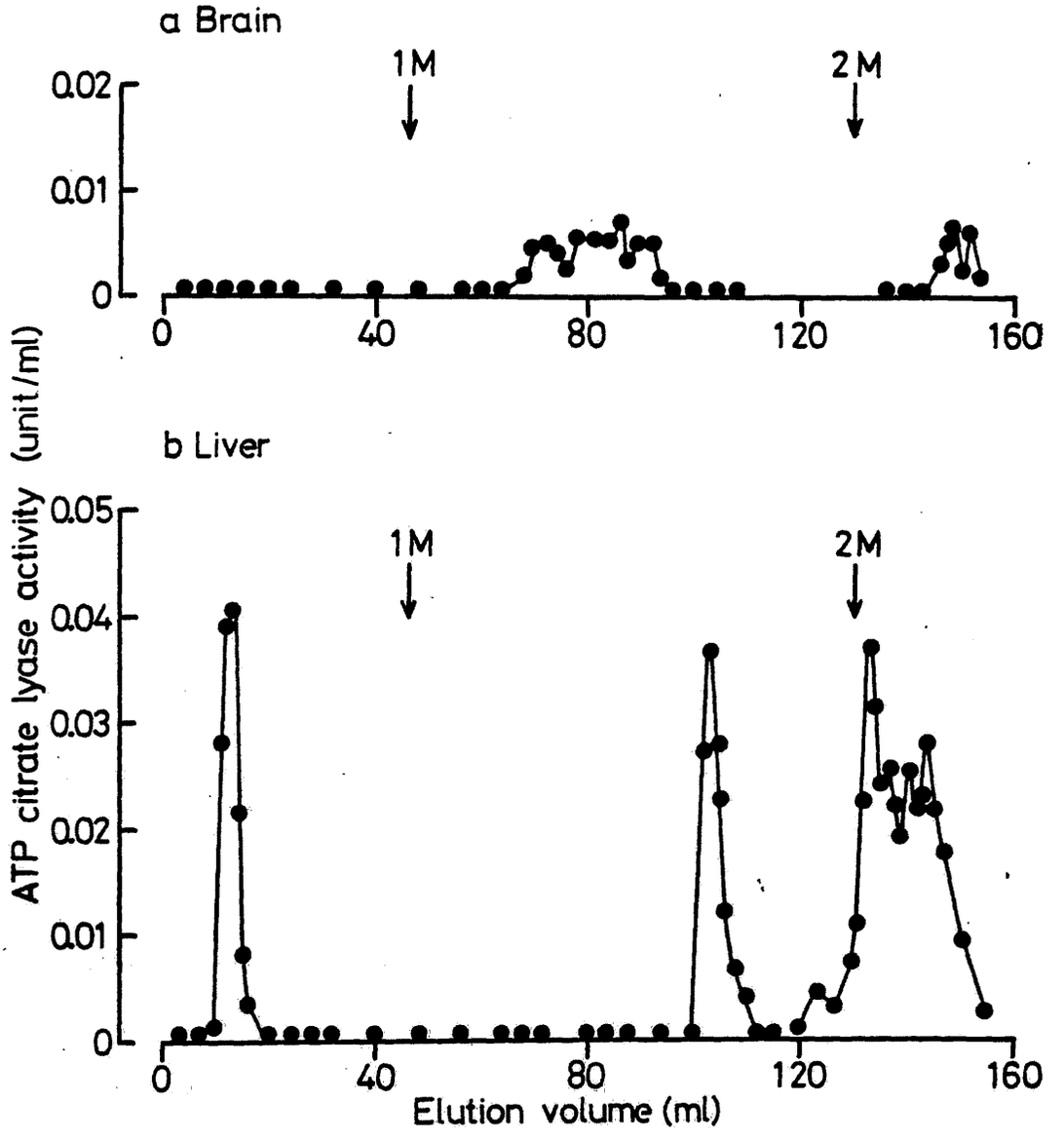
The heterogeneity of liver and brain ATP citrate lyase was also investigated by cation-exchange chromatography on phosphocellulose columns. The liver enzyme from starved and refed rat was separated into three peaks of activity (Fig. 13b). A small percentage of the recovered activity, 17%, was eluted immediately without retention, but the possibility that this represents column overload cannot be excluded by the present experiments. The major proportion of the enzyme was strongly adsorbed by the column and was eluted after application of a 2M NaCl step gradient. A third peak, comprising 19% of the recovered activity, was eluted by the linear gradient at about 0.45M NaCl. However, the elution profile of the enzyme from a duplicate column, using the same supernatant, did not show any activity at this position. The apparent absence of this peak could be explained by the instability of the enzyme, as column fractions were assayed a day later that those from the duplicate column which had activity. This is supported by the fact that the recovery of activity from this column (52%) was lower than that of the other column (62%) by an amount of activity equivalent to that found in the second eluted peak.

Phosphocellulose ion-exchange chromatography of brain supernatants revealed two peaks of ATP citrate lyase activity (Fig. 13 a). The first peak, which contained the major proportion of the recovered activity, was eluted by the linear gradient over the range 0.2 - 0.4M NaCl. This activity is therefore distinct from the second eluted peak of the liver enzyme which was eluted at 0.45M NaCl. The second peak of brain ATP citrate lyase activity was eluted after application of a 2M NaCl step gradient and contained about 20% of the recovered activity. However, total recovery of activity from two duplicate columns was <30%, and therefore quantitative comparison with

FIG. 13

Phosphocellulose Cation-Exchange Chromatography of ATP Citrate Lyase Activity of Crude Supernatants from Liver and Brain

Dialysed supernatants (a, 11.5ml; b, 2ml) prepared from a) brains of 10 normal fed rats, and b) liver of one starved and refed rat, were applied to phosphocellulose columns, 1.6 x 6cm (12 ml bed volume). ATP citrate lyase activity was eluted with 20mM Tris/HCl buffer pH 7.0, containing 1mM EDTA and 1mM dithiothreitol, followed firstly by a linear NaCl gradient (0-1M), and then a step gradient of 2M NaCl. 1ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 7.0, containing 10mM dithiothreitol. Results are from a single experiment and the elution profiles shown are from one of duplicate columns. All four columns were packed using the same batch of pre-cycled phosphocellulose. The arrows indicate the start of the respective gradients.


a Brain
1M
2M
0.02
0.01
0
0
40
80
120
160
b Liver
1M
2M
0.05
0.04
0.03
0.02
0.01
0
0
40
80
120
160
ATP citrate lyase activity (unit/ml)
Elution volume (ml)

the results from the liver enzyme are not feasible.

These results, although only preliminary, indicate that heterogeneity of both liver and brain ATP citrate lyase can be demonstrated by cation-exchange chromatography as well as anion-exchange chromatography. Furthermore, in contrast to DEAE-Sephadex ion-exchange chromatography where a quantitative difference was observed between the liver and brain enzyme (i.e. the amount of activity in each peak), phosphocellulose column chromatography revealed the presence of ATP citrate lyase peaks unique to either liver or brain. Hence, a qualitative difference was observed. Further experiments are required however, to determine the relationships between the chromatographic peaks obtained from these two ion-exchange columns.

3. Gel Filtration of Crude Supernatants

i) Sepharose 6B

a) Liver

Gel filtration of crude liver supernatants was performed using a Sepharose 6B column. The molecular weight fractionation range of this gel for globular proteins is 10^4 - $4x10^6$, and it is therefore suitable for gel filtration chromatography of ATP citrate lyase, which has a molecular weight of the order of 10^5.

ATP citrate lyase was eluted as two peaks of activity when crude supernatants of liver from starved and refed rats were applied to a Sepharose 6B column (Fig. 14). A minor component, comprising 10% of the recovered activity, was eluted at the void volume of the column, followed by a second peak containing the major proportion of the activity. Similar elution profiles were obtained for the enzyme in crude liver supernatants from fed, and starved rats (Tables 8 and 9, and Fig. 15).

FIG. 14

Gel Filtration of Crude Liver Supernatant on Sepharose 6B

Crude supernatant (5ml) prepared from liver of a starved and refed rat was applied to a Sepharose 6B column, 2.26 x 27.5cm (110ml bed volume), and was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. ● ATP citrate lyase activity; o lactate dehydrogenase activity. The arrow indicates the void volume (V_o). The elution profile is from a single experiment and is typical of four.

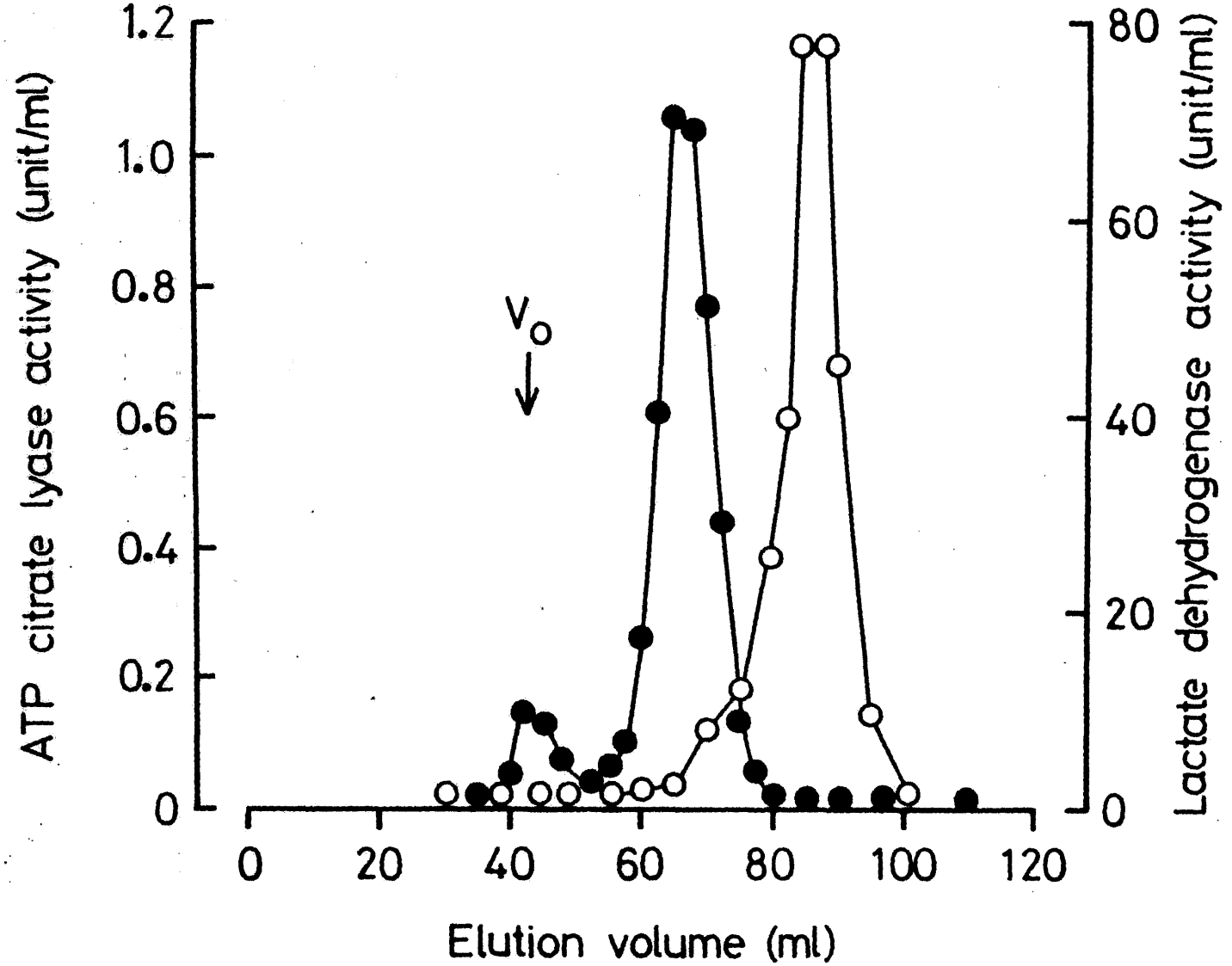

ATP citrate lyase activity (unit/ml)
Lactate dehydrogenase activity (unit/ml)
Elution volume (ml)
V_o
1.2
1.0
0.8
0.6
0.4
0.2
0
80
60
40
20
0
0
20
40
60
80
100
120

TABLE 8

% Total Recovered Activity found in the First-Eluted Peak from Sepharose Gel Filtration of Liver and Brain Supernatants

Crude Supernatant	% Total Recovered Activity Sepharose 6B	Sepharose 2B
Liver		
Normal fed	7.9 ± 4.2 (3)	-
Starved/refed	10.8 ± 1.6 (4)	7.2 ± 1.6 (3)
Starved	8.5 (2)	-
Brain	0.8 ± 0.8 (3)*	-

* Significantly different from starved/refed liver, $p < 0.05$ (Mann-Whitney U test).

Sepharose gel filtration of crude liver and brain supernatants was performed as described in Methods. The total enzyme activity (units) in the high molecular weight peak was calculated from the symmetry of the major peak (see Figs. 14, 17, 18), and is expressed as a percentage of the total recovered activity. Results are means ± SEM for the number of experiments in brackets. Brains were from normal fed rats.

TABLE 9

Elution Volumes of ATP Citrate Lyase in Crude Supernatants of Liver and Brain from Sepharose 6B and Sepharose 2B

Source of ATP Citrate Lyase	V_e ml	K_{av}	Estimated Molecular Weight ($\times 10^{-5}$)
Sepharose 6B			
Liver starved/refed	68.5 ± 0.9 (4)	0.38 ± 0.01 (4)	4.1
normal fed	77.5 ± 1.2 (3)*	0.52 ± 0.02 (3)	2.7
starved	64.3 (2)	0.32 (2)	4.9
Brain	67.9 ± 0.4 (3)	0.38 ± 0.01 (3)	4.1
Sepharose 2B			
Liver starved/refed	89.9 (2)	0.69 (2)	-

* Significantly different from starved/refed liver, and brain, Sepharose 6B $p \leq 0.05$ (Mann-Whitney U test).

Crude supernatants of liver (5ml) and brain (10-13ml) were applied to a Sepharose 6B or Sepharose 2B gel filtration column (bed volumes, V_t = 110ml). ATP citrate lyase activity was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, and the elution volume, V_e, was determined. The void volumes of the columns, V_o, were measured by the elution of Blue Dextran 2000 (Sepharose 6B, V_o = 43.0 ± 0.6ml(7); Sepharose 2B, V_o = 45.8ml ± 0.3(3)). K_{av} was calculated from $\frac{V_e - V_o}{V_t - V_o}$, where V_e is the elution volume of ATP citrate lyase, V_o is the void volume, and V_t is the total bed volume. Results are means ± SEM for the number of experiments in brackets. Brains were from normal fed rats.

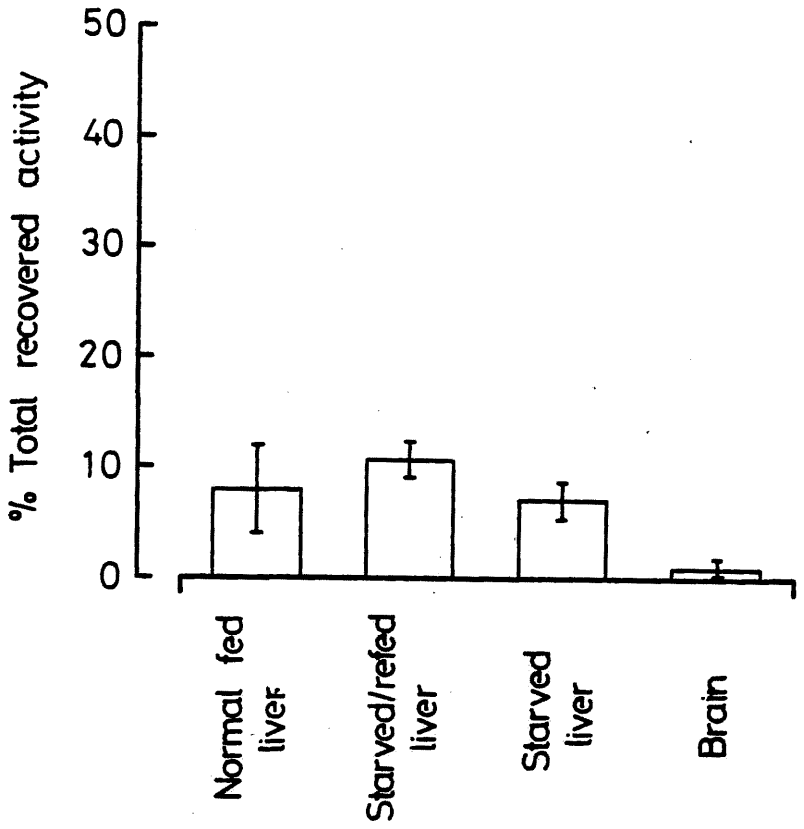


FIG. 15

% Total Recovered ATP Citrate Lyase Activity found in the First-Eluted Peak from Sepharose 6B Gel Filtration of Liver and Brain Supernatants

As for Table 8

Column fractions from Sepharose 6B gel filtration of liver supernatant were assayed for the cytoplasmic marker enzyme, lactate dehydrogenase. The elution profile (Fig. 14) shows that no peak of activity was present corresponding to the minor component of ATP citrate lyase activity. Thus, the void volume peak of ATP citrate lyase cannot be explained by non-specific entrapment of cytoplasmic enzymes by membrane fragments in the supernatant. In fact, a small amount of lactate dehydrogenase, representing only 0.5% of the total recovered activity, was detected in the void volume fractions, and indicates that such a phenomenon does occur. However, essentially all of this cytoplasmic enzyme was eluted as a single peak after the major peak of ATP citrate lyase activity, in accordance with its lower molecular weight. Total recovery of activity was 100%.

The Sepharose 6B column was calibrated by the method of Andrews (1965) in order to estimate the molecular weight of the major peak of ATP citrate lyase activity (Fig. 16). The value obtained for the enzyme from starved and refed rat liver, M_r 4.1 x 10^5, is in good agreement with literature values, obtained for the purified enzyme from the same source, which vary from 4.2 x 10^5 to 5.0 x 10^5 (Inoue *et al.*, 1966; Singh *et al.*, 1976; Linn and Srere, 1979; Redshaw and Loten, 1981). This result therefore establishes that the major peak of enzyme activity corresponds to the native, tetrameric enzyme. Thus, the minor component of activity represents a high molecular weight associated form of the enzyme. Since it is eluted at the void volume of the column, it must have a molecular weight greater than 4 x 10^6, the exclusion limit of the gel.

The major proportion of liver ATP citrate lyase from starved rat was eluted from the Sepharose 6B column with the same elution volume as that from starved and refed rat (Table 9), and therefore has

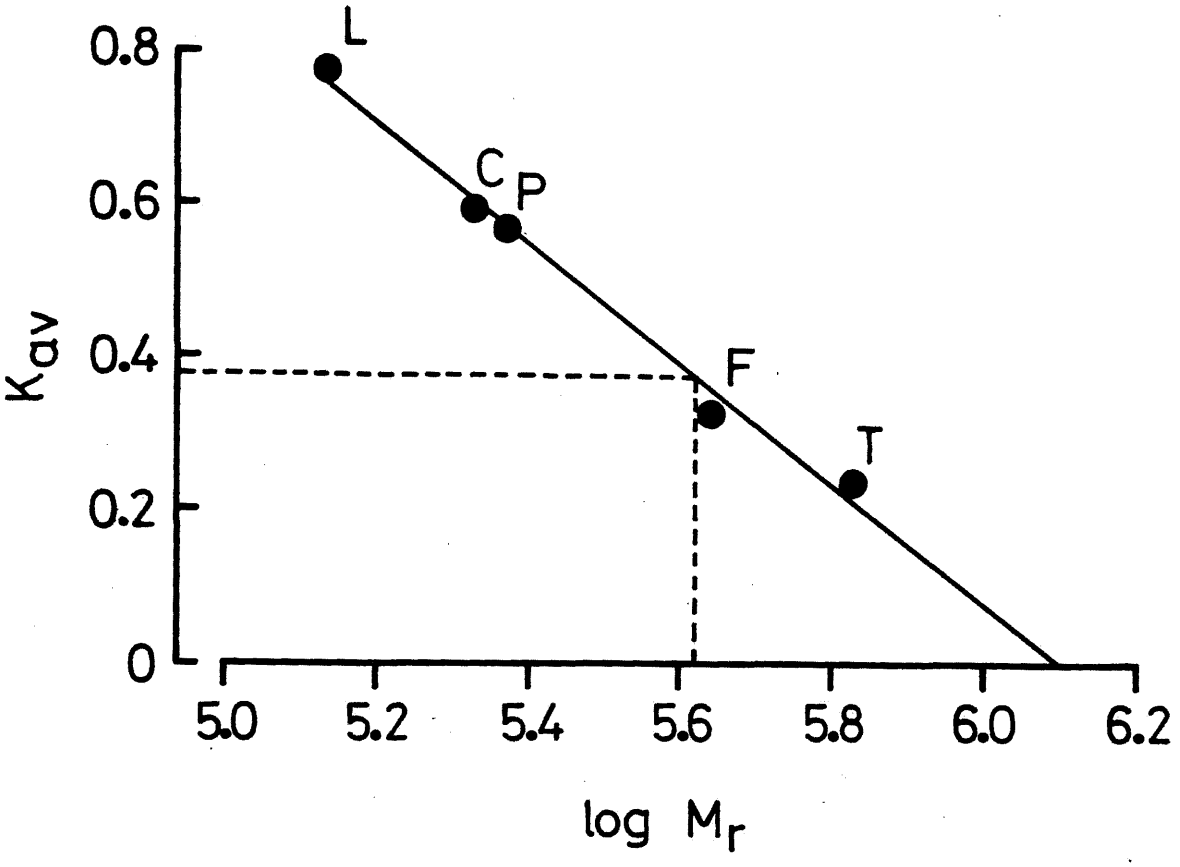


FIG. 16

Estimation of the Molecular Weight of the Major Peak of Liver ATP Citrate Lyase from Sepharose 6B Gel Filtration

Calibration standards (•): L, lactate dehydrogenase (M_r 134,000); C, catalase (M_r 210,000); P, pyruvate kinase (M_r 237,000); F, ferritin (M_r 440,000); T, thyroglobulin (M_r 669,000). The continuous line was plotted by linear regression analysis. Broken lines indicate the observed K_{av} value for the major ATP citrate lyase peak of liver (see Fig. 14) and the estimated log M_r value. K_{av} was calculated for ATP citrate lyase and for molecular weight standard proteins as described in the legend to Table 9.

the same molecular weight. However, although gel filtration of liver supernatants from normal fed rats produced a similar elution profile, the major peak of activity was eluted with a larger elution volume (Table 9), corresponding to a smaller molecular weight. From the calibration curve (Fig. 16), the molecular weight was estimated to be 2.7×10^5, corresponding to an apparent 34% decrease in the size of the enzyme.

b) Brain

Gel filtration of brain supernatants, using the same Sepharose 6B column, revealed only one peak of ATP citrate lyase activity (Fig. 17). In contrast to the liver enzyme, no peak of activity was found in the void volume fractions. Absence of ATP citrate lyase in this region cannot be explained by instability of the enzyme, since no activity was detected even when fractions were assayed immediately after elution from the column.

The elution volume of the enzyme, 68ml (Table 9), corresponds to a molecular weight of 4.1×10^5 (Fig. 16). Hence, ATP citrate lyase of brain has the same molecular weight as the liver enzyme from starved, and starved and refed rats. This is the first report of an estimation of the brain enzyme molecular weight. Furthermore, direct comparison with that of the liver enzyme indicates that ATP citrate lyase from these two tissues is the same with respect to molecular size.

ii) Sepharose 2B

In an attempt to estimate the size of the high molecular weight component of liver ATP citrate lyase, gel filtration of crude extracts was performed on Sepharose 2B, which has an exclusion limit of 4×10^7 molecular weight for globular proteins. As seen in Fig. 18, the minor, associated state of the enzyme was not eluted as a single peak, but as a spread of activity from just after the void volume up to the

FIG. 17

Gel Filtration of Crude Brain Supernatant on Sepharose 6B

Crude supernatant (10ml) prepared from brains of six normal fed rats was applied to a Sepharose 6B column, 2.26 x 27.5cm (110ml bed volume), and was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The arrow indicates the void volume (V_o). The elution profile is from a single experiment and is typical of three.

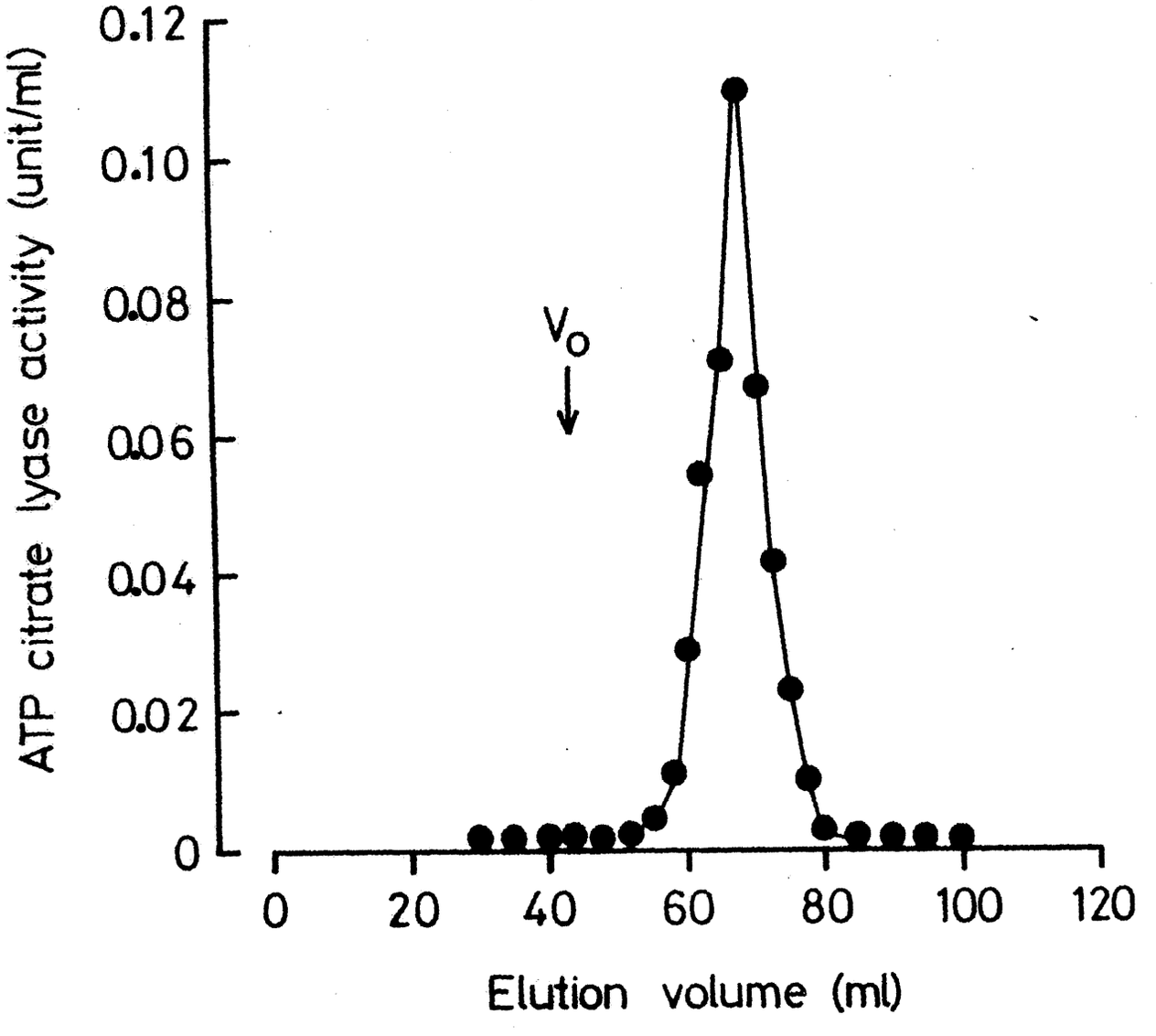
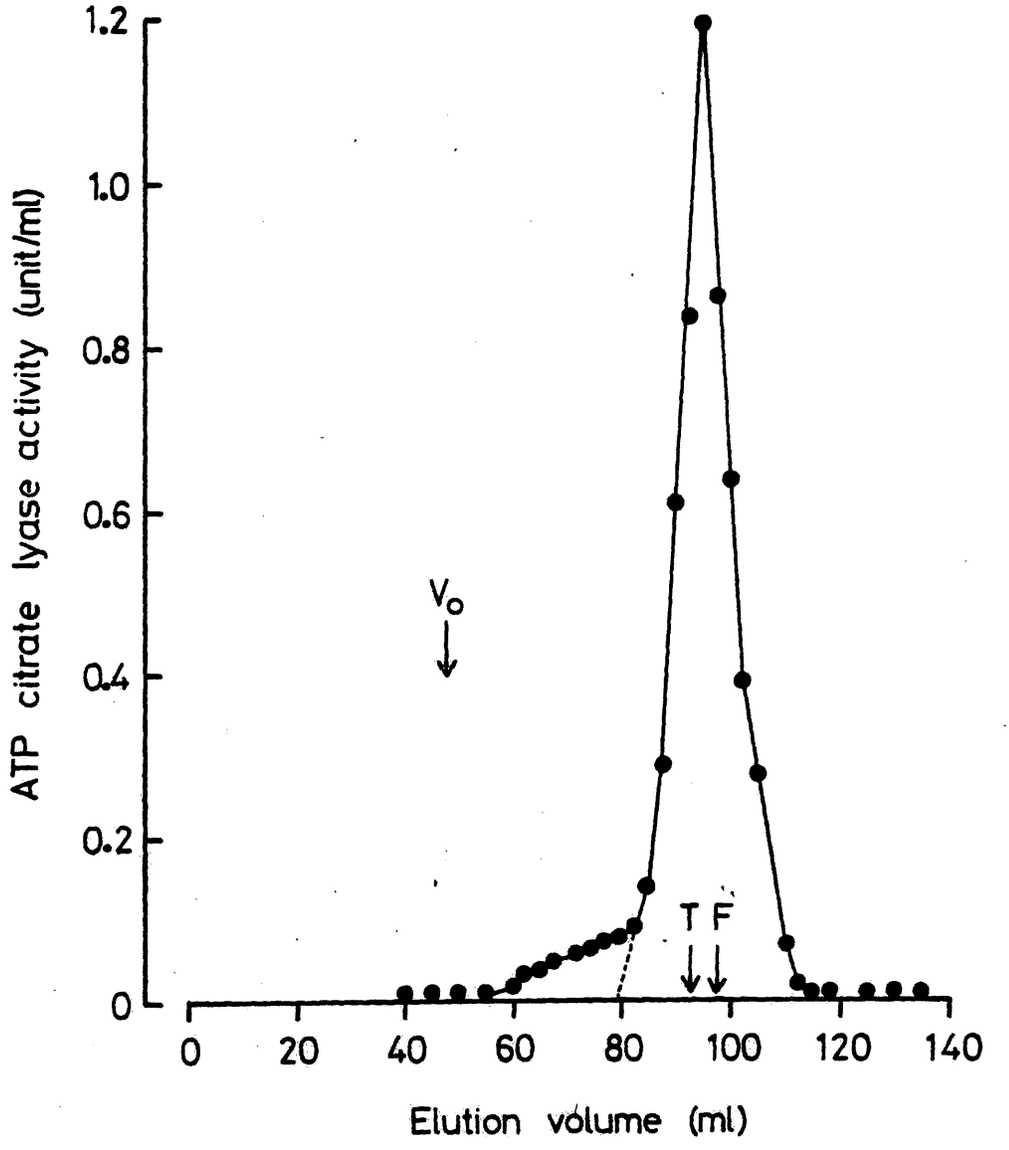

ATP citrate lyase activity (unit/ml)
0.12
0.10
0.08
0.06
0.04
0.02
0
V_o
0
20
40
60
80
100
120
Elution volume (ml)

FIG. 18

Gel Filtration of Crude Liver Supernatant on Sepharose 2B

Crude supernatant (5ml) prepared from liver of a starved and refed rat was applied to a Sepharose 2B column, 2.26 x 27.5cm (110ml bed volume), and was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The arrows indicate the void volume (V_o), and the elution of the molecular weight standard proteins, thyroglobulin, T(M_r 669,000) and ferritin, F(M_r 440,000). The dashed line represents the separation of the two components of the activity and is drawn according to the symmetry of the major component. The elution profile is from a single experiment and is typical of three.


ATP citrate lyase activity (unit/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0
V_o
T
F
0
20
40
60
80
100
120
140
Elution volume (ml)

major peak of ATP citrate lyase. Although this column was not calibrated, due to lack of very high molecular weight marker proteins (i.e. $M_r > 10^6$), the elution volumes of thyroglobulin and ferritin (see Fig. 18) confirmed that the major peak of activity was the tetrameric enzyme, and thus corresponds to the major peak obtained from the Sepharose 6B column. The shape of the elution profile observed for the high molecular weight component of activity, suggests that this associated state of the enzyme exists as a range of sizes, the molecular weight of which was estimated to be around 10^7.

The amount of activity present in the high molecular weight form was estimated by assuming that the major peak was symmetrical and calculating the amount of activity outside this peak (see dotted line Fig. 18). Thus, about 7% of the total recovered activity was contained in this high molecular weight region (Table 8), which is of the same order as that observed for Sepharose 6B gel filtration.

Gel filtration experiments were routinely run overnight and column fractions were assayed the day after preparation of the crude tissue extracts. Recovery of activity from both Sepharose 6B and Sepharose 2B was very high, an average of $147 \pm 8\%$ for 29 experiments. As discussed in a later section, liver ATP citrate lyase appears to undergo some sort of activation after DEAE-Sephadex ion-exchange chromatography under certain conditions. Hence, a similar phenomenon could be responsible for the high recovery of activity observed here.

4. Investigation of the Nature of ATP Citrate Lyase Heterogeneity

i) Rechromatography Experiments

In order to determine whether the various peaks of liver ATP citrate lyase show consistent chromatographic behaviour, and to ascertain the relationships, if any, between these different forms, a series of rechromatography experiments was performed.

When the non-retained ATP citrate lyase activity obtained by ion-exchange chromatography of dialysed supernatants was applied to a second DEAE-Sephadex column, all the activity was adsorbed on the column, and was eluted by the salt gradient (Table 10). The simplest interpretation of this result is that the enzyme not retained by the first column represented overload. However, such a possibility has already been excluded, and therefore, it would appear that the basic, non-retained form of the enzyme changes into the more acidic form which is eluted by the gradient, and corresponds to the peak observed by Hoffmann et al. (1979b). The same result was obtained when the enzyme was applied either immediately after elution from the first column, or after a 2h dialysis against the column buffer. Therefore, the minor, basic component of ATP citrate lyase changes rapidly into the major form of the enzyme. In contrast however, when crude supernatants were either aged or dialysed for 24h prior to column chromatography, the two-peak elution profile of the enzyme was still obtained. Hence, the basic, non-retained form appears to be stable in crude supernatants.

Rechromatography on DEAE-Sephadex was also performed with the different molecular weight forms of ATP citrate lyase obtained from Sepharose 6B gel filtration of liver supernatants. For both the tetrameric enzyme, and the high molecular weight associated state of ATP citrate lyase, all the applied activity was eluted by the salt

TABLE 10

Rechromatography of the Various Chromatographic Fractions of Liver ATP Citrate Lyase on DEAE-Sephadex

Source of Sample		Activity Applied Units	Activity Recovered Units		Total Recovery of Activity %
			Basic Non-Retained Peak	Acidic Retained Peak	
DEAE-Sephadex					
Basic, non-retained peak					
	1.	0.31	0.00	0.21	68
	2.	0.72	0.00	1.0	144
	3.	0.16	0.00	0.10	63
Sepharose 6B					
Void volume peak	4a	0.15	0.00	0.15	100
Major peak (tetrameric enzyme)	4b	1.2	0.013	0.63	54

TABLE 10 - Continued

Fractions containing maximum ATP citrate lyase activity of the various chromatographic peaks were applied to DEAE-Sephadex ion-exchange columns. Columns were washed with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, followed by a step gradient of 0.4M KCl in the same buffer. 1. Sample dialysed against 100 vol. of above buffer for 2.25h. 6ml applied to column, 1.6 x 9cm (bed volume, 18ml), 1.6ml fractions collected. 2. Sample dialysed against 100 vol. of above buffer for 2h. 7.5ml applied to column, 1.6 x 7cm (bed volume, 14ml), 1.3ml fractions collected. 3. Sample applied immediately after elution from first column, without dialysis. 1ml applied to column, 0.9 x 5cm, (bed volume, 3ml), 1ml fractions collected. 4. Samples, 2ml, applied immediately, without dialysis, to columns, 0.9 x 4.5cm (bed volume, 3ml), 1ml fractions collected. All fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. Results are from single experiments. Experiments 1 and 2 were performed with liver from normal fed rats, experiments 3, and 4 with liver from starved and refed rats.

gradient (Table 10), except for a small amount of the tetrameric enzyme which was eluted before the gradient. The absence of any significant amount of the basic, non-retained form indicates that the unstable nature of this enzyme state extends to ATP citrate lyase partially purified by gel filtration. Thus, the relationships between the two molecular weight forms, and the two ionic states of the enzyme cannot be determined from such rechromatography experiments.

As shown in Table 10, the recovery of activity from the rechromatography columns was variable. This can be explained by the availability of only small amounts of enzyme in these experiments, and also by the instability of ATP citrate lyase activity.

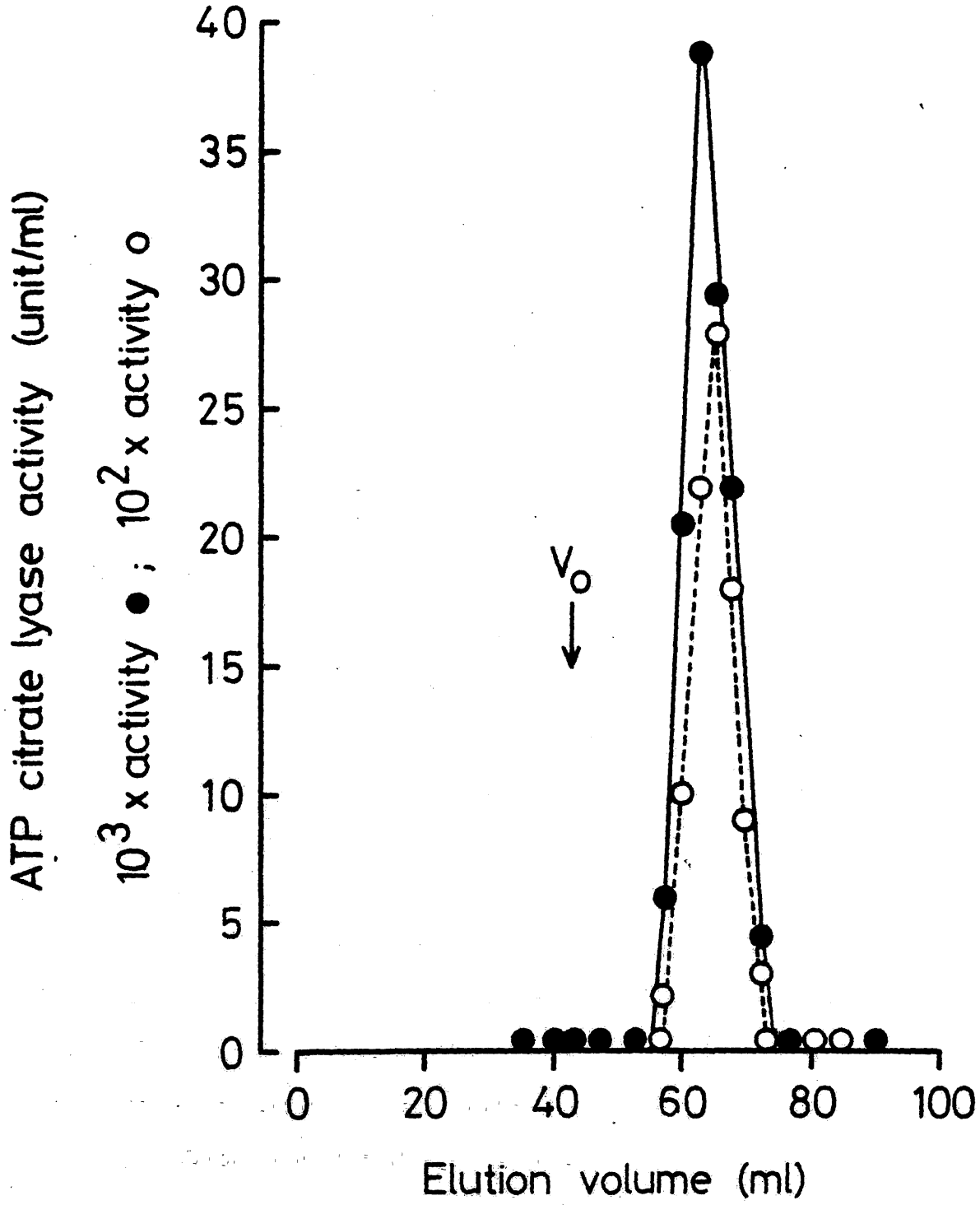
When the high molecular weight ATP citrate lyase obtained from Sepharose 6B gel filtration of supernatants was re-applied to the same column, all the activity was recovered as a single peak which eluted as the tetrameric enzyme (Fig. 19). Hence, this associated state of ATP citrate lyase also appears to be unstable, breaking down to produce the tetrameric enzyme. Rechromatography of the major peak of ATP citrate lyase activity indicated that this molecular weight form is stable since it was eluted with unchanged elution volume, as shown by comparison of Fig. 19 with Fig. 14.

Sepharose 6B gel filtration of the basic, non-retained ATP citrate lyase activity, obtained from DEAE-Sephadex ion-exchange chromatography of supernatants, was not feasible because of the instability of this enzyme state. Rechromatography experiments on DEAE-Sephadex columns had already demonstrated that after the initial chromatographic separation of the enzyme, the non-retained, basic form was rapidly converted to the major acidic form. Thus, the unstable nature of the basic state of ATP citrate lyase prevented direct determination of its molecular weight by Sepharose 6B gel filtration. Moreover, as noted

FIG. 19

Rechromatography of the Major Tetrameric, and the Minor High Molecular Weight Forms of ATP Citrate Lyase obtained from Sepharose 6B, on the Same Column

The two molecular weight forms of liver ATP citrate lyase obtained from the experiment shown in Fig. 14 by Sepharose 6B gel filtration of crude liver supernatant, were re-applied separately to the same column. 4ml samples of first the high molecular weight activity (2.25ml of fractions 17 and 18 combined) (●), and then the tetrameric form (2.25ml of fractions 26 and 27 combined) (o), were applied to the column and each was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The arrow indicates the void volume (V_o). The elution profiles are from a single experiment.


ATP citrate lyase activity (unit/ml)
10^3 x activity ● ; 10^2 x activity o
40
35
30
25
20
15
10
5
0
V_o
0
20
40
60
80
100
Elution volume (ml)

earlier, this form of the enzyme was absent when both molecular weight forms were applied to DEAE-Sephadex columns, again indicating the instability of this ionic state of the enzyme.

ii) Effect of High-Speed Centrifugation on Ion-Exchange and Gel Filtration Elution Profiles of Liver ATP Citrate Lyase

The high molecular weight ($M_r \sim 10^7$) of the minor component of liver ATP citrate lyase present in an associated state suggested the possibility that it could be removed from the supernatant by centrifugation. Hence, high-speed supernatants were prepared, from liver of starved and refed rats, by centrifugation of homogenates at 150,000g for 90 min, instead of the routinely employed 45,000g for 30 min. Gel filtration of such supernatants on Sepharose 6B revealed only one peak of ATP citrate lyase activity, corresponding to the tetrameric enzyme (Fig. 20); the high molecular weight form of the enzyme was absent. When control supernatants, prepared by centrifugation of a sample of the same homogenate at 45,000g for 90 min, were applied to Sepharose 6B column, both the tetrameric and the high molecular weight forms of the enzyme were observed. This therefore, indicates that the associated state of ATP citrate lyase had been removed by the increased centrifugation.

In conjunction with the loss of the high molecular weight enzyme, there was a decrease in the total ATP citrate lyase activity of the supernatant. This corresponded to a reduction in supernatant activity of about 20%, which is the same order of magnitude as the proportion of the enzyme activity found in the high molecular weight peak on gel filtration of 45,000g supernatants (see Table 8).

DEAE-Sephadex ion-exchange chromatography of high-speed liver supernatants containing only tetrameric ATP citrate lyase revealed the presence of both the minor, non-retained peak, and the major peak eluted

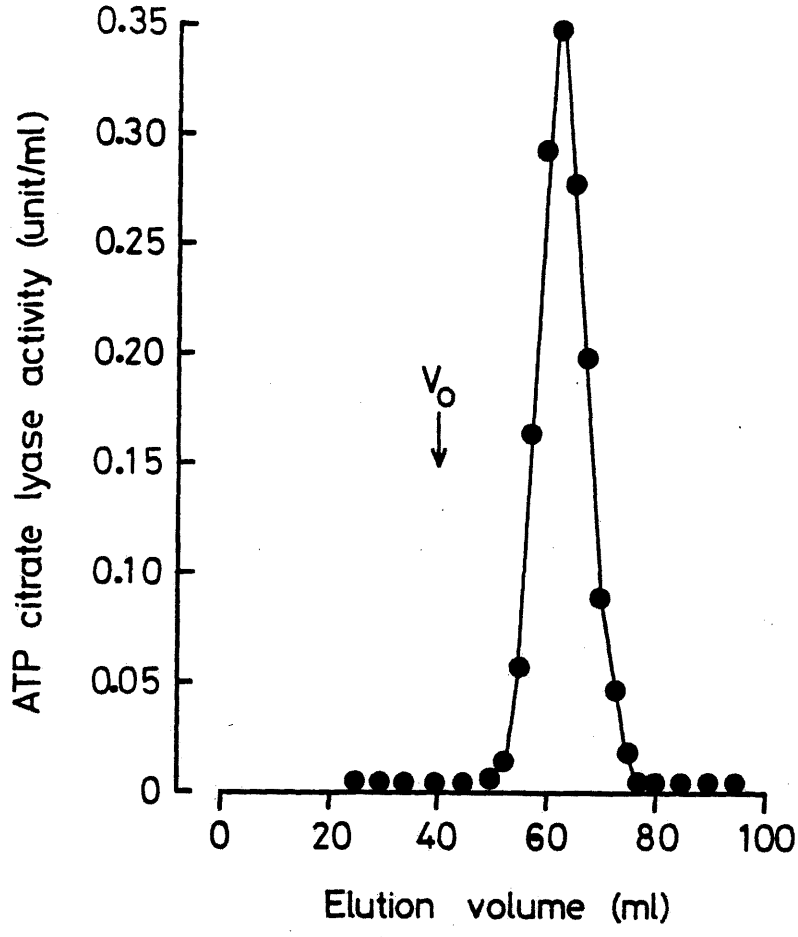


FIG. 20

Effect of High-Speed Centrifugation on the Sepharose 6B Gel Filtration Elution Profile of ATP Citrate Lyase

Crude supernatant was prepared from livers of two starved and refed rats by centrifugation of the combined homogenates at 150,000g for 90 min at 4°C. 5ml of supernatant was applied to the Sepharose 6B column and was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The arrow indicates the void volume (V_o). The elution profile is from one experiment and is typical of two.

by the gradient (Table 11). Therefore, the tetrameric enzyme is comprised of both the basic and acidic forms of ATP citrate lyase. The fact that the non-retained, basic form was found to be present in the absence of high molecular weight ATP citrate lyase, establishes that these two minor fractions of the enzyme activity do not correspond to the same, single form of the enzyme i.e. one which is basic and has a high molecular weight.

In an attempt to determine the nature of the activity lost from the supernatant after high-speed centrifugation, the elution profile of ATP citrate lyase in such supernatants from DEAE-Sephadex columns was compared with that of control, low-speed supernatants prepared from the same homogenate. As shown in Table 11, the total activity of the acidic peak obtained from the high-speed supernatants was reduced by an amount similar to the decrease in the supernatant activity. This therefore, suggests that the high molecular weight associated state of ATP citrate lyase is normally adsorbed by the column and eluted as the acidic peak.

However, in addition to this loss of activity from the acidic peak, there was also an anomalous increase of almost 100% in the activity present in the non-retained basic peak (Table 11). Consequently, any decrease in activity corresponding to the loss of the high molecular weight ATP citrate lyase, would not be detectable. The possibility that some high molecular weight activity is also basic in nature cannot therefore be excluded.

The reason for the increase in the activity of the basic peak is unknown. Since the loss of activity from the acidic peak observed after high-speed centrifugation is greater than that lost from the supernatant, it may be partly explained by a transfer of activity from the acidic to the basic form of the enzyme. However, this would not account for all the increase. Although the total recoveries of activity

TABLE 11

Effect of High-Speed Centrifugation on DEAE-Sephadex Ion-Exchange Chromatography Elution Profile of Liver ATP Citrate Lyase

	Total Applied Activity	Recovered Activity				Total Recovered Activity	
		Non-Retained Peak		Retained Peak			
	Units	Units	% Applied	Units	% Applied	Units	% Applied
Control Supernatant (45,000g)	1.2	0.24	19	0.89	73	1.1	92
High-Speed Supernatant (150,000g)	0.93	0.46	49	0.53	57	0.99	106
Increase or Decrease in Total Activity (Units)	-0.27	+0.22		-0.36		-0.11	

TABLE 11 - Continued

Livers from two starved and refed rats were homogenised in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose. The homogenates were combined and centrifuged at 2,000g for 10 min. The supernatant obtained was divided into two portions; one was centrifuged at 45,000g for 90 min and the second at 150,000g for 90 min. Supernatants were dialysed against 50 vol. of the above buffer for 2h, followed by application of 1.5ml samples to DEAE-Sephadex columns, 0.9 x 4.5cm (bed volume, 3ml). Columns were washed with 8ml homogenising buffer without sucrose, prior to application of step gradients of 0.4M KCl (14ml). 1ml fractions were collected into 0.125ml 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. Results are averages for duplicate columns, and are typical of three experiments.

from both columns are comparable, it may be significant that a higher yield of the activity was obtained from column chromatography of the high-speed supernatant. Thus, some form of activation of ATP citrate lyase in the basic peak could account for the unexpected result. The possibility of such an activation will be discussed later.

iii) Elution Profiles of Fatty Acid Synthetase and Acetyl CoA Carboxylase in Crude Liver Supernatants, from Sepharose Gel Filtration Columns

The possibility that the high molecular weight form of ATP citrate lyase represents an association with other lipogenic enzymes was investigated by measuring the activity of the enzymes fatty acid synthetase and acetyl CoA carboxylase, in the column fractions from Sepharose gel filtration experiments.

Fatty acid synthetase was eluted from a Sepharose 2B gel filtration column with an elution volume of 90 ml (Fig. 21). The enzyme co-eluted with the tetrameric ATP citrate lyase activity as would be expected from the molecular weight, 5.4×10^5 (Burton et al., 1968). However, no activity was found to elute with the minor, high molecular weight ATP citrate lyase activity. It is interesting to note that the activity of fatty acid synthetase was only 10% that of ATP citrate lyase. Hence, under the conditions used, a rate-limiting role for ATP citrate lyase in fatty acid biosynthesis would appear unlikely.

Acetyl CoA carboxylase, EC 6.4.1.2, was assayed by another student using the method of Inoue and Lowenstein (1975), which involves measurement of [^{14}C] malonyl CoA formation from $NaH^{14}CO_3$ and acetyl CoA. The enzyme activity in column fractions from Sepharose 6B gel filtration of liver supernatant from starved and refed rat was determined. It was found that acetyl CoA carboxylase co-eluted with the tetrameric form of

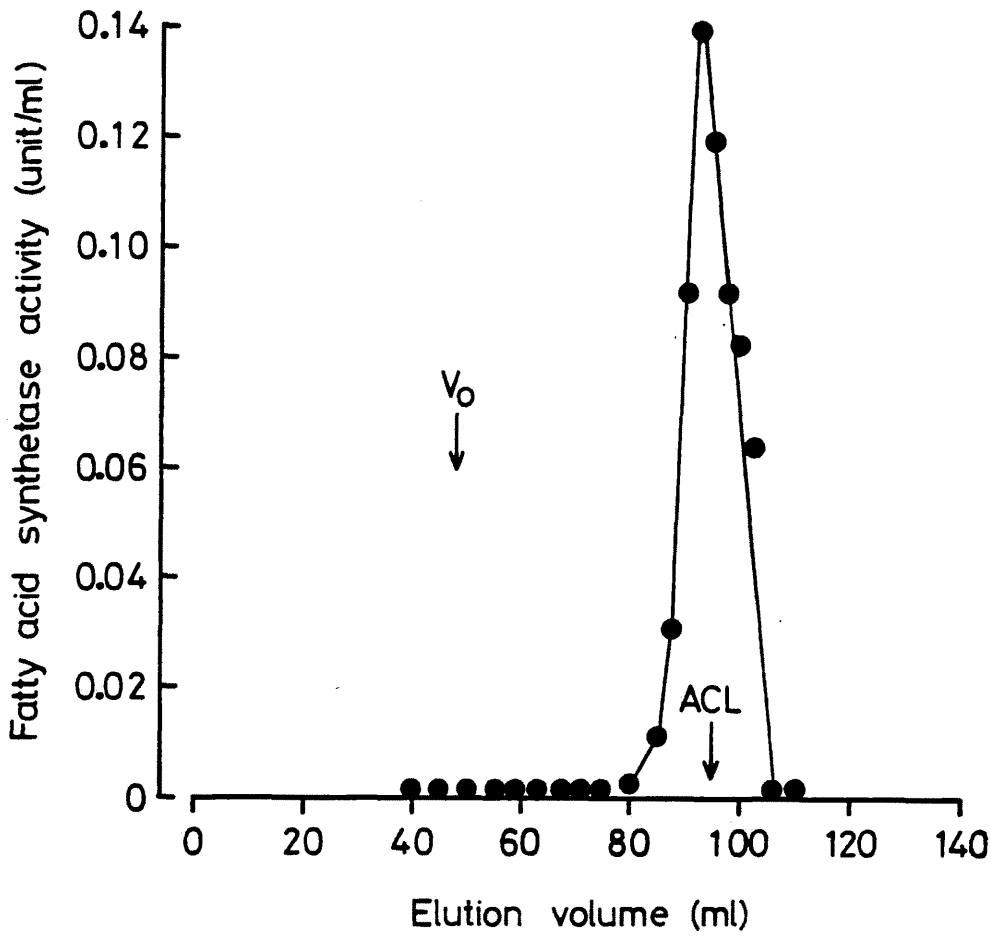


FIG. 21

Elution Profile of Fatty Acid Synthetase obtained by Sepharose 2B Gel Filtration of Crude Liver Supernatant

Fatty acid synthetase activity was measured in column fractions obtained from the experiment in Fig. 18. The arrows indicate the void volume (V_o) and the elution of the major tetrameric peak of ATP citrate lyase (ACL). The elution profile is from a single experiment.

ATP citrate lyase in accordance with its molecular weight of 4 x 10^5 (Gregolin et al., 1966). No activity was found in fractions containing the high molecular weight associated state of ATP citrate lyase (C. Rich unpublished results).

Hence, there is no evidence to suggest that ATP citrate lyase can exist as a multienzyme complex with either acetyl CoA carboxylase or fatty acid synthetase, the two subsequent enzymes of the lipogenic pathway.

iv) Elution Profiles of Marker Enzymes from DEAE-Sephadex Ion-Exchange and Sepharose Gel Filtration Chromatography of Liver Supernatants

Membrane association of ATP citrate lyase was investigated as another possible explanation for the high molecular weight form, especially in view of recent work suggesting that the enzyme can bind to mitochondrial membranes (Janski and Cornell, 1980a,b; Janski and Cornell, 1982; Ranganathan et al., 1980). Thus, the elution profiles of various marker enzymes from the different chromatographic columns were determined.

a) Lactate Dehydrogenase

Lactate dehydrogenase in crude supernatants of liver was eluted from a Sepharose 6B gel filtration column after the major peak of ATP citrate lyase activity (Fig. 14). A minor peak of activity, which was eluted at the void volume, represented only 0.5% of the recovered activity and is probably due to enzyme trapped by membrane fragments in the supernatant. This is in contrast to the significantly larger void volume peak of ATP citrate lyase activity, corresponding to ~10% of the recovered activity.

b) Rotenone-Insensitive NADH Cytochrome c Reductase

A marker enzyme for outer mitochondrial membranes, rotenone-

insensitive NADH cytochrome c reductase (RIDCR) (Sottocasa et al., 1967; Schnaitman and Greenawalt,1968), was assayed in column fractions from Sepharose 6B gel filtration of crude liver supernatant. A single peak of activity was eluted at the void volume, with the high molecular weight ATP citrate lyase activity (Fig. 22).

Further investigation using a Sepharose 2B column also revealed a peak of activity at the void volume (Fig. 23). The presence of a high and variable background activity prevented detection of any small peaks of activity. However, there was no evidence to suggest co-elution of RIDCR with the high molecular weight associated state at ATP citrate lyase.

The elution of this membrane marker enzyme from a DEAE-Sephadex ion-exchange column was also determined. All the recovered activity (78% of applied activity) was eluted as a single peak by the salt gradient after the elution of the major peak of ATP citrate lyase activity (Fig. 24). Hence, there was no co-elution of the membrane marker enzyme with either of the two peaks of ATP citrate lyase activity observed by ion-exchange chromatography. The apparent 10-fold higher activity of RIDCR in this experiment compared to gel filtration elution profiles cannot be explained.

c) NADPH Cytochrome c Reductase

Liver NADPH cytochrome c reductase, which is exclusively microsomal (Phillips and Langdon, 1962), was eluted at the void volume of the Sepharose 6B gel filtration column (Fig. 22). Since the molecular weight of this enzyme purified from rat liver, is only 57,700 (Phillips and Langdon, 1962), the elution of activity at the void volume therefore represents the membrane-bound enzyme, and hence indicates the presence of microsomes in the liver supernatants.

d) Succinate Dehydrogenase

Crude supernatants of liver contained no detectable activity

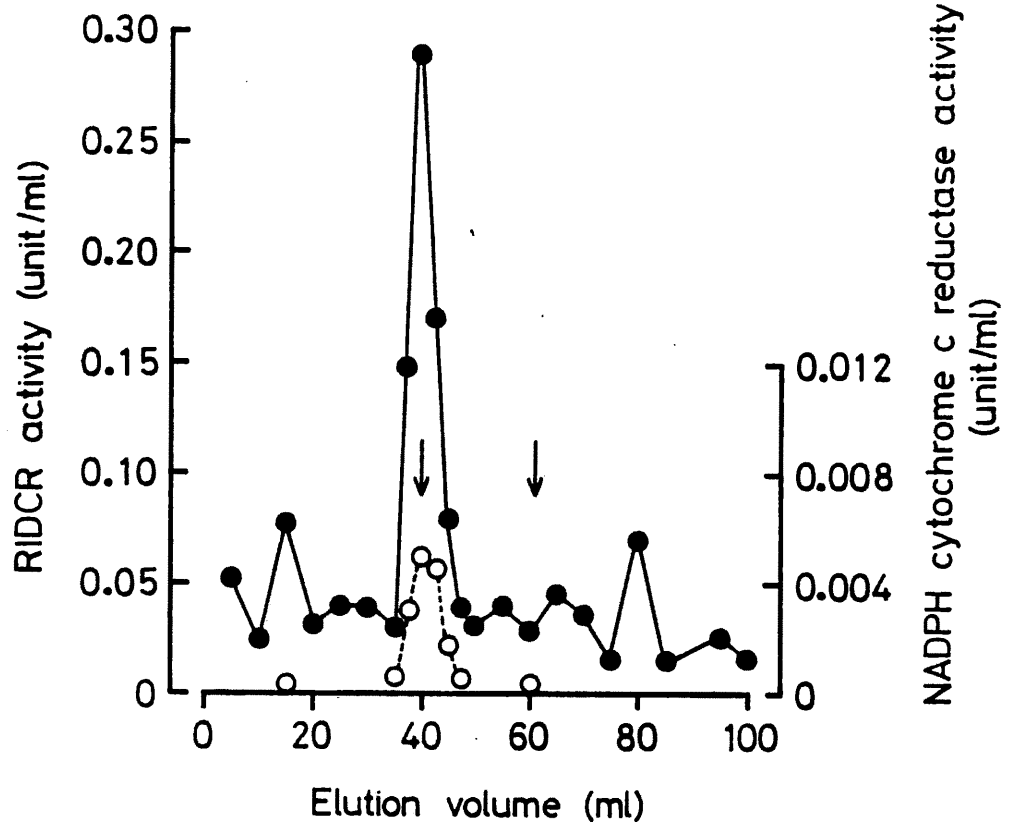


FIG. 22

Elution Profiles of RIDCR and NADPH Cytochrome c Reductase obtained by Sepharose 6B Gel Filtration of Crude Liver Supernatant

The activities of RIDCR (●) and NADPH cytochrome c reductase (o) were measured in column fractions obtained from Sepharose 6B gel filtration of crude supernatant under the same conditions as described in Fig. 14 legend. The first arrow indicates the void volume, and the elution of the high molecular weight ATP citrate lyase activity. The second arrow indicates the elution of tetrameric ATP citrate lyase. The results are from a single experiment.

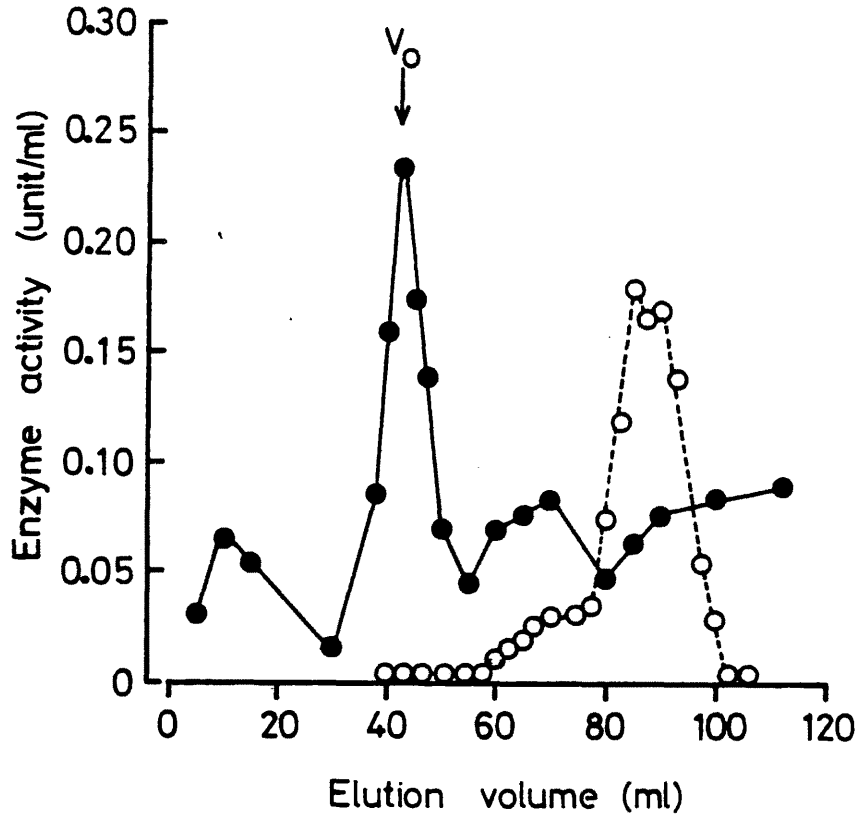


FIG. 23

Elution Profile of RIDCR obtained by Sepharose 2B Gel Filtration of Crude Liver Supernatant

RIDCR activity (•) was measured in column fractions obtained from Sepharose 2B gel filtration of crude liver supernatant under the same conditions described for Fig. 18. (o) ATP citrate lyase activity; the arrow indicates the void volume (V_o). The RIDCR elution profile is from a single experiment and is typical of two.

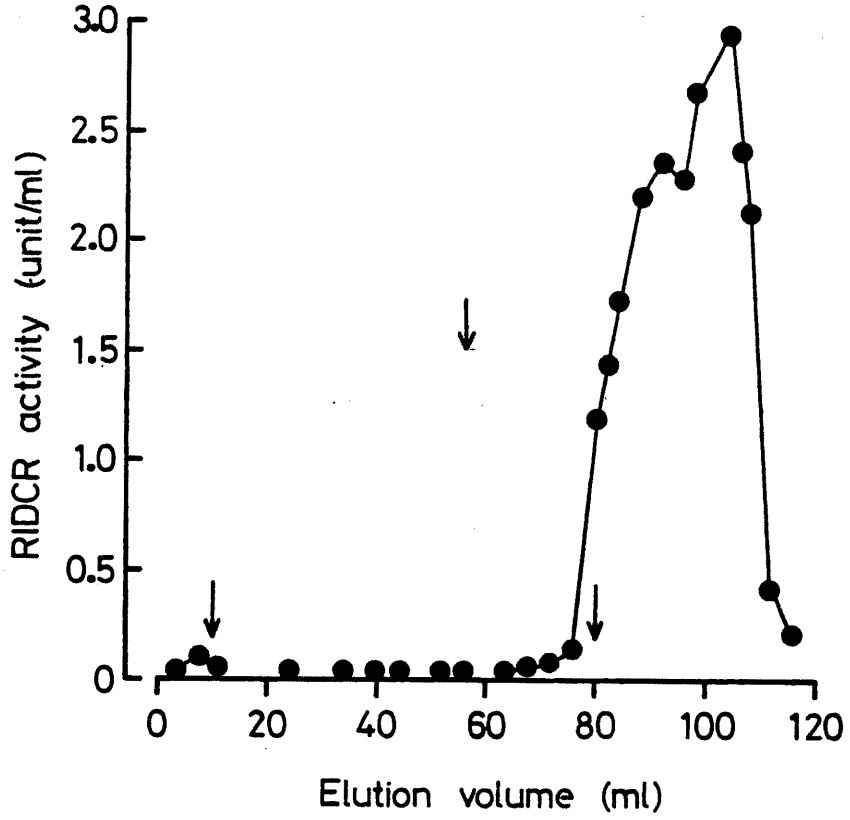


FIG. 24

Elution Profile of RIDCR obtained by DEAE-Sephadex Ion-Exchange Chromatography of Crude Liver Supernatant

RIDCR activity was measured in column fractions obtained from DEAE-Sephadex ion-exchange chromatography of dialysed liver supernatant under similar conditions to those described for Fig. 9 except that supernatants were dialysed for 24h instead of the usual 2h. The sample (8.5ml) was applied to a 1.6 x 11cm column (22ml bed volume). The two lower arrows show the positions of the two peaks of ATP citrate lyase activity i.e. the minor, non-retained peak and the major, retained peak. The raised arrow indicates the start of the linear KCl gradient (0-0.4M). The elution profile is from a single experiment.

of the inner mitochrondrial membrane enzyme succinate dehydrogenase. However, rapid reduction of the artificial electron acceptor DCPIP (2,6-dichlorophenol indophenol) present in the assay occurred in the absence of the substrate succinate. Therefore, although no succinate dehydrogenase activity would be expected in a 45,000g supernatant, due to sedimentation of the mitochondria, the presence of this high endogenous activity prevented conclusive proof of this.

Sepharose 6B column fractions containing the high molecular weight liver ATP citrate lyase were also assayed for succinate dehydrogenase. In contrast to the crude supernatants, these fractions showed no endogenous DCPIP reduction. Moreover, no succinate dehydrogenase activity was detected. Therefore, in the absence of endogenous activity, no succinate dehydrogenase activity was found in the void volume fractions. If this membrane-bound enzyme had been present in the crude supernatants it would have been eluted at the void volume of the Sepharose 6B column because of its size. These results therefore indicate that succinate dehydrogenase is not present in crude supernatants, and hence it is concluded that liver supernatants do not contain inner mitochondrial membrane fragments.

v) Subcellular Fractionation of Crude Liver Supernatants

In an attempt to study more directly the possibility of an association of liver ATP citrate lyase with mitochondria, crude mitochondrial fractions were prepared by differential centrifugation. ATP citrate lyase activity of the various supernatants and pellets obtained was determined. However, as shown in Table 12, no activity was detected in the crude mitochondrial fractions. Assay of the mitochondria-free 15,000g supernatant showed that 100% recovery of homogenate activity was obtained in this fraction. Therefore, these results indicate that the high molecular weight form of ATP citrate lyase is not the result of association of the enzyme with mitochondria.

TABLE 12

Activity of ATP Citrate Lyase and Two Marker Enzymes in Various Subcellular Fractions from Liver

Enzyme	Experiment	Total Activity Units					
		Homogenate	600g Centrifugation		15,000g Centrifugation		Crude Mitochondrial Fraction
			Supernatant	Pellet	Supernatant	Pellet	
ATP Citrate Lyase							
	1	28.4	19.7	0	19.1	0	0
	2	5.1	6.9	-	7.8	0	0
	3	11.0	11.7	-	10.9	-	0
	4	17.6	13.3	-	17.2	-	0
Lactate Dehydrogenase							
	1	5075	2784	770	2993	67	5
RIDCR							
	1	756	331	147	-	-	23
	2	420	331	-	250	55	73

TABLE 12 - Continued

Subcellular fractionation of liver homogenates was performed as described in Methods. Pellets were resuspended by homogenisation in volume of homogenising buffer equal to volume pelleted from. The crude mitochondrial fraction is the pellet obtained from the 15,000g centrifugation after two further washes, and was resuspended in 3-5ml of buffer. Results are from single experiments, and represent the total enzyme activity (units) in each fraction, determined from duplicate assays. Results for lactate dehydrogenase and RIDCR were obtained using the fractions from the corresponding experiments for ATP citrate lyase. Differences between ATP citrate lyase activity of the homogenate, and each of the supernatant fractions, are not statistically significant. Livers were from normal fed rats.

Marker enzyme assays indicated the presence of the outer mitochondrial membrane enzyme rotenone-insensitive NADH cytochrome c reductase (RIDCR) and the inner membrane enzyme, succinate dehydrogenase, in the crude mitochondrial fraction. For RIDCR, two experiments yielded 3% and 17% of the homogenate activity in the mitochondrial fraction (Table 12).The reason for the large difference between the homogenate activity of the two samples is unknown. However, the relatively large and variable background activity present in some samples (see Figs. 22 and 23) may partly explain this discrepancy. Succinate dehydrogenase activity of the mitochondrial fraction averaged 0.32 ± 0.06 units/ml for five preparations. No activity was detected in the 15,000g supernatant, but difficulties with the assay when using crude supernatant and homogenate samples, prevented determination of recovery of activity in the mitochondrial fraction. In contrast to these results, <0.1% of the homogenate activity of the cytoplasmic enzyme lactate dehydrogenase was recovered in the mitochondrial fraction.

vi) Sepharose 2B Gel Filtration of Crude Liver Supernatants Prepared in Sucrose-Free Buffer

Homogenisation of liver in buffer without sucrose was performed in an attempt to disrupt the mitochondria and thereby increase the proportion of membrane fragments in the supernatant. Thus, gel filtration of such supernatants enabled investigation of the effect of an increased mitochondrial membrane content on the amount of ATP citrate lyase activity in the high molecular weight form.

The activity of the mitochondrial matrix enzyme, citrate synthase, was 10-fold higher in supernatants prepared in the absence of sucrose, compared to control samples obtained from the same tissue (0.013 units/ml and 0.0014 units/ml respectively). Hence, this indicates that disruption of the mitochondria had occured in the sucrose-free supernatants.

However, gel filtration of such supernatants showed that there was no change in the elution profile of ATP citrate lyase (Fig.25). Comparison with results from gel filtration of extracts prepared using the routinely employed sucrose buffer indicated that there was no increase in the proportion of the total activity present in the high molecular weight form (Table 13). Furthermore, there was no significant difference between the total ATP citrate lyase activity of supernatants prepared from the same tissue in the presence or absence of sucrose (Table 13).

vii) Sepharose 6B Gel Filtration of Liver Supernatants in the Presence of Mg^{2+}

Since it has been shown that Mg^{2+} stabilises the binding of brain hexokinase to mitochondria (Rose and Warms, 1967), the effect of Mg^{2+} on the Sepharose 6B gel filtration profile of liver ATP citrate lyase was investigated by addition of 10mM $MgCl_2$ to the homogenisation and column elution buffers. As shown in Fig. 26b, rather than stabilising the high molecular weight form of ATP citrate lyase, the minor peak of the enzyme was completely lost. In contrast, the elution profile of ATP citrate lyase was not affected by the presence of Na^+, at the same ionic concentration (i.e. 20mM); liver supernatants prepared and eluted with buffer containing 20mM NaCl, contained both the high molecular weight and the tetrameric forms of the enzyme (Fig. 26a). The elution volume of the tetrameric enzyme was not affected by the presence of either Mg^{2+} or Na^+.

This loss of the high molecular weight component of liver ATP citrate lyase activity was accompanied by the disappearance of an A_{280nm} peak, which in control experiments co-eluted with the void volume peak of enzyme activity. ATP citrate lyase was unaffected by the presence of Mg^{2+} in the homogenising buffer, or by addition of Mg^{2+}

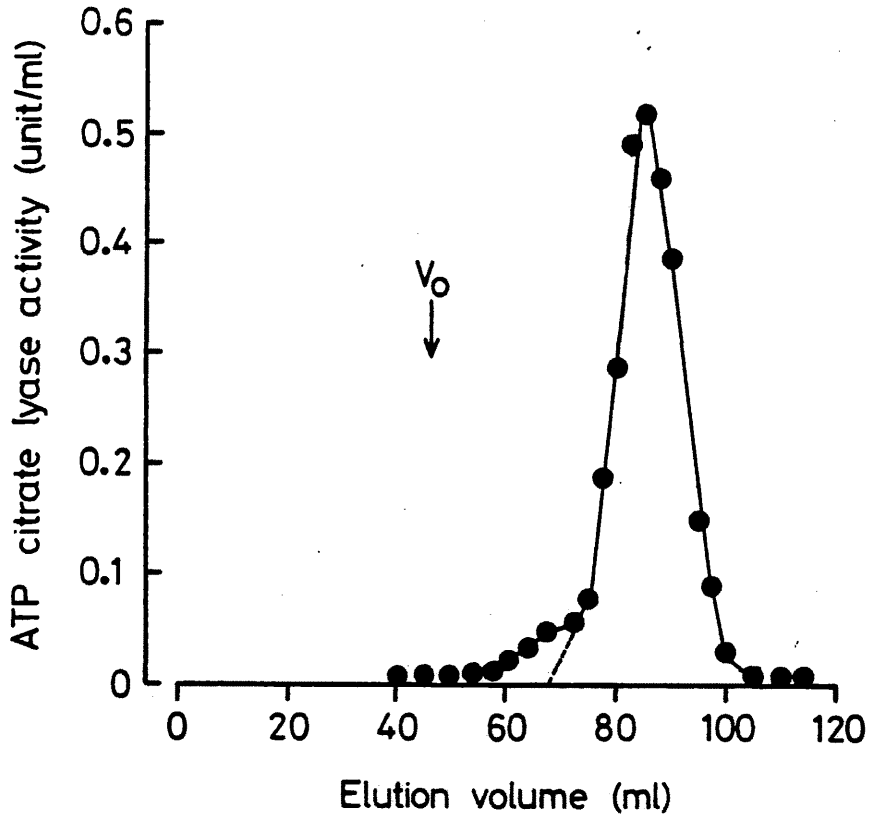


FIG. 25

Sepharose 2B Gel Filtration of Crude Liver Supernatant Prepared in Sucrose-Free Buffer

Crude supernatant was prepared from liver of a starved and refed rat using homogenising buffer without sucrose. The sample (5ml) was applied to a Sepharose 2B column, 2.26 x 27.5cm (110ml bed volume), and was eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol. The arrow indicates the void volume (V_o), and the broken line represents the separation of the two components of the enzyme activity which is drawn according to the symmetry of the major component. The elution profile is from one experiment and is typical of three.

TABLE 13

Effect of Supernatant Preparation in Sucrose-Free Buffer on ATP Citrate Lyase Activity and Recovery of High Molecular Weight Form from Sepharose 2B Gel Filtration

	+ sucrose	- sucrose
Supernatant Activity	0.84 ± 0.11 (6)	0.94 ± 0.15 (6)
% Recovered Activity in High MW Peak	7.2 ± 1.6 (3)	5.7 ± 1.7 (3)

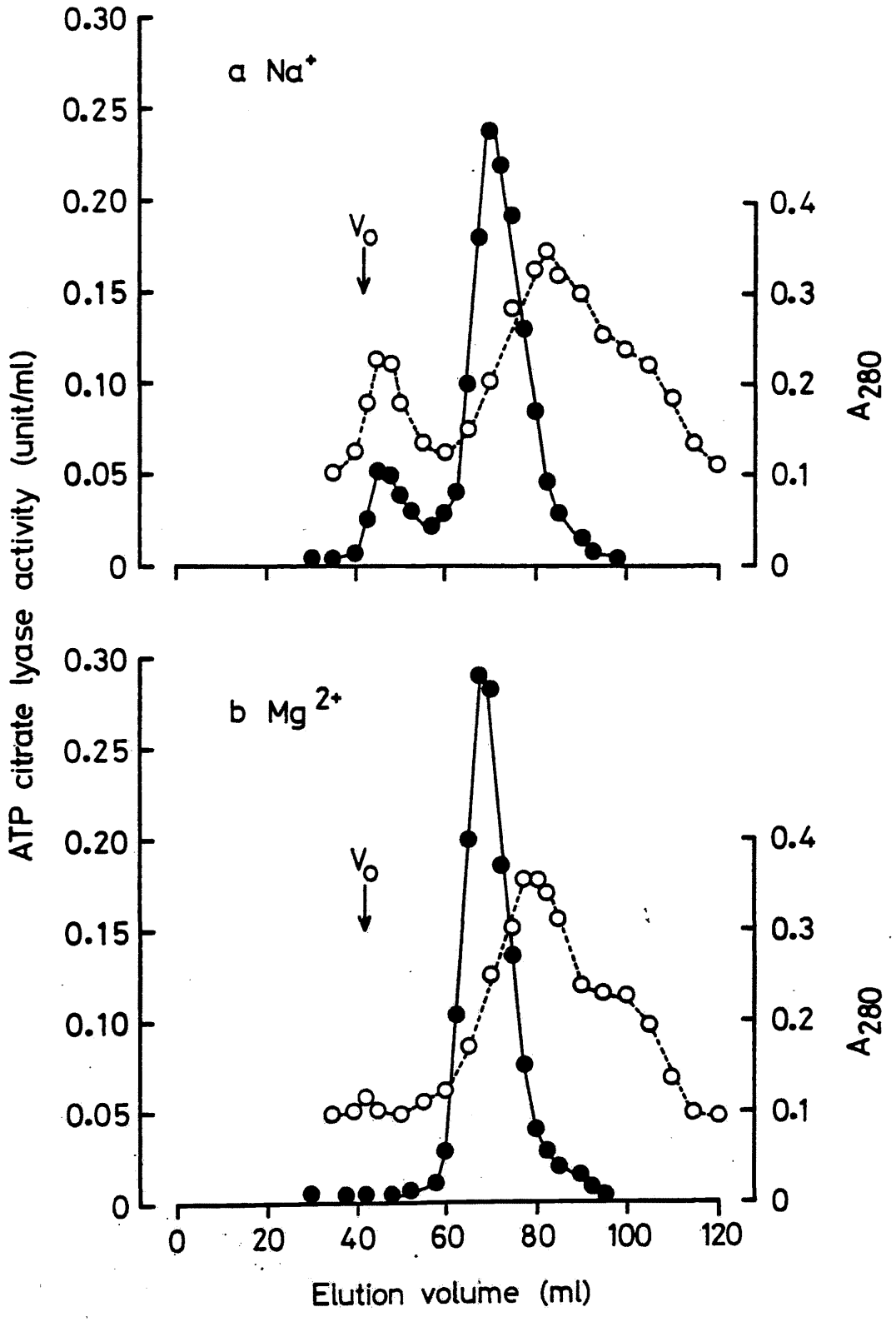
Livers from starved and refed rats were minced separately with scissors and then each was divided into two portions which were homogenised in 4 vol. 20 mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol, either with or without sucrose (0.25M). Homogenates were centrifuged at 2,000g for 10 min, and the supernatants obtained were centrifuged at 45,000g for 30 min. ATP citrate lyase activity of the 45,000g supernatants was determined as described in Methods. Results are means ± SEM for 6 separate determinations, using one liver for each.

Supernatants prepared as above were applied to a Sepharose 2B gel filtration column (2.26 x 27.5cm, bed volume 110ml), and eluted with homogenising buffer without sucrose. The percentage of the total recovered activity which was eluted as the high molecular weight form of the enzyme was calculated from the symmetry of the major peak (see Fig. 18). Results are means ± SEM for 6 separate experiments, 3 with supernatants prepared in sucrose buffer and 3 with supernatants prepared in sucrose-free buffer.

FIG. 26

Effects of Mg^{2+} and Na^{+} Ions on the Elution of Liver ATP Citrate Lyase from Sepharose 6B

Crude supernatants were prepared from livers of starved and refed rats using homogenising buffer containing either a) 20mM Na^{+} or b) 10mM Mg^{2+}. Supernatants (5ml) were applied to Sepharose 6B columns, 2.26 x 27.5cm (110ml bed volume), and were eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and a) 20mM Na^{+} or b) 10mM Mg^{2+}. 2.5ml fractions were collected into 0.125 vol. 1M Tris/HCl buffer pH 8.0, containing 10mM dithiothreitol and either 20mM Na^{+} or 10mM Mg^{2+}. ● ATP citrate lyase activity; o absorbance at 280nm, measured by dilution of fractions 1:9 (v/v) with distilled water. The arrow indicates the void volume (V_o). The elution profiles are from two separate experiments and are typical of two for each ion.


a Na+
b Mg 2+
Vo
Vo
ATP citrate lyase activity (unit/ml)
A280
A280
Elution volume (ml)
0.30
0.25
0.20
0.15
0.10
0.05
0
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120

(10mM) to supernatants prepared in the absence of Mg^{2+} (Table 14). In addition, no enzyme activity could be detected in resuspended pellets from the homogenate centrifugation. Hence, the evidence suggests that in the presence of Mg^{2+}, the associated state of ATP citrate lyase is disrupted, releasing the tetrameric enzyme.

viii) Effect of Prolonged Dialysis of Liver Supernatants on Ion-Exchange Chromatography Elution Profile of ATP Citrate Lyase

Crude liver supernatants were routinely dialysed for about 2h against 30-50 vol. homogenisation buffer prior to DEAE-Sephadex ion-exchange chromatography. When this dialysis period was increased to 24h, with no change of dialysis buffer, ATP citrate lyase from liver of normal fed rats was still eluted from the DEAE-Sephadex column as two peaks of activity; a basic, non-retained peak and an acidic peak eluted by the gradient. However, comparison with the elution profiles obtained from chromatography of 2h dialysed supernatants, revealed a large increase in the proportion of activity in the basic peak. Thus, 40% of the recovered activity was eluted as the basic, non-retained form of the enzyme (Table 15 exp.1), compared to an average 14% obtained using supernatants from normal fed rats dialysed for only 2h (see Table 5). The high recovery of activity (176%) which accompanied this increase in the first-eluted peak suggested that some form of enzyme activation had taken place, rather than a simple transfer of enzyme from the acidic to the basic peak. Moreover, no increase in the dialysed supernatant activity was detected.

Further experiments revealed that this apparent activation phenomenon also occurs with ATP citrate lyase from starved and refed rats (Table 15, exps. 2 and 3; Fig. 27). Hence, after prolonged dialysis a 3-fold increase in the activity of the basic peak was observed when

TABLE 14

Effect of Mg^{2+} on Liver ATP Citrate Lyase Activity

Experiment	ATP Citrate Lyase Activity Units/ml	
	$-Mg^{2+}$	$+Mg^{2+}$
1	1.0 ± 0.2	1.0 ± 0.2
2	0.83 ± 0.19	0.91 ± 0.21

Livers from 4 starved and refed rats were minced separately with scissors, and each divided into two portions which were then homogenised in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, with or without 10mM $MgCl_2$. Homogenates were centrifuged at 2,000g for 10 min and the supernatants obtained were centrifuged at 45,000g for 30 min. ATP citrate lyase activity of the final supernatants were determined as described in Methods (experiment 1). The supernatants prepared in the absence of $MgCl_2$ (from experiment 1) were assayed for enzyme activity after addition of 10mM $MgCl_2$, or for controls, an equal volume of buffer without $MgCl_2$ (experiment 2). Results are means $\pm$ SEM for the 4 experiments.

TABLE 15

Effect of 24h Dialysis of Crude Liver Supernatants on ATP Citrate Lyase Activity in the Two Peaks observed by DEAE-Sephadex Ion-Exchange Chromatography

Experiment	ATP Citrate Lyase Activity Units		% Total Recovery	ATP Citrate Lyase Activity Units		% Total Recovered in Peak 1
	Applied	Recovered		Peak 1	Peak 2	
1. Normal fed						
24h	1.7	3.0	176	1.2	1.8	40
2. Starved/refed						
2h	0.78	1.0	128	0.050	0.95	5.0
24h	0.75	1.1	147	0.17	0.90	16
3. Starved/refed						
2h	0.86	0.92	107	0.055	0.87	6.0
24h	0.86	0.80	93	0.15	0.65	19
4. Starved/refed						
2h	2.6	2.2	85	0.67	1.5	31
24h	2.7	1.9	70	0.63	1.3	33

TABLE 15 - Continued

For each experiment, one rat liver was homogenised in 4 vol. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, and the homogenate was centrifuged at 45,000g for 30 min at 4°C. Samples of the supernatants obtained were then dialysed against 50 vol. homogenising buffer at 4°C for 2h or 24h, with no change of dialysis buffer. The dialysed supernatants were applied to DEAE-Sephadex columns (experiments 1 and 4, 1.6 x 11cm, bed volume 22ml; experiments 2 and 3, 0.9 x 5cm, bed volume 3 ml), and eluted as described in Methods. Sample volumes were as follows: exp 1, 8.5ml; exp 2, 0.5ml; exp 3, 1.5ml; exp 4, 2.0ml. Results are from single experiments.

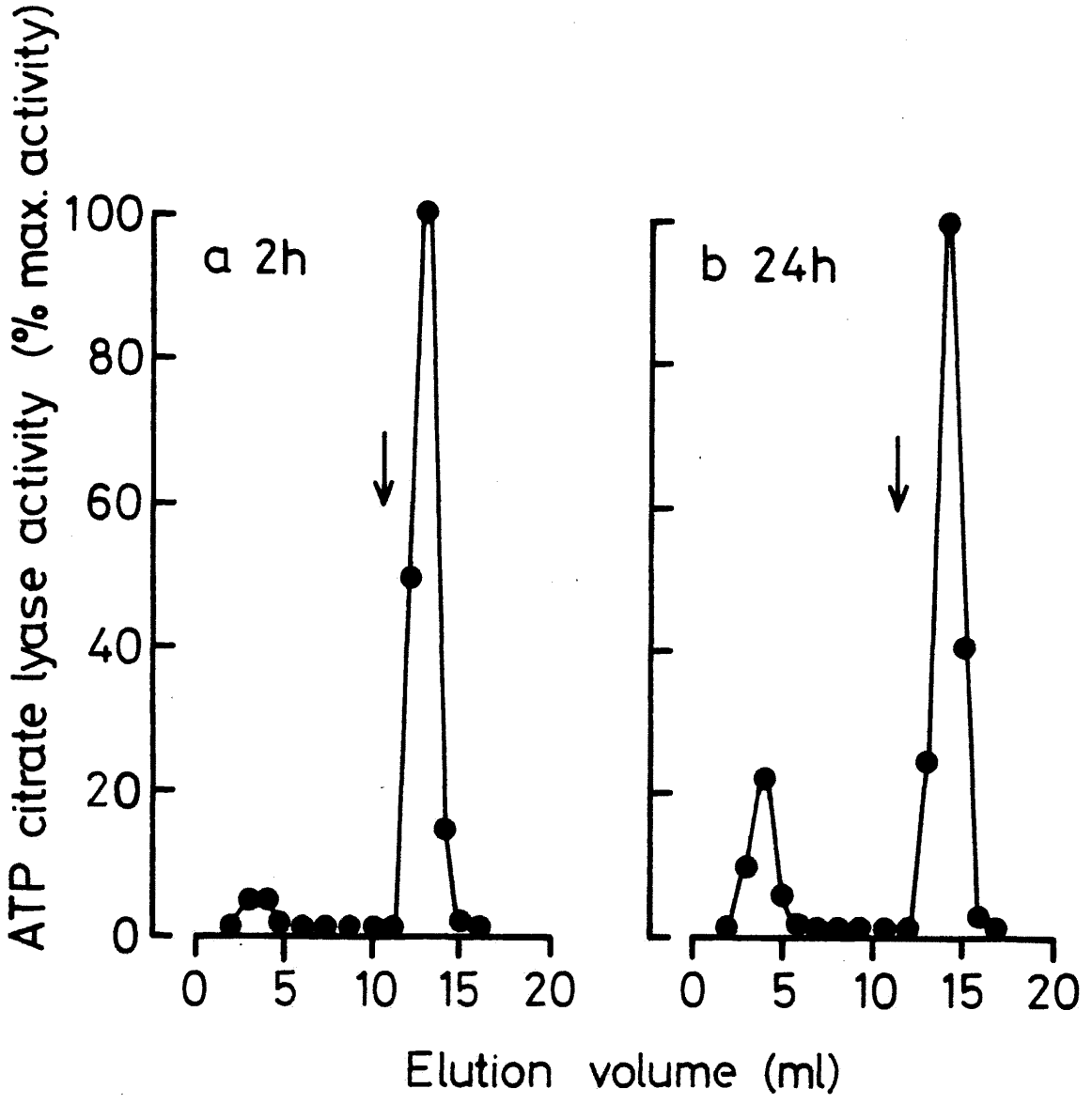


FIG. 27

Effect of 24h Dialysis of Crude Liver Supernatant on the Elution of ATP Citrate Lyase from a DEAE-Sephadex Column

The elution profiles shown are those obtained for experiment 3, Table 15. The crude supernatant samples were dialysed for a) 2h and b) 24h against homogenising buffer. The arrows indicate the start of the salt gradients.

compared to results from chromatography of the same supernatant dialysed for only 2h. In addition, there was a small decrease in the activity of the acidic peak which suggests that the possibility of conversion of the enzyme from one form to the other cannot be excluded. Moreover, the total recovery of activity was not exceptionally high in these experiments but this may be explained by the use of only small amounts of activity. Although the percentage of recovered activity in the basic peak was low for the control experiments (i.e. 6% compared to average value of 14%), it has previously been found that results from duplicate columns are very reproducible. Therefore, the increase in the proportion of recovered activity eluted in the basic peak after 24h dialysis is not thought to be due to variation between columns.

As shown in Table 15, experiment 4, the increase in the activity of the basic peak could not always be demonstrated. However, it is noteworthy that in this experiment, the proportion of recovered activity in the non-retained peak was high (30%) for both the control and the 24h dialysed supernatants.

These results suggests that under certain conditions specific activation of the basic, non-retained form of ATP citrate lyase occurs. This phenomenon was only apparent after partial purification of the enzyme on DEAE-Sephadex; no increase in activity was detected in the dialysed supernatants. Incubation of DEAE-Sephadex column fractions containing either the basic or acidic form of ATP citrate lyase with 5mM dithiothreitol at 4°C overnight did not produce any increase in enzyme activity. This therefore indicates that activation is not an artefact due to enzyme reactivation by the sulphydryl reagent.

5. Inhibition of ATP Citrate Lyase Activity by L-Glutamate

Inhibition of rat liver and brain ATP citrate lyase activity by L-glutamate was reported by Szutowicz et al. (1974a). They found

that the susceptibility of the enzyme to such inhibition was dependent on the tissue, and on the age and dietary condition of the animals used. This was the first report of any difference between the liver and brain enzyme and was therefore investigated in this study.

i) Effect of Glutamate on ATP Citrate Lyase of Crude Supernatants

ATP citrate lyase activity in crude supernatants of liver and brain was measured after a 30 min preincubation at 37°C in the presence or absence of 10mM glutamate (Table 16). Using the standard assay (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), glutamate was found to inhibit the liver enzyme from normal fed, and 48h starved rats, by 60%. A similar degree of inhibition was observed for brain ATP citrate lyase from adult normal fed rats. However, the enzyme from liver of starved and refed rats was inhibited by only 36%, and the brain enzyme from newborn rats by 11%.

The effect of glutamate on ATP citrate lyase activity was also determined using the modified assay (1mM ATP, 2mM citrate, 0.2mM CoA and 13mM $MgCl_2$) employed by Szutowicz et al. (1974a). In control assays preincubated for 30 min in the absence of glutamate, enzyme activity was only about 40% of the corresponding values using the standard assay. However, the inhibitory effect of glutamate was more pronounced under these changed conditions. Thus, inhibition of the liver enzyme from rats of all three dietary conditions, was twice that observed using the standard assay in many cases, and a 3-fold increase in the degree of glutamate inhibition was found for the newborn rat brain enzyme. In contrast, inhibition of ATP citrate lyase from adult rat brain was not significantly different using this modified assay compared to the standard assay.

Szutowicz et al. (1974a), using partially purified ATP citrate lyase (40-fold purification), reported inhibition of the enzyme from liver of

TABLE 16

Inhibition of ATP Citrate Lyase Activity of Crude Supernatants by 10mM L-Glutamate

Crude Supernatant	% Inhibition by L-Glutamate		
	Standard Assay	Modified Assay	Szutowicz et al. (1974a)
Liver			
Normal fed	60.4 ± 6.3 (5)	100 ± 0 (3)***	-
Starved/refed	35.6 ± 1.4 (7)*	55.5 (2)***	+3
Starved	59.5 (2)	100 (2)	38
Brain			
Adult a	57.0 ± 1.0 (3)	64.0 (1)	
b	57.3 ± 5.5 (3)	63.0 ± 2.5(3)	79
Newborn	10.5 (2)**	37.5 (2)	+1

TABLE 16 - Continued

* Significantly different from all other supernatants, standard assay $p < 0.05$ (Mann-Whitney U test).

** Significantly different from liver, normal fed, and starved/refed, and from adult brain (a+b), standard assay $p < 0.05$ (Mann-Whitney U test).

*** Significantly different from standard assay $p < 0.05$ (Mann-Whitney U test).

\+ Indicates % increase in activity.

Crude supernatants, except adult brain, b, were obtained by centrifugation of the tissue in 4 vol. (liver and newborn brain) or 2 vol. (adult brain, a) of 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose, followed by centrifugation of the homogenates at 45,000g for 30 min at 4°C. The second method used for preparation of crude supernatants of adult brain (b) was the same as that used by Szutowicz et al. (1974a); brains were homogenised in 2 vol. 5mM Tris/HCl pH 7.4, containing 0.2M KCl and 1mM dithiothreitol, followed by centrifugation of homogenates at 30,000g for 30 min at 4°C. ATP citrate lyase activity was measured at 37°C as described in Methods, using either the standard assay conditions (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), or the modified assay conditions (1mM ATP, 2mM citrate, 0.2mM CoA and 13mM $MgCl_2$). Assays were preincubated for 30 min at 37°C in the presence or absence of 10mM glutamate. Results are means $\pm$ SEM for the number of determinations in brackets; the results of Szutowicz et al. (1974a), which were obtained under the modified assay conditions, are shown for comparison.

starved rats, and from brain of adult rats by glutamate. However, they found no inhibition of the liver ATP citrate lyase from starved and refed rats, or the enzyme from newborn rat brain (see Table 16). Furthermore, the degree of inhibition observed for the adult brain enzyme was twice that for the liver enzyme from starved rats.

Therefore, although inhibition of ATP citrate lyase by glutamate has been demonstrated, as shown in Table 16, the results do not agree quantitatively with those of Szutowicz and co-workers. However, in addition to the fact that only crude tissue extracts were used, compared to the partially purified samples used by Szutowicz, the supernatants were also prepared by a different method (procedure of Hoffmann et al., 1979b - see Methods Section 5).

ii) Effect of Glutamate on the Different Chromatographic Forms of ATP Citrate Lyase

The effect of glutamate on ATP citrate lyase activity was further investigated using the various chromatographic forms of the enzyme. Liver ATP citrate lyase eluted from DEAE-Sephadex columns by the salt gradient (major peak) retained its susceptibility to inhibition by glutamate (Table 17). The enzyme from both normal fed, and starved and refed rats, was inhibited by about 50% using the modified assay. In contrast however, the enzyme of the non-retained peak was not significantly affected by glutamate. For the adult brain enzyme, both the basic, non-retained, and the acidic, retained forms of ATP citrate lyase were inhibited by 65-70% in the presence of glutamate (modified assay).

Glutamate also inhibited the major tetrameric form of the liver enzyme eluted from Sepharose 6B and Sepharose 2B gel filtration columns (Table 18). The low levels of the high molecular weight enzyme activity prevented accurate determination of the effect of glutamate

TABLE 17

Inhibition by 10mM L-Glutamate of ATP Citrate Lyase Activity Eluted from DEAE-Sephadex Ion-Exchange Columns

Source of Enzyme	% Inhibition by L-Glutamate			
	Activity in Non-Retained Peak		Activity in Adsorbed Peak	
	Standard Assay	Modified Assay	Standard Assay	Modified Assay
Liver				
Normal fed	5.0 ± 9.1 (4)	7.7 ± 3.5 (3)	41.2 ± 8.0 (5)*	51.0 ± 3.3 (5)*
Starved/refed	11.3 (2)	5.5 (2)	24.0 (2)	49.5 (2)
Brain				
Adult	56.5 (2)	64.5 (2)	66.0 (2)	69.5 (2)

* Significantly different from corresponding assay of non-retained peak activity

$p < 0.02$ (Mann-Whitney U test).

DEAE-Sephadex ion-exchange chromatography of liver and brain supernatants was performed as described in Methods. ATP citrate lyase activity in fractions containing maximum activity of each peak was determined after 30 min preincubation at 37°C in the presence or absence of 10mM glutamate. Enzyme activity was measured using either the standard assay (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), or the modified assay (1mM ATP, 2mM citrate, 0.2mM CoA and 13mM $MgCl_2$). Results are means ± SEM of the percentage inhibition of enzyme activity by glutamate, for the number of determinations in brackets.

TABLE 18

Inhibition by 10mM L-Glutamate of Liver ATP Citrate Lyase Activity Eluted from Sepharose Gel Filtration Columns

Source of Enzyme	% Inhibition of Tetrameric ATP Citrate Lyase by L-Glutamate	
	Standard Assay	Modified Assay
Sepharose 6B		
Normal fed	21.0 ± 3.1 (3)	42.3 ± 9.4 (3)*
Starved/refed	-	35.5 (2)
Starved	25.5 (2)	43.0 (2)
Sepharose 2B		
Starved/refed	22.0 (2)	50.5 (2)

* Significantly different from standard assay $p = 0.05$ (Mann-Whitney U test).

Sepharose 6B and Sepharose 2B gel filtration of crude liver supernatants was performed as described in Methods. ATP citrate lyase activity in fractions containing the maximum activity of the major, tetrameric form of the enzyme was determined after 30 min preincubation at 37°C in the presence or absence of 10mM glutamate. Enzyme activity was measured using either the standard assay (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), or the modified assay (1mM ATP, 2mM citrate, 0.2mM CoA and 13mM $MgCl_2$). Results are means ± SEM of the percentage inhibition of enzyme activity by glutamate, for the number of determinations in brackets.

in most cases. However, it appeared to have no effect on this associated state of the enzyme.

iii) Investigation of the Nature of the ATP Citrate Lyase Inhibition by Glutamate

a) Effect of Preincubation Time

For determination of ATP citrate lyase inhibition by glutamate, assays were preincubated for 30 min in the presence of glutamate, prior to starting the enzyme reaction by the addition of CoA. This procedure was the method employed by Szutowicz et al. (1974a), who reported that the inhibition was time dependent. They observed maximum inhibition of the enzyme after a 60 min preincubation,but routinely used a 30 min preincubation period which resulted in a 20% decrease in the inhibition. Therefore, to enable comparison of results the 30 min preincubation was also used in this study.

The time-dependent nature of the enzyme inhibition by glutamate was observed with crude supernatants of liver and brain (Fig. 28). The degree of inhibition was increased between 1.5 - 5.5-fold when the preincubation time was increased from the routinely used 7 min to 30 min. Hence, this time-dependency of the inhibition observed for partially purified samples of the brain enzyme by Szutowicz et al. (1974a), also appears to be present in crude supernatants.

Control assays performed in the absence of glutamate indicated that the increased preincubation time did not significantly affect ATP citrate lyase activity except for the liver enzyme assayed by the modified assay (Table 19). Supernatants from normal fed, starved, and starved and refed, consistently showed lower activity after the prolonged preincubation period, with an average decrease in activity of 62%. ATP citrate lyase activity of the same samples measured under the standard assay conditions was also reduced after 30 min preincubation in some

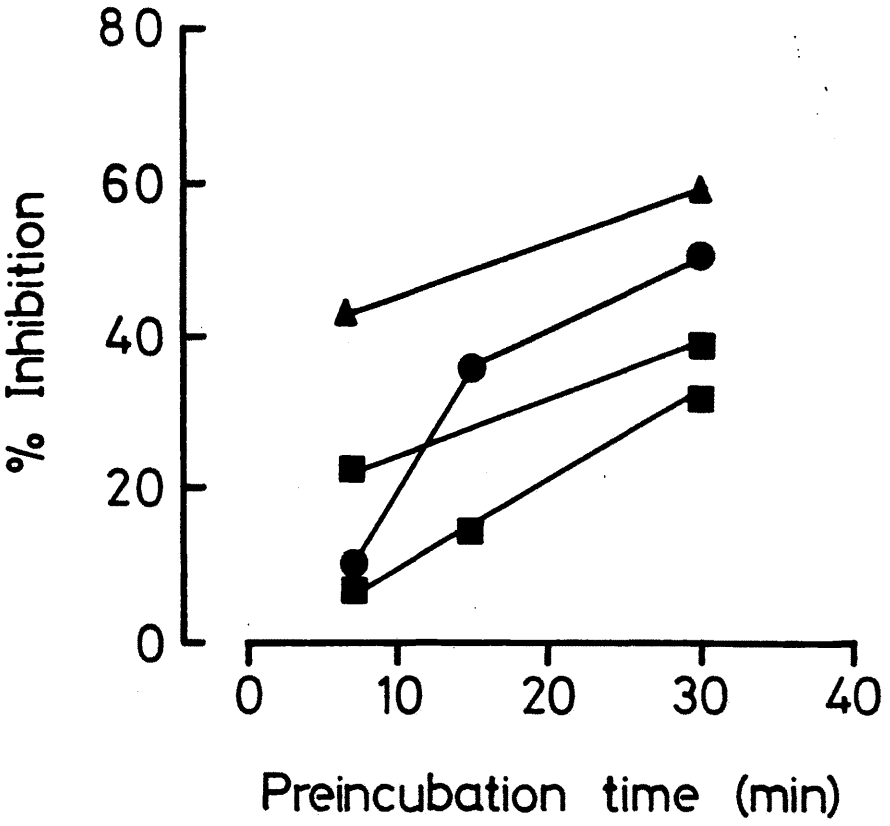


FIG. 28

Effect of Preincubation Time on the Inhibition of Liver ATP Citrate Lyase Activity by L-Glutamate

Crude supernatants of liver and brain were prepared as described in Methods. ATP citrate lyase activity was measured in the presence and absence of 10mM glutamate. Assays were preincubated at 37°C with or without glutamate for various times as indicated. The reaction was then started by the addition of CoA, and the inhibition of enzyme activity by glutamate compared to controls preincubated for the same time. Results are from single experiments; ● supernatant from normal fed rat; ■ supernatant from starved and refed rat; ▲ supernatant from adult rat brain.

TABLE 19

Effect of Increased Preincubation Time on ATP Citrate Lyase Activity of Crude Liver Supernatants

Crude Supernatant	Standard Assay			Modified Assay		
	Preincubation Time min		% Change	Preincubation Time min		% Change
	7	30		7	30	
Normal fed	0.15	0.092	-39	0.040	0.0081	-80
	0.083	0.064	-23	0.017	0.0071	-58
Starved/refed	0.16	0.17	+6	0.069	0.035	-49
	0.49	0.37	-24	0.19	0.097	-49
Starved	0.036	0.032	-11	0.012	0.0061	-49
	0.051	0.041	-20	0.012	0.0015	-88
Average % change			-19 ± 6			-62 ± 7*

* Significantly different from standard assay p = 0.05 (Wilcoxin test)

TABLE 19 - Continued

Crude liver supernatants were prepared by the routine method described in Methods. ATP citrate lyase activity of the supernatants was determined at 37°C using either the standard assay (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), or the modified assay (1mM ATP, 2mM citrate, 0.2mM CoA and 13mM $MgCl_2$). Assays were preincubated at 37°C for 7 or 30 min. Results are from single experiments, and each value is the average of duplicate assays. The % change is the percentage change in activity after 30 min preincubation compared to control assays preincubated for 7 min; the average % change shown is the average obtained from all experiments.

cases, but the average decrease was only 19%.

b) Effect of Inhibitors

The inhibition of liver ATP citrate lyase activity by glutamate was measured in the presence of a transaminase inhibitor, aminooxyacetate (Hopper and Segal, 1962). Addition of 2mM aminooxyacetate to the preincubation mixture reduced the inhibition of enzyme activity by glutamate (Table 20). Thus, for crude supernatants, the degree of inhibition of the enzyme was 50% lower in the presence of the transaminase inhibitor; for the partially purified enzyme (activity eluted by the salt gradient from DEAE-Sephadex), only a 20% decrease was observed. This reduction in the inhibitory effect of glutamate on ATP citrate lyase suggests that transamination of glutamate may be partly involved in the enzyme inhibition. When the preincubation time was decreased from 30 min to 15 min, the lower degree of inhibition of the enzyme by glutamate observed with control samples, was also observed when aminooxyacetate was present. Thus the transaminase inhibitor did not appear to have any appreciable effect on the time-dependency of the inhibition by glutamate.

The presence of the protease inhibitor phenylmethylsulphonyl fluoride (0.2mM) appeared to have no effect on the inhibition of liver ATP citrate lyase by glutamate. In a single experiment, liver supernatants were prepared from liver of one starved and refed rat, either in the presence or absence of phenylmethylsulphonyl fluoride. Glutamate inhibited the ATP citrate lyase of both supernatants by 33% when measured under the standard assay conditions (results not shown).

c) Effect of Sephadex Gel Filtration

In order to investigate the possibility that the inhibition of ATP citrate lyase by glutamate requires the presence of one or more low molecular weight cellular components, crude supernatants of liver

TABLE 20

Effect of Aminooxyacetate (2mM) on the Inhibition of Liver ATP Citrate Lyase Activity by L-Glutamate

Experiment	Sample	Preincubation Time min	% Inhibition by Glutamate	
			-AO	+AO
1	Crude Supernatant	15	36	17
		30	51	30
	DEAE-Sephadex column fraction	15	14	12
		30	45	33
2	Crude Supernatant	30	50	18

The inhibition of ATP citrate lyase activity by glutamate was determined in the presence and absence of 2mM aminooxyacetate (AO) which was added to the preincubation mixture. Assays were preincubated for 15 or 30 min as indicated, in the presence or absence of 10mM glutamate. The reaction was started by the addition of CoA, and ATP citrate lyase activity was measured using the standard assay conditions (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$). Crude liver supernatants were prepared by the routine method (see Methods) from normal fed rats. In experiment 1, the crude supernatant was applied to a DEAE-Sephadex ion-exchange column, and the 'DEAE-Sephadex column fraction' represents ATP citrate lyase eluted from the column by the salt gradient. Results are from two experiments as shown and all assays were performed in duplicate.

and brain were applied to Sephadex G25 or Sephadex G100 gel filtration columns. As shown in Table 21, ATP citrate lyase eluted from such columns was inhibited by the same amount as the enzyme of the crude supernatants, with the possible exception of the brain enzyme eluted from the Sephadex G100 column; in this case the degree of inhibition by glutamate appeared to be reduced by gel filtration. Sephadex G100 gel filtration of both liver and brain supernatants did not affect the time-dependent nature of the enzyme inhibition. Therefore, removal of low molecular weight components of crude tissue extracts does not appear to have any appreciable effect on the inhibition of either liver or brain ATP citrate lyase by glutamate.

d) Effect of Supernatant Dilution

Crude supernatants of liver from starved and refed rats were diluted with homogenising buffer, and the inhibition of ATP citrate lyase activity by glutamate was determined for each dilution. It was found that as the supernatant concentration was decreased (i.e. with increasing dilution), the degree of inhibition of the enzyme also decreased (Fig. 29) It could, in fact, be completely abolished by sufficient dilution. This reduction and eventual removal of the inhibitory effect of glutamate by supernatant dilution indicates that a direct allosteric interaction is not involved since this would not be affected by the enzyme concentration. Furthermore, the complete loss of the inhibition provides strong evidence against such a mechanism.

TABLE 21

Effect of Sephadex G25 and G100 Gel Filtration of Crude Liver and Brain Supernatants on Inhibition of ATP Citrate Lyase Activity by 10mM L-Glutamate

Experiment	Sample	Preincubation Time min	% Inhibition by Glutamate
1. Liver	Crude Supernatant	30	33
	G25 fraction	30	26
2. Liver	Crude Supernatant	30	39
	G100 fraction	30	36
		7	11
3. Brain	Crude Supernatant	30	57
	G25 fraction	30	59
4. Brain	Crude Supernatant	30	59
	G100 fraction	30	37
		7	23

Crude supernatants of liver and brain, prepared by the routine method, were applied to Sephadex G25 or Sephadex G100 gel filtration columns and eluted with 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA and 1mM dithiothreitol. Inhibition of ATP citrate lyase activity by 10mM glutamate was measured under standard assays conditions (5mM ATP, 20mM citrate, 0.2mM CoA and 10mM $MgCl_2$), using samples of the crude supernatants, or column fractions containing maximum enzyme activity. Results are from single experiments and are averages of duplicate assays; liver was obtained from starved and refed rats, brain was from normal fed rats. Experiments 2 and 4 were performed with the same Sephadex G100 column, bed volume 13ml (1.6 x 6.5cm), sample volumes 2ml and 1.5ml respectively; experiment 1, Sephadex G25 column, bed volume 19ml (1.6 x 9.5cm), sample volume 4ml; experiment 3, Sephadex G25 column, bed volume 19ml (1.6 x 9.5cm), sample volume 4ml.

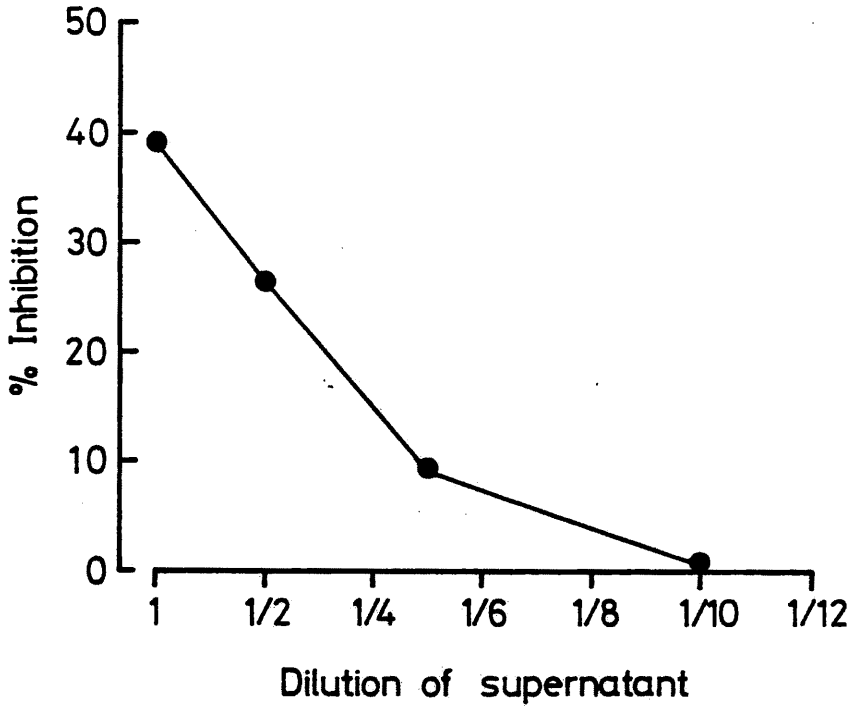


FIG. 29

Effect of Supernatant Dilution on the Inhibition of Liver ATP Citrate Lyase Activity by L-Glutamate

Crude supernatant was prepared from liver of a starved and refed rat as described in Methods, and was diluted as indicated with homogenising buffer i.e. 20mM Tris/HCl buffer pH 8.0, containing 1mM EDTA, 1mM dithiothreitol and 0.25M sucrose. 50µl samples of each dilution were added to assays and preincubated for 30 min at 37°C in the presence or absence of 10mM glutamate. ATP citrate lyase activity was determined at 37°C using the standard assay conditions (5mM ATP, 20mM citrate, 0.2mM CoA, 10mM $MgCl_2$). The dilution of the supernatant is expressed as the volume of supernatant/total volume. Results are from one experiment and are typical of three.

DISCUSSION

1. ATP Citrate Lyase Assays

i) Spectrophotometric Assay

ATP citrate lyase activity was assayed using the coupled spectrophotometric assay which was first developed by Srere (1959), and is the most widely used method. One of the main advantages of this assay is the relatively high sensitivity; under the conditions used in this study, rates corresponding to as little as 3×10^{-4} µmoles NADH oxidised per min could be detected. Furthermore, the reaction can be followed continuously in the cuvette, thus providing a relatively quick and easy procedure. However, the major disadvantage was the non-specific NADH oxidation which occurred, resulting in a background reaction in the absence of any one of the substrates. This problem was overcome by measuring the activity of NADH oxidation prior to starting the ATP citrate lyase reaction, and subtracting the result from the rate obtained with the complete reaction mixture. Although this proved to be satisfactory in most cases, determination of brain ATP citrate lyase activity in crude supernatants presented some difficulties due to the high non-linear nature of the background.

Under certain conditions, assays of ATP citrate lyase activity are subject to interference by the enzyme citrate synthase. This enzyme catalyses the formation of citrate from acetyl CoA and oxaloacetate and is therefore the reverse of the ATP citrate lyase reaction. There are several reports in the literature where such interference has been found and appropriate correction was necessary (Hayashi and Kato, 1978; Sterri and Fonnum, 1980; Szutowicz et al., 1977; Tucek, 1967b). ATP citrate lyase activity was determined by various different assays and correction for

reverse citrate synthase activity was made by performing the assay in the absence of ATP and/or Mg^{2+}.

The effect of citrate synthase activity on the apparent ATP citrate lyase activity of a sample would depend on the direction of the reaction catalysed by the enzyme.

$$\text{acetyl CoA + oxaloacetate} \underset{2}{\overset{1}{\rightleftharpoons}} \text{citrate + CoA}$$

Hence, if the enzyme was active in the forward direction (1), the ATP citrate lyase detected would be lower than the true activity, since oxaloacetate produced by the latter reaction would be utilised by citrate synthase, rather than the coupling enzyme of the assay, malate dehydrogenase. Conversely, if citrate synthase was active in the reverse direction (2), this would increase the apparent ATP citrate lyase activity detected, as production of oxaloacetate by both enzymes would lead to increased NADH oxidation.

However, the possibility of interference by this enzyme is unlikely in the crude liver and brain extracts used in this study. Citrate synthase is an exclusively mitochondrial enzyme, and it cannot be assayed using intact mitochondria. Since the crude supernatants were obtained by centrifugation of homogenates at 30,000g - 45,000g, all the mitochondria should have been pelleted. Indeed, assay of liver supernatants for citrate synthase activity revealed that they contained negligible activity even after sonication to disrupt any mitochondria present. Supernatants prepared in the absence of isotonic sucrose however contained 10-fold higher activity, indicating the release of the enzyme caused by disruption of the mitochondria in the hypotonic buffer. Nevertheless, the conditions of the assay itself would make interference by citrate synthase unlikely even if it was present. The

presence of excess exogenous malate dehydrogenase prevents the products of the ATP citrate lyase reaction, acetyl CoA and oxaloacetate, being available for citrate synthase acting in the forward direction. Furthermore, the citrate synthase reaction is inhibited by ATP (Shepherd and Garland, 1966), which is present in all assays as a substrate for ATP citrate lyase. It is noteworthy that in all the literature reports of citrate synthase interference, ATP citrate lyase was measured in subcellular fractions of brain to which detergent had been added to solubilise mitochondrial enzymes. Hence, citrate synthase would be present in such preparations.

Another possible source of error in determination of ATP citrate lyase activity would be loss of activity due to oxidation of essential sulphydryl groups on the enzyme. Hence, the sulphydryl reagent dithiothreitol was routinely added to all homogenisation and column buffers prior to use. In addition, further protection was provided by the presence of mercaptoethanol in the enzyme assay. That ATP citrate lyase is sensitive to sulphydryl group oxidation was demonstrated by reactivation of aged samples by incubation with dithiothreitol (Fig.4), as had previously been demonstrated by Cottam and Srere (1969).

ii) Radiochemical Assay

The radiochemical assay for ATP citrate lyase was developed in an attempt to eliminate the problem of the endogenous NADH oxidation obtained with the coupled assay. Furthermore, it was hoped that it would provide a more sensitive assay which would be particularly useful for measuring the activity of the brain enzyme. However, as already discussed (see Results Section 1ii), the assay was only of limited use since it could not be used with crude supernatants of liver or brain.

The fact that the activity of the partially purified enzyme from both liver and brain could be determined, suggested that the failure of the assay when using crude extracts was due to utilisation of the [^{14}C] oxaloacetate produced during the incubation period.

In addition to the limited use of this assay, a high and variable background activity was observed. Unlike the endogenous NADH oxidation of the coupled spectophotometric assay which varied as a percentage of the total activity (Table 2), the high background count rate of the radiochemical assay showed no relation to the ATP citrate lyase activity of the sample. It was in fact independent of the presence of sample and was found to be the result of decarboxylation of the radioactive substrate, [1,5-^{14}C] citrate. Hence, the effect of this background on the total amount of radioactivity determined was increased when the ATP citrate lyase activity of the sample was low.

However, before further investigation of this background radioactivity, the sensitivity of the radiochemical assay compared to the spectrophotometric assay was considered. Under the conditions of the malate dehydrogenase coupled system (20mM citrate, 5mM ATP, 0.2mM CoA), the specific radioactivity of [^{14}C] carbon dioxide is 40-fold less than that of [1,5-^{14}C] citrate. This is due to the high citrate concentration of the assay, and the fact that only one of the two labelled carbon atoms of citrate gives rise to carbon dioxide. Therefore, taking into account this dilution of the radioactivity, and the high background count, the minimum amount of [^{14}C] carbon dioxide which could be detected was estimated to be 9×10^{-2} µmoles CO_2, using 0.2 µCi [1,5-^{14}C] citrate per assay (i.e. conditions of the assay described in Methods 3ii). This compares with a minimum detectable rate of 3×10^{-4} µmoles NADH oxidised per min for the spectrophotometric assay. Therefore,

to measure the same activity obtained with the spectrophotometric assay, the radiochemical assay would require an incubation period of 5h. Clearly, under these conditions the spectrophotometric assay is far more sensitive.

However, there are two methods by which the sensitivity of the radiochemical assay could be increased. Firstly, by reducing the large isotopic dilution of the radioactive substrate. This could be achieved most effectively by decreasing the citrate concentration from, for example, 20mM to 2mM, which would increase the specific radioactivity 10-fold. However, in view of the unusual kinetics of this enzyme with respect to citrate (Plowman and Cleland, 1967), and the possibility of its involvement in the heterogeneity of the enzyme observed in this study, such a modification was undesirable. An alternative would be to use [1-^{14}C] citrate instead of [1,5-^{14}C] citrate as the radioactive substrate, resulting in a 2-fold increase in the specific radioactivity of carbon dioxide. However, this form of labelled citrate is not commercially available.

The second way of increasing sensitivity is to increase the amount of radioactivity per assay; a 10-fold increase would enable detection of the minimum rate of ATP citrate lyase activity measured by the coupled assay, after a 30 min incubation period. Greater sensitivity could be achieved by increasing the incubation time of the assay, although this will be limited by time-period over which the assay remains linear, and by practical considerations. However, even after a 20-fold increase in the amount of radioactivity in the assay, and an incubation period of 1h, the sensitivity of the radiochemical assay would only be 5 times greater than that of the spectrophotometric assay. Therefore, considering the cost of the label, and the time involved, this increase in sensitivity was not sufficient to justify further investigation of the assay, which

was required to try to eliminate the background count, and determine the reason for the failure of the assay with crude extracts.

2. Heterogeneity of Rat Liver and Brain ATP Citrate Lyase

ATP citrate lyase has been shown to exist as multiple forms in crude extracts of rat liver and brain. The liver enzyme was found to be heterogenous in both size and ionic charge; in contrast, heterogeneity of brain ATP citrate lyase was observed only with respect to ionic charge.

i) DEAE-Sephadex Anion-Exchange Chromatography

ATP citrate lyase from both liver and brain was separated into two peaks of activity by DEAE-Sephadex ion-exchange chromatography of crude supernatants. Hence, there appear to be two charge forms of the enzyme in each of these tissues; one which was not retained by the column but was eluted immediately, and a second, which was adsorbed on the column and was eluted by the salt gradient. However, although the two peaks of brain ATP citrate lyase activity were eluted in the same respective positions to those of the liver enzyme, the distribution of recovered activity was different. Thus, whilst only a minor proportion of the liver activity was eluted in the first peak (16%), for the brain enzyme the activity was almost equally distributed between both peaks, with 40% of the recovered activity in the first-eluted peak. Therefore, a possible difference between the properties of ATP citrate lyase of liver and brain is indicated.

Further investigation of the non-retained ATP citrate lyase activity from liver indicated that it is not the result of column overloading. Moreover, proteolytic degradation of the enzyme does not appear to be involved since chromatography in the presence of

phenylmethylsulphonyl fluoride did not affect the elution profile. Phenylmethylsulphonyl fluoride inhibits the group of proteases known as the serine proteases which includes chymotrypsin and trypsin (Fahrney and Gold, 1963; Gold and Fahrney, 1964).

The basic, non-retained form of liver ATP citrate lyase was, however, found to be very unstable, and was rapidly converted to the more acidic, retained form as shown by rechromatography experiments. This therefore suggests that the observed heterogeneity of liver ATP citrate lyase does not represent the existence of distinct forms such as isoenzymes, but rather different states in which the enzyme may exist.

The method used for ion-exchange chromatography was essentially the same as that developed by Hoffmann et al. (1979b), but with two minor changes. Firstly, the homogenisation conditions were changed from the 1:5 w/v homogenates used for the liver enzyme by Hoffmann et al. (1979b) to 1:4 w/v and 1:2 w/v homogenates for liver and brain respectively. Secondly, dithiothreitol was used as the sulphydryl reagent instead of dithioerythritol. Furthermore, there is no indication from Hoffmann's paper that supernatants were dialysed before application to the column, a step which was routinely used in this work.

Hoffmann et al. (1979b) used DEAE-Sephadex ion-exchange chromatography of crude supernatants as the first step in the purification of liver ATP citrate lyase. They found only a single peak of enzyme activity which was eluted by the salt gradient; no elution of the enzyme before the gradient was reported. Therefore, the two-peak elution profile of ATP citrate lyase revealed in this work is in contrast to their results. However, elution of the second peak was in good agreement with the elution of the only peak of activity found by Hoffmann and co-workers. This was taken as an indication that the chromatographic procedure had been correctly reproduced. It is possible that as the non-retained, minor peak of liver ATP citrate lyase represented only 15-20% of the total

recovered activity, Hoffmann and his group may have disregarded such activity as column overload, since they were only interested in the purification of the enzyme. In fact, there was no evidence from their presentation of the results that they had assayed column fractions before the gradient for enzyme activity.

There are several reports in the literature of the use of anion-exchange chromatography on DEAE-substituted columns in the purification of liver ATP citrate lyase. However, the two-peak profile obtained for both the liver and brain enzyme has not, as far as is known, been previously observed. Examination of the literature indicates that the behaviour of the enzyme on ion-exchange chromatography is very sensitive to the exact conditions used. Thus, liver ATP citrate lyase was adsorbed on DEAE-cellulose in the presence of 20mM potassium phosphate buffer, pH 7.5 (Linn and Srere, 1979), or 5mM Tris/HCl buffer, pH 7.4-7.8 (Inoue et al., 1966; Singh et al., 1976). In contrast, Plowman and Cleland (1967) and Redshaw and Loten (1981) used DEAE-cellulose chromatography under conditions where liver ATP citrate lyase was not adsorbed to the column, namely 5mM Tris/HCl buffer pH 7.4 plus 0.05M KCl, and 10mM sodium phosphate buffer, pH 7.5 respectively. These different conditions therefore illustrate that binding of the enzyme is sensitive to the ionic conditions over a small pH range (pH 7.4 - 7.8). In fact, as shown in Figs. 9 and 12, it was found that both liver and brain ATP citrate lyase started to elute from the column almost as soon as the ionic concentration increased after application of the gradient. That this was a true elution of adsorbed enzyme protein was demonstrated by increasing the wash volume of the column 2-fold, before application of the gradient. No ATP citrate lyase activity was eluted until the gradient had been applied, indicating that elution of the second peak did not simply represent some sort of retention of the enzyme, rather than adsorption to the column. These results therefore confirm the literature reports that liver ATP citrate lyase is only weakly bound to DEAE-ion-exchangers and is thus highly

sensitive to changes in the ionic strength of the buffers used. The fact that the conditions employed are critical may explain why the heterogeneity of ATP citrate lyase has not been previously reported. It should also be mentioned that the results of DEAE-cellulose ion-exchange chromatography of ATP citrate lyase reported in the literature were obtained using samples which had been partially purified (e.g. by $(NH_4)_2SO_4$ precipitation), and therefore the minor component of activity may have already been lost. However, it is interesting to note here that Takeuchi et al. (1981) recently reported a two-peak elution profile of ATP citrate lyase from infected root tissue of sweet potato, using DEAE-cellulose chromatography. They observed elution of enzyme activity both before and after the gradient; the major proportion of activity was adsorbed on the column and eluted by the gradient.

Liver and brain ATP citrate lyase were found to have different elution profiles on DEAE-Sephadex chromatography with respect to the proportion of the total activity which was eluted in the first, non-retained peak. This therefore suggested that there may be a difference in the properties of the enzyme from these two tissues, resulting in a different distribution of activity between the same two ionic states of the enzyme. However, since in both cases, the enzyme of the first-eluted peaks is not adsorbed by the column, it is not possible to detect any differences in the charge properties which may exist. Therefore the possibility that the non-retained activity from each tissue corresponds to two distinct forms of ATP citrate lyase cannot be excluded.

One possible criticism of the quantitative comparison of these elution profiles of ATP citrate lyase from liver and brain would be the fact that the homogenising conditions were different for the two tissues. Liver was homogenised in 4 vol. buffer as this provided a suitable homogenate concentration which enabled even homogenisation of the tissue, and resulted in an adequate level of enzyme activity. However, for brain, homogenisation was performed in only 2 vol. buffer since the lower activity

of ATP citrate lyase in this tissue (Table 1) prevented use of a more dilute homogenate. Nevertheless, such a difference in homogenate concentration would only be important if the different elution profiles did in fact represent only a different distribution of activity as described above. Liver and brain are, however, very different in cellular composition, and therefore it would first be necessary to demonstrate that the amount of activity present in the first, non-retained peak was dependent on the homogenate concentration. This could easily be done by performing DEAE-Sephadex ion-exchange chromatography of liver supernatants obtained by homogenisation of the tissue in only 2 vol. buffer. Thus, if the higher proportion of brain ATP citrate lyase activity in the non-retained peak was due to the higher homogenate concentration compared to the liver, a similar increase would be expected in the percentage of the recovered activity of the liver enzyme in this peak. It is interesting to note here however, that induction of liver ATP citrate lyase by starving and refeeding had no effect on the elution profile of the enzyme (Table 5). Therefore, despite a 2-7-fold increase in activity, which has been shown to be the result of an equivalent increase in the amount of enzyme protein (Suzuki et al., 1967), no change was observed in the distribution of activity between the two peaks. This suggests that the two-peak profile is independent of the ATP citrate lyase concentration of the tissue.

Further investigation of the non-retained, basic form of ATP citrate lyase using DEAE-Sephadex ion-exchange chromatography was not possible for two reasons. Firstly, this minor form of the enzyme is not adsorbed on the column and therefore represents a non-specific separation. Hence, the effects of different experimental conditions on the elution of this enzyme state cannot easily be determined. For example, certain conditions may affect the adsorption of the major, acidic form of the enzyme which would then be eluted without retention and appear to represent

an increase in the amount of the basic form. Secondly, this enzyme state was found to be unstable; rechromatography of the first-eluted peak on DEAE-Sephadex columns revealed that the enzyme had been converted to the major, acidic form. Therefore attempts to study the properties of the basic form of ATP citrate lyase, possibly involving further purification, were not feasible.

Cation-exchange chromatography of crude supernatants was performed in an attempt to specifically separate the minor component of activity, from the major acidic form of ATP citrate lyase.

Since the minor, basic component of liver ATP citrate lyase was not adsorbed by the positively charged DEAE-Sephadex column, ion-exchange chromatography of crude supernatants was performed using a negatively charged cation-exchange column, in an attempt to specifically separate this activity from the major, acidic form of ATP citrate lyase. Although cation-exchange chromatography using a CM-Sephadex column was not successful, chromatography on a phosphocellulose column produced interesting results which will now be discussed.

ii) Phosphocellulose Cation-Exchange Chromatography

Phosphocellulose cation-exchange chromatography of liver and brain supernatants revealed interesting preliminary results. The elution profiles of ATP citrate lyase from these two tissues were found to be different. Thus, whilst both liver and brain extracts contained some activity which was strongly adsorbed to the column, and was eluted by a 2M NaCl step gradient, a second peak of activity was also adsorbed by the column in each case, but was eluted by the linear salt gradient at different ionic strengths. For the liver enzyme, this additional peak of activity was eluted at about 0.45M NaCl, whereas that of the brain enzyme was eluted at a lower ionic concentration, between 0.2-0.4M NaCl.

Therefore, in contrast to DEAE-Sephadex anion-exchange chromatography which revealed only a quantitative difference between the enzyme from liver and brain, cation-exchange chromatography on phosphocellulose indicated a qualitative difference.

Phosphocellulose ion-exchange chromatography therefore appears to be a very useful column system since it revealed the presence of two charge forms of ATP citrate lyase, one in liver and one in brain, which were both adsorbed to the column but were eluted at different points in the gradient. Moreover, it demonstrates that the existence of charge heterogeneity of ATP citrate lyase observed by DEAE-Sephadex ion-exchange chromatography is not merely an artefact of that column system. However, the relationships between the various chromatographic peaks from these two column systems cannot as yet be deduced. The low recovery of activity from the phosphocellulose columns, especially with regard to the brain enzyme (55% for liver; <30% for brain), prevented comparison of the amount of activity in each peak between the liver and brain enzyme, and also with the results from DEAE-Sephadex chromatography.

A possible modification of phosphocellulose chromatography would be the use of an anionic buffer such as acetate or glycine, rather than the cationic Tris/HCl buffer used in the present experiments. Since Tris is positively charged it may participate in the ion-exchange process, and thus result in local pH changes. Furthermore, small changes in buffer concentrations and pH may improve the recovery of activity. Although the buffer used in these experiments was not found to have any effect on the activity of liver ATP citrate lyase of crude supernatants, this may not necessarily be true for the different forms of the enzyme separated by the chromatographic procedure.

No previous reports of phosphocellulose ion-exchange chromatography of ATP citrate lyase are known. However, the results obtained are

particularly interesting in view of the recent discovery by Linn and Srere (1979) that liver ATP citrate lyase is a phosphoenzyme, containing structural phosphates in addition to catalytic phosphates directly involved in the enzyme reaction. Therefore, since the enzyme has binding sites for phosphates in addition to the catalytic binding site, adsorption of ATP citrate lyase on phosphocellulose column may occur at these specific phosphate sites rather than a simple electrostatic interaction. The observation that a proportion of the total ATP citrate lyase activity of both liver and brain was strongly adsorbed on the column and required a high ionic concentration (>2M NaCl) for elution, suggests that such a mechanism may be involved.

This phosphorylation of ATP citrate lyase could also be responsible for the charge heterogeneity of the enzyme observed on DEAE-Sephadex and phosphocellulose chromatography. Thus, binding of phosphates may produce local changes in the ionic state of the enzyme molecule, or indeed may result in small conformational changes of the protein leading to different charge properties. Despite intense investigation no function has yet been ascribed to this phosphorylation mechanism (Guy et al., 1981; Janski et al., 1979; Linn and Srere, 1979; Ranganathan et al., 1980). More recently however, Janski and Cornell (1982) reported indirect evidence in support of a relationship between phosphorylation of the enzyme and its binding to mitochondria.

The possible involvement of phosphorylation of ATP citrate lyase in the observed heterogeneity of the enzyme therefore requires further investigation since this may represent a physiological role. Ion-exchange chromatography could be performed using supernatants prepared in buffer containing fluoride, an inhibitor of phosphatase enzymes. However, the concentration of fluoride required would be relatively high (50-100mM), and it would therefore be necessary to

dialyse it out of the supernatant sample before ion-exchange chromatography. Another experiment would be to treat the crude supernatant with a phosphatase enzyme such as that used by Guy et al. (1981), which would ensure that the ATP citrate lyase was present in the dephosphorylated state. Ion-exchange chromatography of such supernatants would therefore represent the elution profile of the dephosphorylated enzyme only.

The recent finding that brain ATP citrate lyase is not phosphorylated (Szutowicz and Srere, 1983) is particularly interesting in view of the different elution profile of this enzyme, compared to that of liver, observed here by DEAE-Sephadex ion-exchange chromatography. Thus, there appears to be some evidence that the liver and brain enzymes may have different properties.

iii) Gel Filtration Chromatography

The size heterogeneity of liver ATP citrate lyase was demonstrated by gel filtration chromatography on Sepharose 6B and Sepharose 2B. Thus, the enzyme from crude liver extracts was separated into two molecular weight components. The major proportion of the activity was eluted from the Sepharose 6B column with an elution volume which corresponded to an estimated molecular weight of 4.1×10^5 (Fig.16). Comparison with literature values indicates that this represents the native tetrameric enzyme. However, in addition to the tetrameric form, a minor proportion of the activity, comprising about 10% of the total recovered activity, was eluted as a high molecular weight form at the void volume of the column.

In an attempt to determine the molecular weight of this peak of activity, gel filtration of crude supernatants was performed on Sepharose 2B which has a higher exclusion limit for proteins than Sepharose 6B. The results indicated that the high molecular weight ATP citrate

lyase activity represented a range of sizes, and the molecular weight was estimated to be of the order of 10^7. Therefore, because of this high molecular weight, a simple aggregation of the tetrameric enzyme can be excluded. Hence, it would appear to represent some sort of highly associated state of ATP citrate lyase.

The two-peak profile of liver ATP citrate lyase was found to be independent of the dietary state of the animals used; liver from normal fed, starved, and starved and refed, all possessed a minor component of the high molecular weight activity. In contrast, gel filtration of brain extracts revealed only the tetrameric form of ATP citrate lyase. Therefore, in addition to a tissue difference with respect to the ionic charge heterogeneity of the enzyme, liver and brain extracts also differ in that the high molecular weight associated state of ATP citrate lyase was only found in liver.

Several authors have commented on the presence of high molecular weight protein in their purified ATP citrate lyase preparations. Trace contamination of purified mammary gland enzyme by a high molecular weight protein was observed by Guy et al. (1981) during sedimentation velocity experiments. From their results they suggested that this minor component represented an octomeric form of the the enzyme. Redshaw and Loten (1981) noted the presence of an additional, minor band of high molecular weight seen with some of their purified liver ATP citrate lyase preparations on SDS polyacrylamide gel electrophoresis. Linn and Srere (1979) reported that the enzyme from liver tends to polymerise, but this was found to result in loss of activity. Using the same purification method, Ramakrishna and Benjamin (1979) observed a high molecular weight peak of ATP citrate lyase activity on gel electrophoresis of the pure liver enzyme, under non-denaturing conditions. In contrast, phosphoproteins purified by a different method from liver and adipose tissue, which they

identified as ATP citrate lyase, migrated as single protein bands. These authors suggested that the high molecular weight enzyme may represent aggregation of ATP citrate lyase similar to that reported by Linn and Srere (1979). However, the fact that enzyme activity was retained makes this unlikely since Linn and Srere found that aggregation resulted in considerable loss of activity. Although no estimation of the molecular weight of the minor component was given, it appeared to contain a similar proportion of the total ATP citrate lyase activity as observed for the high molecular weight, void volume peak of the enzyme demonstrated here by Sepharose 6B gel filtration.

However, all these observations were made during experiments with purified ATP citrate lyase and therefore may represent artefacts of the purification procedure. In contrast, the high molecular weight ATP citrate lyase observed in the present work was obtained using crude liver supernatants which correspond more closely to the *in vivo* situation, although this does not exclude the possibility of artefact.

Although gel filtration of ATP citrate lyase has previously been used during purification of the enzyme, this is the first known report of its use in the estimation of the enzyme molecular weight. The major component of liver ATP citrate lyase from starved and refed rats, was found to have a molecular weight of 4.1×10^5, which is in good agreement with literature values (Inoue *et al.*, 1966; Linn and Srere, 1979; Redshaw and Loten, 1981; Singh *et al.*, 1976). In contrast, the enzyme from liver of normal fed rats consistently showed a lower molecular weight of 2.7×10^5. It is not possible however to draw any conclusions from this interesting observation since the gel filtration system used is not sensitive enough to accurately measure such relatively small changes in molecular weight. However, no previous comparison has been made of the molecular weight of liver ATP citrate lyase from rats of

different dietary conditions. Therefore, it is possible that closer examination by a more sensitive method, such as polyacrylamide gel electrophoresis, may reveal significant differences which in turn could be involved in the regulation of the enzyme.

Determination of the molecular weight of brain ATP citrate lyase revealed that it was the same as the liver enzyme (starved, and starved and refed), when measured under the same conditions. This therefore provides a direct comparison of the size of the enzyme from these two tissues. No previous estimation of brain ATP citrate lyase molecular weight had been reported, until recently, when Szutowicz and Srere (1983) confirmed by SDS gel electrophoresis, that the subunit molecular weight of the purified brain enzyme was identical to that of liver enzyme from starved and refed rats. Interestingly, they also reported a high specific activity for the pure brain enzyme; 21.4 units/mg compared to their previous value of 0.12 units/mg (Szutowicz et al., 1975).

iv) Relationship between the Charge Heterogeneity and the Two Molecular Weight Forms of Liver ATP Citrate Lyase

The high molecular weight state of ATP citrate lyase was found to be unstable since it was eluted as the tetrameric enzyme on rechromatography on Sepharose 6B. Both molecular weight forms of the enzyme were adsorbed on DEAE-Sephadex columns and were eluted by the gradient. However, the instability of the associated state of the enzyme may effect the behaviour of the enzyme when rechromatographed on an ion-exchange column. This result cannot therefore be taken as evidence that the high molecular weight form is acidic in nature.

Rechromatography experiments were initially performed in an attempt to determine the relationships between the two charge states of

the liver enzyme from DEAE-Sephadex ion-exchange chromatography, and the two molecular weight forms, observed on Sepharose gel filtration. This did not however prove possible due to the instability of both the basic, non-retained form, and the high molecular weight associated form of the enzyme. Nevertheless, the fact that these two minor components of ATP citrate lyase contained similar proportions of the total activity (compare Tables 5 and 8), together with the similarities in the instability of both enzyme states, and the apparent resistance to inhibition by glutamate (see Tables 17 and 18), suggested that they represented the same form of the enzyme. Comparison with the literature indicated that the major peak of activity observed on gel filtration, and that obtained by ion-exchange chromatography, both represented the native form of the enzyme. Hence, the estimated molecular weight of the major peak of activity was in agreement with literature values for the tetrameric enzyme, and the enzyme which was adsorbed on the DEAE-Sephadex column was eluted as described by Hoffmann et al. (1979b). This interpretation of the results, that the heterogeneity of liver ATP citrate lyase represented the existence of two forms of the enzyme, the native tetrameric form which is acidic, and the additional minor component which has a high molecular weight and is basic in nature, was published in a preliminary report (Corrigan and Rider, 1981). However, subsequent experiments revealed a more complex situation. The use of high-speed centrifugation (150,000g) to prepare supernatants resulted in loss of the high molecular weight ATP citrate lyase activity. DEAE-Sephadex ion-exchange chromatography of such supernatants, containing only tetrameric ATP citrate lyase, revealed the presence of both charge forms of the enzyme. Thus, the high molecular weight enzyme and the basic, non-retained form could not represent the same single state of ATP citrate lyase. Further investigation indicated that high molecular weight ATP citrate lyase is acidic in nature,

since a loss of activity from the retained peak was observed corresponding to the loss of the associated form of the enzyme. This result, incidentally, agrees with that from earlier rechromatography experiments.

Therefore, there appear to be at least two forms of liver ATP citrate lyase, in addition to the native acidic tetrameric enzyme; a tetrameric form which is basic, and an acidic high molecular weight form. In addition, the possibility that some of the high molecular weight enzyme is also basic in nature cannot be excluded from the present results.

In contrast to liver, the heterogeneity of brain ATP citrate lyase is less complex. Thus, although both the basic, non-retained and the acidic, retained forms were observed on ion-exchange chromatography of crude extracts, gel filtration of the same supernatants revealed only the tetrameric enzyme. Therefore the brain enzyme appears to exist in only two forms, one basic and the other acidic, both of which are tetrameric. The high molecular weight associated state of ATP citrate lyase found in liver was absent from brain extracts. Moreover, although a basic, tetrameric form of the enzyme was found in both tissues, the proportion of the total activity it contained differed, and there was no positive evidence to indicate that it did in fact represent the same form.

A diagramatic representation of the various chromatographic forms of both liver and brain ATP citrate lyase is shown in Fig.30.

v) Further Investigation of the Nature of the High Molecular Weight ATP Citrate Lyase

There are three possible types of association which could account for the high molecular weight ATP citrate lyase; aggregation of the enzyme, association with other enzymes forming a multi-enzyme complex, association with membranes.

a) Aggregation of ATP Citrate Lyase

The high molecular weight ($M_r 10^7$) of the minor component of liver ATP citrate lyase excludes the possibility that it represents a simple

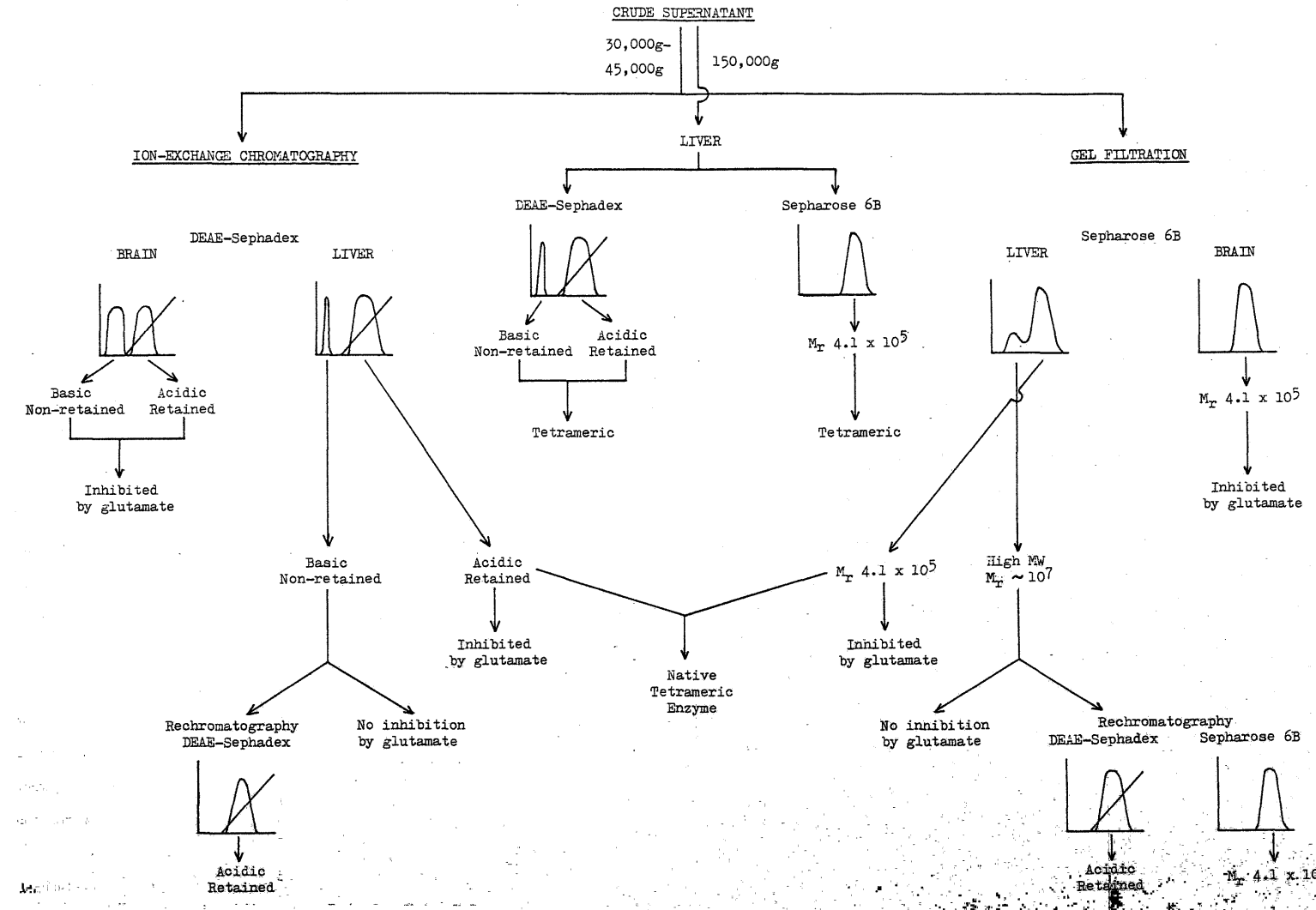


FIG. 30

Summary of the Heterogeneity of Rat Liver and Brain ATP Citrate Lyase

aggregation of the native, tetrameric enzyme (M_r 4.1 x 10^5), such as the dimer observed for the mammary gland enzyme by Guy et al. (1981). Polymerisation of the tetrameric enzyme however could produce this high molecular weight form. Acetyl CoA carboxylase, the next enzyme in the pathway of fatty acid biosynthesis, does undergo such polymerisation to form long linear chains (for review see Lane et al., 1974). Hence, the tetrameric acetyl CoA carboxylase, which has a molecular weight of around 5 x 10^5, similar to tetrameric ATP citrate lyase, is activated in the presence of citrate to form linear chains consisting of 10-20 tetrameric enzyme molecules. This polymeric form of the enzyme has a molecular weight which ranges from 4 x 10^6 - 8 x 10^6 (Gregolin et al., 1966), and is therefore comparable to the size of the high molecular weight form of ATP citrate lyase. However, in contrast to ATP citrate lyase, acetyl CoA carboxylase only has enzyme activity when in the polymeric form; the tetrameric enzyme is inactive. Therefore, since both molecular weight forms of ATP citrate lyase possess enzyme activity, the possibility of a regulatory control mechanism for the enzyme by tetramer-polymer transitions, such as that found for acetyl CoA carboxylase, is not apparent. One important consideration here is that ATP citrate lyase activity was determined under optimal assay conditions and therefore any change in activity which may occur under physiological conditions may not be detected. Interestingly, Linn and Srere (1979) commented on the fact that purified liver ATP citrate lyase does tend to polymerise when concentrated but with subsequent loss of activity.

The polymerisation of acetyl CoA carboxylase by citrate can be demonstrated on sucrose density gradients (Gregolin et al., 1968). In the absence of citrate, only the tetrameric enzyme is observed, but when citrate is present in the buffers, the enzyme is eluted as the polymeric

form. Gel filtration of ATP citrate lyase in the presence of citrate may therefore reveal whether such enzyme polymerisation is involved in the formation of the high molecular weight form. Citrate has in fact been reported to be an activator of liver ATP citrate lyase (Hoffmann et al., 1979b; Szutowicz and Angielski, 1970) although no physiological function for this has been determined. It is of interest to note here, with respect to the possible involvement of citrate in polymerisation of ATP citrate lyase, that Redshaw and Loten (1981) performed Sepharose 6B gel filtration of partially purified liver enzyme in the presence of citrate-phosphate buffer (50mM sodium citrate). The enzyme was eluted as one peak of activity corresponding to tetrameric ATP citrate lyase. This would therefore suggest that a citrate-activated polymerisation of the enzyme does not occur, although obviously a more detailed investigation is required.

b) Multi-Enzyme Aggregate

Fatty acid biosynthesis in the cytoplasm is catalysed by three soluble enzymes of similar molecular weight, ATP citrate lyase, acetyl CoA carboxylase, and fatty acid synthetase. All three enzymes undergo similar changes in activity, brought about by changes in the amount of enzyme protein, as a result of dietary and hormone induced alterations in the rate of lipogenesis (for reviews see Lane and Moss, 1971; Rosmos and Leveille, 1974; Volpe and Vagelos, 1973; 1976). Furthermore, ATP citrate lyase and fatty acid synthetase in particular appear to have very similar properties, as indicated by co-purification of the two enzymes (Guy et al., 1981; Plowman and Cleland, 1967). In fact this similarity was at one stage considered as evidence for the existence of a single enzyme protein which catalysed both enzyme reactions. However, it was later proved that the ATP citrate lyase and fatty acid synthetase

activities are on separate proteins (Inoue et al., 1966; Suzuki et al., 1967).

There are many examples in the literature of enzyme-enzyme complexes (Beckman and Johansson, 1976; Fahien et al., 1977; Halper and Srere, 1977; Ovadi and Keleti, 1978). Furthermore, there have also been reports presenting evidence for the existence of multi-enzyme aggregates consisting of all the glycolytic enzymes, in both bacterial and mammalian cells (Clarke and Masters, 1973; Mowbray and Moses, 1976). Thus, the possibility of the high molecular weight, associated state of ATP citrate lyase representing a multi-enzyme aggregate of all three lipogenic enzymes was investigated. As with other enzyme-enzyme complexes, such aggregation would appear to be advantageous due to localisation of the consecutive reactions of a biochemical pathway.

A simple aggregation of one of each enzyme protein, ATP citrate lyase, acetyl CoA carboxylase and fatty acid synthetase complex, is excluded because of the size of the associated state of ATP citrate lyase. Furthermore, since acetyl CoA carboxylase does not polymerise under the conditions employed (i.e. citrate is required for polymerisation), an aggregation of ATP citrate lyase and fatty acid synthetase with the polymeric form of acetyl CoA carboxylase cannot be invclved. Hence, if the high molecular weight ATP citrate lyase activity does represent an aggregation of these enzymes, it would have to consist of an assembly of several of each enzyme protein. However, no activity of either acetyl CoA carboxylase or fatty acid synthetase was found in column fractions containing the minor component of high molecular weight ATP citrate lyase. In fact, both enzymes co-eluted with the major, tetrameric form of ATP citrate lyase, as would be expected from the similar molecular weights. Gillevet and Dakshinamurti (1982) however, have recently presented evidence in favour of a high molecular weight lipogenic enzyme complex. They found co-elution of the three enzymes on a sucrose density gradient

at a position corresponding to a molecular weight greater than that expected for the native enzymes (i.e. $\sim 5 \times 10^5$). Although they gave no estimate for the molecular weight of the proposed complex, it was much less than that of the polymeric form of acetyl CoA carboxylase used as a marker. Therefore, since the molecular weight of polymeric acetyl CoA carboxylase is of the same order as that of the associated state of ATP citrate lyase observed in this work, it would seem that the high molecular weight ATP citrate lyase activity of the proposed lipogenic enzyme complex is not the same as the associated form of the enzyme described here.

c) Membrane Association

Association of liver ATP citrate lyase with mitochondria has recently been demonstrated (Janski and Cornell, 1980a,b; Ranganathan et al., 1980). Janski and Cornell (1980a) measured the release of cytosolic and mitochondrial enzymes from isolated rat hepatocytes by the non-ionic detergent digitonin. They reported that 23% of the ATP citrate lyase in hepatocytes from starved rats was released like the mitochondrial enzyme citrate synthase, whilst the remaining enzyme was released like the soluble enzyme, lactate dehydrogenase. In a subsequent paper (Janski and Cornell, 1980b) they extended this work and concluded that a small proportion of ATP citrate lyase (25%) exists as an association with mitochondria, probably the outer membrane. This association was found to be stabilised by Mg^{2+}, whereas release of the enzyme was enhanced by the substrates and products of the enzyme reaction. Using a more direct approach, they reported that 2.2% of the total hepatic ATP citrate lyase activity was present in isolated mitochondria. Mitochondrial binding of liver ATP citrate lyase has also been demonstrated by Ranganathan et al.(1980), who found 10% binding of the pure liver enzyme

when incubated with isolated liver mitochondria. Such an association of ATP citrate lyase with mitochondria would seem advantageous since it would localise the enzyme at the site of substrate formation i.e. release of citrate from the mitochondria.

Therefore, the possibility that the high molecular weight ATP citrate lyase represents an association of the enzyme with mitochondria was considered. However, liver tissue was routinely homogenised in buffer containing isotonic sucrose (0.25M), and thus the mitochondria should remain intact and be sedimented by the centrifugation of the homogenates at 45,000g for 30 min. That this was in fact the case was demonstrated by the absence of any appreciable activity of the mitochondrial matrix enzyme, citrate synthase, compared to supernatants prepared from the same tissue but in the absence of sucrose. The small amount of activity that was detected probably results from leakage of the enzyme from the mitochondria during homogenisation. Sonication of supernatants to disrupt any intact mitochondria which may have been present did not produce any increase in citrate synthase activity. In addition, no activity of the inner mitochondrial membrane enzyme succinate dehydrogenase was detected in crude supernatnats. It would therefore appear unlikely that the high molecular weight ATP citrate lyase represents enzyme bound to mitochondria. Nevertheless, such a possibility was further investigated to determine whether this minor, high molecular weight component of activity was the result of binding to fragments of the outer mitochondrial membrane stripped off during homogenisation.

Supernatants and column fractions from Sepharose 6B and Sepharose 2B gel filtration of supernatants were assayed for the outer mitochondrial membrane enzyme rotenone-insensitive cytochrome c reductase (RIDCR). The activity present in the supernatant was eluted from the

Sepharose 6B column at the void volume, apparently eluting with the high molecular weight ATP citrate lyase. However, this only serves to demonstrate that the marker enzyme is membrane-bound and was therefore eluted at the void volume because of exclusion from the column. Further investigation using the Sepharose 2B column revealed that the enzyme was also eluted at the void volume of this column, and there was no evidence for any co-elution with high molecular weight ATP citrate lyase.

Although RIDCR was used as a marker enzyme for the outer mitochondrial membrane, the results obtained may be misleading due to the fact that the enzyme is also found on microsomal membranes (Sottocasa et al., 1967). Since microsomes would be present in the 45,000g supernatants used for gel filtration experiments, the elution profile of NADPH cytochrome c reductase, an enzyme found exclusively in the microsomes (Phillips and Langdon, 1962), was determined in an attempt to estimate the proportion of the total RIDCR activity due to the microsomal enzyme. NADPH cytochrome c reductase was eluted at the void volume of the Sepharose 6B column indicating that the activity represented the membrane-bound enzyme. Sottocasa et al. (1967) measured the activities of RIDCR and NADPH cytochrome c reductase, in microsomal and mitochondrial fractions obtained by differential centrifugation of 10% liver homogenates from starved rats. They reported that the specific activity of RIDCR was 24-fold greater than that of NADPH cytochrome c reductase in the same microsomal fraction. Therefore, as the activity of RIDCR here was found to be 60-fold greater than that of the exclusively microsomal enzyme in the Sepharose 6B column fractions, it would appear unlikely that it represented only activity of the microsomal enzyme. This therefore suggests that outer mitochondrial membrane fragments are present in the crude liver supernatants. However, it must

be noted that whereas Sottocasa et al. (1967) used the same assay buffer for determination of both enzyme activities, the two enzyme assays were performed in this study using two different buffers (see Methods 8iv and 8vi). This was due to the fact that NADPH cytochrome c reductase activity was not reproducible under the buffer conditions used initially for the assay of RIDCR.

Since there was some evidence from these marker enzyme assays to suggest that outer mitochondrial membrane fragments are present in the crude liver supernatants, the possible binding of ATP citrate lyase to such membrane fragments was further investigated using a more direct approach. Hence, ATP citrate lyase activity was determined in the crude mitochondrial fraction obtained from differential centrifugation of liver homogenates. If the high molecular weight ATP citrate lyase activity does represent enzyme bound to membrane fragments, a higher proportion of the total activity would be expected in the isolated mitochondrial fraction than the 10% observed in the minor peak on gel filtration. This would however assume a reasonable yield of intact mitochondria, and for this reason a loose fitting homogeniser, such as that recommended for mitochondrial preparations, was used. The results obtained using liver from normal fed rats gave no indication of any mitochondrial binding of the enzyme; no ATP citrate lyase activity was detected in the mitochondrial fraction. Activity of both RIDCR and succinate dehydrogenase was present however, indicating the presence of outer and inner mitochondrial membranes respectively.

Further evidence against a mitochondrial association of ATP citrate lyase to explain the high molecular weight enzyme activity was provided by the gel filtration experiments in the presence of Mg^{2+}. Janski and Cornell (1980b) reported that the association of the enzyme with mitochondria was stabilised by magnesium ions, an effect which had

previously been reported for the mitochondrial association of brain hexokinase (Rose and Warms, 1967). Therefore, in an attempt to stabilise and possibly increase the amount of the proposed membrane-associated high molecular weight ATP citrate lyase activity, gel filtration was performed in the presence of Mg^{2+}. Such experiments however revealed that the peak of high molecular weight enzyme activity was completely removed. Thus, magnesium ions appeared to disrupt the high molecular weight form of the enzyme to produce the tetramer as no loss of activity was detected in the pellet from the centrifugation, and magnesium ions had no effect on ATP citrate lyase activity of supernatants. This result therefore indicates that the associated state of liver ATP citrate lyase observed on gel filtration is not the same as the mitochondrial membrane-bound enzyme described by Janski and Cornell (1980a,b). Furthermore, whereas as Janski and Cornell found differences in the proportion of activity bound to mitochondria depending on the dietary state of the animals used, the elution profiles of liver ATP citrate lyase from normal fed, starved, and starved and refed rats, all showed the same distribution of activity between the two molecular weight forms of the enzyme.

The effect of magnesium on the elution profile of liver ATP citrate lyase is interesting since similar experiments in the presence of the same ionic concentration of sodium ions did not change the elution profile of ATP citrate lyase. Hence, disruption of the associated state of the enzyme was not due to the increased ionic strength of the buffer; whether it was specific for magnesium ions or divalent cations in general was not investigated. Nevertheless, the fact that magnesium ions are required for ATP citrate lyase activity, and appear to influence the kinetics of the enzyme reaction (Szutowicz and Angielski, 1970) may be significant.

The possibility of mitochondrial association of ATP citrate lyase was also investigated using supernatants prepared in the absence of isotonic sucrose. This resulted in disruption of the mitochondria, as demonstrated by the presence of citrate synthase activity, and therefore an increase in the amount of outer mitochondrial membrane fragments in the supernatant. However, such treatment did not produce any appreciable change in the gel filtration elution profile of ATP citrate lyase, a further indication that the high molecular weight activity does not represent enzyme bound to mitochondrial membranes.

In addition to the mitochondrial binding reported in the literature, a microsomal association of the enzyme has also been suggested (Tucek, 1967b; Avruch et al.,1976). Tucek (1967b) found a high proportion of brain ATP citrate lyase in the microsomal fraction obtained from sheep caudate nuclei, and Avruch et al.(1976) commented on the presence of a high proportion of a cytoplasmic phosphoprotein, later identified as ATP citrate lyase (Alexander et al.,1979), in the microsomal fraction from adipocytes. The authors indicated that no attempt had been made to verify these observations, and therefore the possibility of non-specific adsorption was not ruled out. More recently Witters et al. (1981) reported activity of the three lipogenic enzymes ATP citrate lyase, acetyl CoA carboxylase, and fatty acid synthetase, in the microsomal fraction from rat hepatocytes. In addition, they also observed the presence of two major phosphoproteins in this fraction, identified by immunoprecipitation as ATP citrate lyase and acetyl CoA carboxylase. They proposed that the microsomes may be a major locus of fatty acid synthesis. However, the properties of the microsomal acetyl CoA carboxylase appeared to be different from those of the other two enzymes, and they therefore suggested that the apparent association of the latter could be the result of non-specific trapping or very weak

association.

From the present results, it does not seem likely that such an association could account for the high molecular weight ATP citrate lyase observed on Sepharose gel filtration; centrifugation of homogenates at 100,000g for 60 min, conditions which are routinely used for sedimentation of microsomes (Sottocasa et al.,1967),did not remove this minor peak of activity (results not shown). Only under more rigorous centrifugation conditions, 150,000g for 90 min, was this achieved. It is concluded therefore that the high molecular weight associated state of ATP citrate lyase revealed by gel filtration of crude liver supernatants, does not represent association of the enzymes with membranes, either mitochondrial or microsomal.

3. Possible Physiological Significance of ATP Citrate Lyase Heterogeneity

The heterogeneity of rat liver and brain ATP citrate lyase which has been demonstrated in this work has not previously been reported. There is at present a lot of interest in the regulatory properties of ATP citrate lyase, and the possible involvement of the enzyme in the control of fatty acid synthesis and/or acetylcholine synthesis. Thus, heterogeneity of the enzyme as described here (see Fig. 30) may represent some form of enzyme regulation.

The distribution of the total activity of liver ATP citrate lyase between the two charge states, and also between the two molecular weight forms, was found to be independent of the dietary state of the animals used (Tables 5 and 8, Figs. 10 and 15). This tends to suggest that neither the ionic charge heterogeneity, nor the size heterogeneity, is directly involved in the long-term regulation of the enzyme in liver. However, as discussed in the introduction,certain properties of the enzyme, which are not yet fully explained, suggest the ATP citrate lyase may be controlled by short-term regulatory mechanisms such as allosteric

control and covalent modification, i.e. phosphorylation of the enzyme and the enzyme kinetics with respect to citrate. Therefore, the observed heterogeneity may play a role in the short-term regulation of enzyme activity.

Comparison of the heterogeneity of liver ATP citrate lyase with that of the brain enzyme reveals that although both charge forms are present in each tissue, the distribution of total activity between these two states differs. In addition, the high molecular weight form of ATP citrate lyase was found only in liver; no such enzyme form was found in brain. Hence, these tissue differences may reflect the different physiological roles of the enzyme. The fact that the associated state of ATP citrate lyase is found only in liver may be an indication of a role for this form in fatty acid biosynthesis. Although ATP citrate lyase is also involved in fatty acid biosynthesis in brain it does not appear to be under dietary or hormonal control like the liver enzyme (see Introduction, Section 2iv). The different distribution of activity of the liver and brain enzyme between the two charge forms of the enzyme may represent the dual function of the enzyme in acetylcholine and fatty acid synthesis in brain compared to only fatty acid biosynthesis in liver.

There are several properties of liver ATP citrate lyase which should be considered in this discussion of the possible physiological function(s) of the enzyme heterogeneity; the kinetics of the enzyme with respect to citrate, structural phosphorylation by cAMP-dependent and cAMP-independent protein kinases (see Introduction, Sections 2iii and 2v, respectively), proteolytic degradation of the enzyme (see Discussion, Section 3iii) and binding of the enzyme to mitochondrial membranes (see Discussion, Section 2v c)). The physiological roles of these properties have not yet been determined. It is therefore interesting to consider to what extent they may be involved in the heterogeneity of the enzyme

observed here.

i) Effect of Citrate on ATP Citrate Lyase Activity

ATP citrate lyase has two K_m values for the substrate citrate and one of the explanations which has been proposed to explain this phenomenon is that there are two forms of the enzyme, each with a different K_m for citrate. It is very unlikely that two forms of an enzyme which differ only in the affinity for one of the substrates could correspond to the two molecular weight forms of ATP citrate lyase. However, a small change in the binding site to produce the different substrate affinity of the enzyme, may affect the charge properties, and hence result in the two forms of the enzyme observed on ion-exchange chromatography. Furthermore, the fact that both the liver and brain enzymes show similar kinetic behaviour with respect to citrate would also be more consistent with the ionic charge rather than the size heterogeneity of ATP citrate lyase, since only the former is found for both enzymes.

An alternative and more favourable explanation for the unusual kinetics of ATP citrate lyase with citrate is that there are two independent binding sites, a substrate binding site and a regulatory binding site. In this hypothesis citrate acts at the latter site as an activator. Since citrate acts as an activator of acetyl CoA carboxylase, resulting in polymerisation of the enzyme, a similar mechanism may also occur with ATP citrate lyase, thus representing the high molecular weight associated form of the enzyme, as has already been discussed (p.188). This possibility requires further investigation.

ii) Phosphorylation of ATP Citrate Lyase

ATP citrate has recently been shown to undergo reversible phosphorylation at a site(s) distinct from the catalytic site

phosphorylation which occurs during the enzyme reaction. The possible involvement of this phosphorylation in the heterogeneity of the enzyme was discussed earlier (p.182).Thus, phosphorylation could change the charge properties of the enzyme resulting in the two forms of ATP citrate lyase observed on ion-exchange chromatography. Alternatively, it could affect the enzyme in such a way as to result in polymerisation to produce the high molecular weight form of the enzyme, possibly by altering the quaternary structure of the enzyme. Although Janski and Cornell (1982) recently presented evidence that phosphorylation of ATP citrate lyase altered the binding of the enzyme to the mitochondrial membrane, and hence the presence of high molecular weight activity, no evidence was found to suggest that the associated state of ATP citrate lyase of this study corresponded to mitochondrially bound enzyme. It is interesting to note in this context of enzyme phosphorylation the recent report of a protein kinase in liver which binds to acetyl CoA carboxylase and also polymerises to produce a high molecular weight aggregate (Lent and Kim, 1982).

There appears to be some disagreement at present as to the number of structural phosphorylation sites on ATP citrate lyase. The enzyme has been shown to be phosphorylated *in vitro*, by both cAMP-dependent protein kinase (Alexander *et al.*, 1981; Guy *et al.*, 1980, 1981; Pierce *et al.*, 1981, 1982; Ramakrishna *et al.*, 1981; Ranganathan *et al.*, 1982; Redshaw and Loten, 1982), and cAMP-independent protein kinase (Alexander *et al.*, 1981; Ramakrishna and Benjamin, 1981; Ramakrishna *et al.*, 1981). In addition, ATP citrate lyase undergoes structural site phosphorylation in the presence of the hormones insulin and glucagon (Alexander *et al.*, 1979; Janski *et al.*, 1979; Pierce *et al.*, 1981). Since glucagon is known to mediate its effects via cAMP, it was suggested that the phosphorylation in response to glucagon represented phosphorylation at the same site as

that brought about by cAMP-dependent protein kinase. In fact, both Alexander et al. (1981) and Pierce et al. (1981) reported evidence to indicate that one site on the enzyme is phosphorylated by cAMP-dependent protein kinase and glucagon. The insulin-stimulated phosphorylation was thought to represent the cAMP-independent mechanism. Therefore, these two hormones, which are known to produce their effects by different mechanisms, may both produce the same effect on ATP citrate lyase i.e. increase in phosphorylation, but by acting at different sites on the enzyme the net effect may be different. Ramakrishna et al. (1981) reported that the catalytic subunit of cAMP- dependent protein kinase phosphorylated different sites of adipose ATP citrate lyase compared to a lyase kinase isolated from liver (i.e. cAMP-independent protein kinase). More recently however, others have observed that both the insulin- and glucagon-stimulated phosphorylation of the enzyme occurs at the same site (Pierce et al., 1982; Swergold et al., 1982). In fact, Pierce and co-workers suggested that the glucagon stimulated phosphorylation did not represent any physiological function but merely corresponded to a non-specific phosphorylation as a result of a general increase in the phosphorylating activity of the cell in response to this hormone. Clearly, further investigation is required in order to resolve this question of the significance of ATP citrate lyase phosphorylation.

iii) Proteolytic Degradation of ATP Citrate Lyase

The limited proteolytic degradation of liver ATP citrate lyase first demonstrated by Singh et al. (1976) has received a lot of attention with regard to a possible role in the enzyme regulation. Incubation of the pure liver enzyme with trypsin resulted in degradation of the enzyme into two smaller, unequal polypeptides (Singh et al., 1976). However, no loss of activity was observed, and the only change detected in the properties of the enzyme was a decreased thermal stability.

It was suggested that each enzyme subunit is composed of two domains connected by a protease sensitive region. Thus, in the presence of trypsin the two domains were separated but retained sufficient interaction to maintain enzyme activity. Further investigation of the thermal and proteolytic degradation of liver ATP citrate lyase was carried out by others (Osterlund and Bridger, 1977; Osterlund et al., 1980; Vogel and Bridger, 1981). They found that degradation of the enzyme could be reduced by the substrates citrate and CoA, by NADPH and by an as yet unidentified peptide stabilising factor. Hence it was suggested that ATP citrate lyase activity may be controlled by a process involving limited proteolysis of the enzyme which could prevent complete enzyme degradation. Furthermore, Osterlund et al. also suggested that phosphorylation may influence the interaction of the enzyme with the proposed stabilising factors. It has recently been demonstrated that the properties of acetyl CoA carboxylase are also affected by proteolytic degradation (Song and Kim, 1981), and this limited proteolysis has been linked to phosphorylation of the enzyme (Guy and Hardie,1981). Therefore, there is some evidence to suggest that proteolytic degradation may have a physiological role in the regulation of enzyme activity. Interestingly, Lill et al. (1982) have recently demonstrated that the two subunit domains of ATP citrate lyase obtained by limited proteolytic degradation are associated with two half reactions. Furthermore, the two types of phosphorylation of ATP citrate lyase (i.e. structural and catalytic) were correlated with these two partial reactions. Hence, they postulated that phosphorylation at the structural site was involved in conformational changes in these subunit fragments and was necessary for complete enzyme activity. This therefore represents a physiological function for the enzyme phosphorylation.

Endogenous proteolysis, resulting in separation of subunit

domains as described above, may alter the ionic charge properties of the enzyme, leading to the observed heterogeneity of ATP citrate lyase on ion-exchange chromatography. The nicked enzyme would have to retain sufficient interaction so as to elute from the Sepharose 6B gel filtration column as the tetrameric enzyme. Evidence for this is the fact that Singh et al. (1976) found that trypsin-treated enzyme showed the same sedimentation behaviour as the untreated enzyme. Ion-exchange chromatography of crude liver supernatants in the presence of the proteolytic inhibitor phenylmethylsulphonyl fluoride, did not, however, reveal any change in the elution profile of ATP citrate lyase (Fig. 11). Therefore, this suggests that proteolytic degradation of the enzyme is not responsible for the charge heterogeneity, although the possibility that enzyme nicking occurs as a result of a specific proteolytic enzyme not affected by this inhibitor cannot be excluded.

The effect of trypsin on brain ATP citrate lyase has not yet been investigated. However, such experiments would be particularly interesting in view of the possible involvement of proteolytic degradation in enzyme regulation.

iv) Mitochondrial Membrane Association of ATP Citrate Lyase

Although the binding of ATP citrate lyase to mitochondrial membranes described by Janski and Cornell (1980a,b) would provide an explanation for the high molecular weight associated state of the enzyme observed on gel filtration, no evidence was found to support this. Furthermore, the elution profile of a mitochondrial membrane marker enzyme from a DEAE-Sephadex ion-exchange chromatography column, indicated that neither of the two charge forms of the enzyme represent mitochondrial association of ATP citrate lyase (Fig. 24).

4. Activation of ATP Citrate Lyase

The apparent activation of the non-retained, basic form of ATP citrate lyase observed after prolonged dialysis of the crude liver supernatants is an interesting phenomenon which could be involved in the short-term regulation of the enzyme. The results suggested that the three-fold increase in the amount of activity eluted as the basic peak after 24h dialysis of liver supernatants was the result of a specific activation of this form of the enzyme. The fact that there was no increase in the total ATP citrate lyase activity of the dialysed supernatant initially suggested that the increase in activity of the non-retained peak may have been the result of transfer of activity from the retained acidic peak. However, no comparable decrease was observed in this second peak, and since the recovery of total enzyme activity was high (>120%), it appeared that activation of the basic form of the enzyme occured and in such a way as to be undetectable in the supernatant. Further investigation is required to determine whether this effect is a true activation of the enzyme, and if so whether it has any physiological significance.

It is interesting to note with reference to this activation, that unusually high recoveries (>120%) have previously been obtained from some chromatographic columns. In particular, gel filtration of liver and brain extracts consistently produced high recovery of total activity. Activation of the enzyme may therefore occur under certain conditions to different degrees resulting in these high yields of activity. For the brain enzyme however, high yields from both ion-exchange chromatography and gel filtration may be partly explained by inaccurate determination of the supernatant activity due to the presence of the high endogeneous rate of NADH oxidation. The anomalous increase observed in the activity eluted as the basic peak from high-speed supernatants (150,000g) may also

be attributable to this activation phenomenon.

Reference to the literature indicates that several authors have noted unusual activation of ATP citrate lyase. Tucek et al. (1967b) consistently obtained high recovery of brain ATP citrate lyase from subcellular fractionation studies. This was explained by the authors as the result of interference of citrate synthase in the assay. Szutowicz et al.(1975) commented on the high yield of the brain enzyme obtained after partial purification by ammonium sulphate precipitations. They suggested that it may be the result of interference by ATPase or by the removal of an inhibitory factor, an idea which is particularly interesting in view of the recent work by Osterlund et al. (1980), who have identified a protein stabilising factor of the enzyme. In addition, Simpson (1981) found that liver homogenate ATP citrate lyase activity was only 5% of the subsequent supernatant activity. This was explained as the result of interference in the assay by other enzymes but it could also be accounted for by the presence of enzyme inhibitors in the homogenate. Results from this study did not suggest any difference in the activity of the liver enzyme in homogenates and the corresponding supernatants however (results not shown).

5. Inhibition of ATP Citrate Lyase Activity by L-Glutamate

The effect of L-glutamate on ATP citrate lyase was investigated following the report by Szutowicz et al. (1974a) that the enzyme from some sources was inhibited by this amino acid. In view of the heterogeneity of both liver and brain ATP citrate lyase demonstrated by ion-exchange and gel filtration chromatography, this inhibition of the enzyme provided another possible method to differentiate the various chromatographic forms.

ATP citrate lyase activity of crude liver and brain supernatants was found to be inhibited by glutamate under the assay conditions used by

Szutowicz et al. (1974a). Although the results obtained were not in complete agreement with those of Szutowicz and co-workers, a similar pattern of inhibition was observed. One important difference however, was that whereas Szutowicz found the inhibition of the brain enzyme to be 2-fold greater than that of the liver enzyme from starved rats, the results presented here indicated similar inhibition to that of the liver enzyme (starved rat). The quantitative differences observed may be attributable to the differences in the enzyme preparation procedure used, and in the state of purity of the enzyme. The use of crude enzyme preparations may lead to misleading results due to the presence of other enzymes. Thus, the exogenous glutamate may be utilised, thereby reducing its inhibitory effect on ATP citrate lyase. Alternatively, conversion of glutamate to a more potent inhibitor would result in an increase in the effect of glutamate. From the results, this latter situation would appear more likely in the case of liver, since inhibition of ATP citrate lyase of crude extracts was found to be greater than that reported for the purified enzyme (see Table 16). Moreover, partial purification of liver ATP citrate lyase, by either DEAE-Sephadex ion-exchange chromatography or Sepharose 6B gel filtration, resulted in a reduced susceptibility of the enzyme to inhibition. The difference in the relative inhibition of the liver and brain enzyme compared to the results of Szutowicz et al. (1974a) could therefore be explained by the presence of different enzymes in crude extracts from these two tissues.

The fact that the inhibition of ATP citrate lyase by glutamate was dependent on the source of the enzyme prompted further investigation of the nature of the inhibition, since the susceptibility of the enzyme may be reflected in the various chromatographic forms of the enzyme.

Indeed, the different forms of the ATP citrate lyase did show different susceptibility to inhibition by glutamate (Tables 17 and 18). Thus, neither the minor, basic form of the liver enzyme from DEAE-Sephadex ion-exchange chromatography, nor the high molecular weight associated state observed on gel filtration of liver supernatants, were inhibited by glutamate. Interestingly, a different pattern was observed for the brain enzyme; both the basic and acidic forms of brain ATP citrate lyase were inhibited by the same amount. Thus, the initial results suggested that the distribution of total activity between the two ionic charge states of the liver and brain enzyme may reflect the different susceptibilities of the enzyme to glutamate, possibly indicating a role for this heterogenous nature of the enzyme. However, the requirement for a long preincubation period suggested that the inhibition was not due to a simple allosteric effect of glutamate, but rather produced as a result of metabolism of the exogenous glutamate. As a further indication of this, no inhibition was obtained when glutamate was incubated with enzyme sample alone, in the absence of the assay components (results not shown). It therefore appears that one or more of the assay ingredients is also required for inhibition.

One of the major routes of glutamate metabolism is transamination to α-ketoglutarate. Therefore, the effect of glutamate on ATP citrate lyase was determined in the presence of a transaminase inhibitor, aminooxyacetate, which inhibits glutamic-alanine transaminase (Hopper and Segal, 1962; Longshaw et al., 1972; Rognstad and Katz, 1970). That the inhibition of ATP citrate lyase by glutamate was reduced in the presence of aminooxyacetate, indicates that such reactions are partly involved in the inhibition observed. In fact, the transamination product of glutamate, α-ketoglutarate, has already been shown to inhibit liver

ATP citrate lyase (Szutowicz et al., 1974b; Simpson, 1981). However, in contrast to the inhibition by glutamate, that of α-ketoglutarate was not found to be time-dependent, and the same effect was produced independent of the age or dietary state of the tissue used (both liver and brain) (Szutowicz et al., 1974b). Furthermore, the authors suggested that it was not of any physiological significance since they reported that the K_i (9mM) was greater than the physiological concentration of α-ketoglutarate (Szutowicz et al., 1975).

If the inhibition of ATP citrate lyase is in fact due to metabolism of glutamate, it is possible that removal of low molecular weight components required for such metabolism would abolish the inhibition. However, the tetrameric enzyme obtained by gel filtration retained its susceptibility to glutamate despite the fact that all low molecular weight components would have been removed. Nevertheless, further investigation was carried out by subjecting crude supernatants of both liver and brain to Sephadex G25 and Sephadex G100 gel filtration. As indicated in Results however, this produced no appreciable change in the susceptibility of the enzyme to such inhibition.

The most convincing evidence indicating an indirect effect of glutamate was provided by the discovery that inhibition was dependent on supernatant concentration. If the inhibition of ATP citrate lyase was due to an effect of glutamate on the enzyme itself, or by its interaction in the enzyme reaction, dilution of the supernatant alone whilst keeping the concentrations of all other assays components and glutamate unchanged, would not be expected to alter the degree of inhibition. However, as already indicated this was not the case. In fact, the inhibition could be completely abolished by sufficient dilution, an effect not due to use of such low enzyme activity that inhibition was not detectable. Therefore, this implies that some component of the

supernatant is required for inhibition of ATP citrate lyase by glutamate.

Szutowicz et al. (1974a) suggested that the inhibition of ATP citrate lyase by glutamate represented a regulatory mechanism for fatty acid biosynthesis. They proposed that in adult brain ATP citrate lyase exists in a greatly inhibited state due to the presence of a high glutamate concentration (10mM compared to a K_i value of 0.3mM for glutamate). This they suggested could explain the low incorporation of citrate into fatty acids in brain. Furthermore, they also suggested that the high ATP citrate lyase activity in liver of starved and refed rats was the result of the presence of enzyme resistant to glutamate inhibition, compared to the low activity in starved animals which represented enzyme susceptible to such inhibition. Therefore, they envisaged a mechanism whereby the presence of glutamate itself affected the activity of ATP citrate lyase, and hence the rate of fatty acid biosynthesis. However, the reason as to why glutamate should be physiologically important in such a mechanism remains unclear.

The results of this study suggest that the inhibition of ATP citrate lyase by glutamate is an indirect effect. Therefore, the different susceptibility of the enzyme from different sources may simply represent differences in the composition of the samples, rather than a property of the enzyme itself. Thus, other components in addition to glutamate are required for inhibition.

As already discussed, transamination of glutamate may play a small part in the observed inhibition of the enzyme. Glutamate is also known to participate in one of the routes of acetyl CoA transport out of the mitochondria (see Introduction, Section 3i b) and Fig. 1), which also involves cleavage of citrate by ATP citrate lyase. However, this pathway is not thought to be of any major significance, and therefore a mechanism whereby glutamate produces inhibition of ATP

citrate lyase by affecting the supply of the precursor citrate seems unlikely. This type of inhibition would not in fact be detected by the assay system used here since citrate is present at saturating concentration. Another possibility is that glutamate indirectly affects the phosphorylation state of the enzyme. Although phosphorylation does not appear to change the activity of ATP citrate lyase, the presence of glutamate may result in the formation of a phosphorylated state in vivo which has not yet been isolated. It is interesting to compare this possible mechanism in which glutamate could affect the phosphorylation of ATP citrate lyase via a protein kinase, with the affect of glutamate on the similar enzyme, citrate lyase from Rhodopseudomonas gelatinosa (Giffhorn et al., 1980). In this case, glutamate inhibits citrate lyase deacetylase by binding to the enzyme, and this in turn prevents the conversion of the active acetylated form of citrate lyase to the inactive, deacetylated form. Clearly, further investigation is required to determine the mechanism of the inhibition of ATP citrate lyase by glutamate before the physiological significance, if any, can be evaluated.

CONCLUSION

ATP citrate lyase has been shown to exist in different forms in crude extracts of rat liver and brain. Furthermore, this heterogeneity of the enzyme is not the same in these two tissues. An investigation of the various chromatographic forms of the liver enzyme did not reveal the nature of the enzyme heterogeneity although a number of possibilities were considered. The results of some of this work have recently been published (Corrigan and Rider, 1983). ATP citrate lyase from rat liver and brain was also shown to be inhibited by glutamate, a property first reported by Szutowicz et al.,(1974a). In contrast to their results, it was concluded that this inhibition is not due to an allosteric effect of glutamate itself.

Recent interest in ATP citrate lyase has focused attention on the possible regulatory properties of the enzyme which is involved in both fatty acid and aceytylcholine biosynthesis. Further investigation of the enzyme heterogeneity and the inhibition by glutamate is therefore required to determine whether either represents a potential regulatory mechanism. Of particular importance in this respect is the role of reversible phosphorylation and the involvement of citrate as an allosteric effector. There is at present some uncertainty as to the physiologically important control mechanism of acetyl CoA carboxylase, following reports of regulation by reversible phosphorylation (Carlson and Kim, 1973; Kim, 1979) and by inhibitor proteins (Abdel-Halim and Yousufzai, 1981, 1982). Hence, in view of this new evidence it is important to consider what role ATP citrate lyase may have in the regulation of fatty acid biosynthesis, since it too undergoes reversible phosphorylation, and as shown in this study it exists in multiple forms. Finally the significance of the tissue differences in the heterogeneity of ATP citrate lyase cannot be determined until more is known of the conditions required to produce the various enzyme states.

APPENDIX

PRD Diet for Laboratory Animals

SPECIES: RATS AND MICE

Developed at Porton by Dr Paterson and Christopher Hill in the 60's. Ideally suitable for breeding and stock holding.

On quality and performance, PRD has a proven record with leading Accredited Breeders, Pharmaceuticals, Universities, etc.

Labsure PRD achievements remain as a tribute to Dr Paterson's original formulation.

Approximate daily feeding rates: Adult Rat 15-20g

Adult Mouse 5g

PRD is manufactured in a 3/8" (9.6mm) Pellet size.

Proximate Analysis	
Crude Oil	2.7%
Crude Protein	19.7%
Crude Fibre	5.3%
Calcium (as Ca)	0.6%
Phosphorus (as P)	0.7%
Salt (as Na Cl)	1.0%
Metabolisable Energy	2568 kcal/kg
Carbohydrate (as %)	53.48

Amino Acids (as percentage of feed)	
Threonine	0.7
Glycine	0.9
Valine	1.0
Cystine	0.2
Methionine	0.3
Isoleucine	0.8
Leucine	1.5
Tyrosine	0.7
Phenylalanine	0.9
Lysine	1.0
Histidine	0.5
Arginine	1.2
Tryptophan	0.2

Trace Elements Added	
Manganese	25 ppm
Copper	7 ppm
Cobalt	0.4 ppm
Iron	30 ppm
Iodine	1.3 ppm
Magnesium	102 ppm

Vitamins Added per kg	
Vitamin A	8,000 iu
Vitamin D_3	1,000 iu
Vitamin B_2	8 mg
Nicotinic Acid	50 mg
Pantothenic Acid	12 mg
Vitamin B_{12}	12 μg
Vitamin E	60 iu
Vitamin K	10 mg
Folic Acid	10 mg
Choline Chloride	200 mg
Vitamin B_1	4 mg
Vitamin B_6	6 mg

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PUBLICATIONS

Some of the results presented in this thesis have already been published, and copies of these publications are enclosed overleaf. Both papers were published under my maiden name of Corrigan.

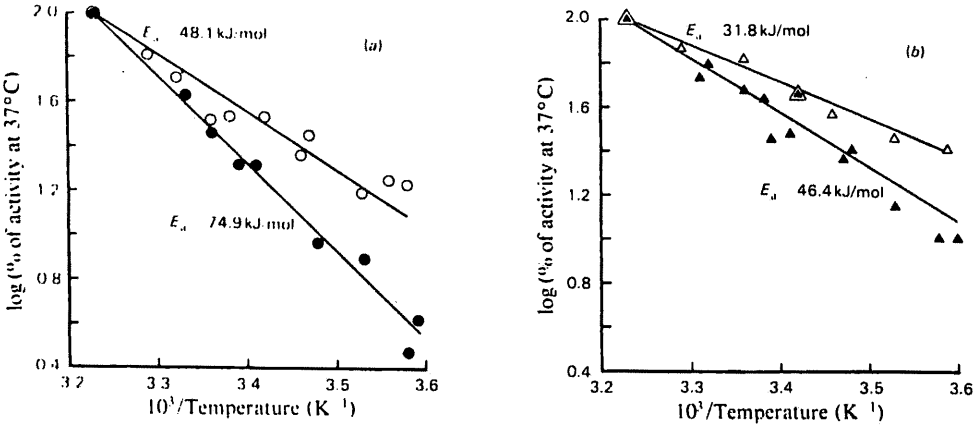

Fig. 2. *Effects of temperature on the reactions of fatty acid synthase*

Arrhenius plots of (*a*) fatty acid synthesis with acetyl-CoA (●) or butanoyl-CoA (○) as primer, and of (*b*) acetoacetyl-CoA reductase (▲) or crotonyl-CoA reductase (△). The lines were drawn with a weighted least-squares procedure, and the slopes were compared with a 'pseudo'-*F* test, both by using the Genstat package (Rothamsted Experimental Station, Herts, U.K.). E_a values were derived from these slopes.

and long-chain fatty acids with the use of acetyl-CoA, acetoacetyl-CoA or crotonyl-CoA as primer. By comparison with the spectrophotometric assay, higher concentrations of substrates and enzyme were used in this radiochemical assay (Kumar & Dodds, 1981), and hence higher concentrations of CoA were necessary to achieve comparable inhibitions. The effects on the incubations with acetyl-CoA or crotonyl-CoA were virtually identical, 29% inhibition being obtained at 400μM-CoA and 56% inhibition at 750μM-CoA. The inhibition was less pronounced with acetoacetyl-CoA as primer, being 7% and 34% inhibition respectively. Radio-g.l.c. analysis of the products of the reaction (Kumar & Dodds, 1981) revealed no difference in the distribution of the various acids formed.

The results of an experiment conducted to determine the Arrhenius activation energies (E_a) of the fatty acid synthase reactions are presented in Fig. 2. With the exception of the acetoacetyl-CoA reductase and butanoyl-CoA-dependent fatty acid synthase pair of lines, the slopes of all the other pairs of lines were significantly different from each other ($P<0.001$). The first condensation reaction (between acetyl-CoA and malonyl-CoA) would appear to have the highest activation energy of all the reactions, but the results could have been affected by different assay conditions, especially pH (Dawes, 1964). In a separate experiment this was shown to be so, when E_a (acetoacetyl-CoA reductase) at pH 7.7 was found to be 51.0 kJ/mol but at pH 7.0 had risen to 69.0 kJ/mol. The presence of 40μM-CoA in this system lowered E_a to 45.2 kJ/mol and 53.6 kJ/mol respectively.

The results presented here are consistent with a speculative mechanism in which both the reductase partial reactions of fatty acid synthase are stimulated by an increase in the negative charge carried by the enzyme, achieved either by raising the pH or by the addition of CoA, which presumably results in allosteric binding. The changes in enzyme charge or conformation, thus effected, result in the lowering of the activation energy of the reductase reactions, while making the condensation reaction less favourable.

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Apparent heterogeneity of ATP citrate lyase from rat liver and brain

ANNE P. CORRIGAN and CHRISTOPHER C. RIDER
Department of Biochemistry, Royal Holloway College, University of London, Egham Hill, Egham, Surrey TW20 0EX, U.K.

ATP citrate lyase (EC 4.1.3.8) produces cytoplasmic acetyl groups in the form of acetyl-CoA. In liver these serve as precursors for lipogenesis (Daikuhara *et al.*, 1968; Watson & Lowenstein, 1970), but in brain they may also be used for acetylcholine synthesis. Since the importance of this enzyme in the latter process is still equivocal despite much investigation (Jope, 1979), it is of interest to compare the properties of ATP citrate lyase from liver and brain.

In order to study brain and liver ATP citrate lyase, partial purification of the enzyme from these two tissues was performed by a method used for the liver (Hoffmann *et al.*, 1979). Whereas they observed only a single chromatographic peak, eluted with KCl, we found an additional peak eluted in advance of the salt gradient and within a single bed volume (Fig. 1*a*). Similar results were obtained for brain extracts, except that whereas the first peak for liver accounts for 10–15% of the recovered activity, for brain it contains nearly 50% (Fig. 1*b*). For the liver enzyme, experiments with smaller sample volumes indicated that this rapidly eluted activity is not an artifact of column overloading. However, on rechromatography of the first peak, activity was eluted only with the gradient, suggesting that the non-retained

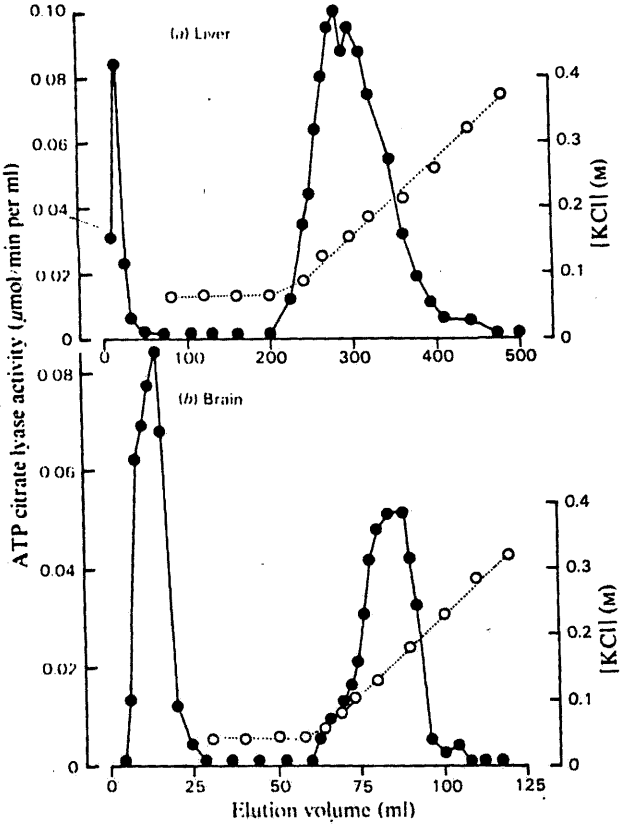

Fig. 1. *Elution of ATP citrate lyase activity in crude extracts of (a) rat liver and (b) rat brain from DEAE-Sephadex A-25 ion-exchanger*

Tissues were homogenized in 4 vol. of 20 mM-Tris/HCl/1 mM-dithiothreitol/1 mM-EDTA/250 mM-sucrose, pH 8.0, and centrifuged at 45000 *g* for 30 min. Supernatants were dialysed in 30 vol. of homogenizing buffer, and samples (*a*) 34 ml and (*b*) 21 ml were applied to columns (*a*) 2.2 cm × 22 cm and (*b*) 1.6 cm × 11 cm and eluted in buffer without sucrose followed by a linear gradient of 0–0.4 M-KCl (○). ATP citrate lyase activity (●) was determined by the method of Szutowicz *et al.* (1974).

enzyme is unstable and is converted into the more acidic form. This may explain why Hoffmann *et al.* (1979) only observed the latter peak.

Gel filtration of liver extracts on Sepharose 6B and 2B also revealed two peaks of lyase activity. The first of these was eluted with an apparent molecular weight of the order of 10^7, whereas the second and major peak corresponded in elution to the accepted M_r value of 450000 (Singh *et al.*, 1976).

The inhibition of lyase activity in the chromatographic peaks separated from liver was investigated by the method of Szutowicz *et al.* (1974), in which the assay mixture was preincubated with L-glutamate (10 mM) for 30 min. For both ion-exchange and gel filtration, the activity of the second peak was inhibited by 40%, whereas that of the first eluted peak was inhibited by 10%.

For liver, the relative proportions of the peaks, and the different effects of L-glutamate, suggest that the high molecular weight activity observed on gel filtration is the same as the more basic, unstable peak separated by ion-exchange. Our results thus indicate that brain and liver ATP citrate lyase exist not only as the free enzyme but also in some form of association. We have been unable to find the activity of the other lipogenic enzymes, fatty acid synthetase and acetyl-CoA carboxylase, in this association. Detergent studies with hepatocytes have indicated that 25% of the ATP citrate lyase activity is associated with mitochondrial membranes (Janski & Cornell, 1980*a*,*b*), and the ability of the purified liver enzyme to bind to mitochondrial preparations has also been demonstrated (Ranganathan *et al.*, 1980). Here we provide direct evidence that such an association is present not only in liver but also in brain. The physiological importance, if any, is at present unclear, but henceforth consideration of the role of ATP citrate lyase in brain must take account of the likelihood that the enzyme has a complex subcellular distribution.

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Disaccharidase activities in the small intestine of lean and obese (*ob/ob*) mice

ANN P. MORTON and PETER J. HANSON
Department of Biological Sciences, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

The activity of the enzymes hydrolysing the disaccharides maltose, sucrose and trehalose is increased in the intestinal mucosa of rats made diabetic by the injection of streptozotocin (Caspary *et al.*, 1972). Human diabetics, provided that they do not also exhibit an insufficiency of exocrine pancreatic function, do not possess elevated intestinal disaccharidase activities (Caspary *et al.*, 1974). Genetically diabetic C57BL/KsJ (*db/db*) mice also have elevated disaccharidase activities in the mucosa of the small intestine, suggesting that the changes seen in streptozotocin-diabetic rats are not a direct [illegible] effects of the drug on the intestine (Ramaswamy & Flint, 1980).

Disaccharidase activities have been measured in the mucosa of the small intestine of Aston C57BL/6J obese (*ob/ob*) mice, since the characteristics of this syndrome represent a milder form of diabetes than that in C57BL/KsJ (*db/db*) mice (Herberg, 1979), and a comparison of the effects of the two syndromes on the intestine is of interest. For example, one possible explanation of the above discrepancy between human diabetics, and streptozotocin-diabetic rats and *db/db* mice, is that in these latter animal models diabetes is much more severe than in man because it is uncontrolled. Thus, if the severity of the diabetic state is a factor influencing disaccharidase activity, there might be a difference [illegible]

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Multiple chromatographic forms of ATP citrate lyase from rat liver

Anne P. CORRIGAN and Christopher C. RIDER*
Department of Biochemistry, Royal Holloway College, University of London, Egham Hill, Egham, Surrey TW20 0EX, U.K.

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ATP citrate lyase is shown to exist as multiple forms in extracts of rat liver. DEAE-Sephadex ion-exchange chromatography of liver supernatants reveals two peaks of activity. A minor, basic, component, comprising 14% of the recovered activity, is eluted without retention, whereas the major, acidic, form is eluted by a KCl gradient. Gel filtration of similar extracts shows the presence of a high-M_r form of ATP citrate lyase (M_r around 10^7) in addition to the tetrameric enzyme (M_r 4.1×10^5). This associated state, which represents 10% of the total activity, is unstable, breaking down to the tetramer, and appears to be disrupted by Mg^{2+}. The basic form changes in the partially purified state to give the acidic form. Most of the high-M_r enzyme is acidic in nature. No evidence could be found for an association of the enzyme with mitochondrial or microsomal membranes. ATP citrate lyase from rat brain also shows two peaks of activity on DEAE-Sephadex ion-exchange chromatography, but the activity is distributed between the peaks in almost equal proportions. However, only the tetrameric enzyme was observed on gel filtration.

ATP citrate lyase [ATP citrate (*pro*-3*S*)-lyase, EC 4.1.3.8] is a cytoplasmic enzyme that in liver and other tissues serves to produce acetyl groups, the precursors for lipogenesis, from citrate exported by mitochondria (Daikuhara *et al.*, 1968; Watson & Lowenstein, 1970). On the metabolic route from carbohydrate to lipid, the ATP citrate lyase reaction is the first enzymic step that is exclusively lipogenic. Because of this position it might be expected that ATP citrate lyase is a regulatory enzyme, particularly since the reaction catalysed is irreversible and consumes ATP. However, it is the next enzyme of the pathway, acetyl-CoA carboxylase, that is usually considered to be the regulatory enzyme of fatty acid biosynthesis.

Interest in the possibility that ATP citrate lyase is regulatory has been stimulated by the discovery that this enzyme is subject to reversible phosphorylation in hepatocytes and adipocytes (Alexander *et al.*, 1979; Ramakrishna & Benjamin, 1979). Both cyclic AMP-dependent and cyclic AMP-independent kinases are involved (Guy *et al.*, 1980; Alexander *et al.*, 1981; Ramakrishna & Benjamin, 1981; Ramakrishna *et al.*, 1981), and this phosphorylation is distinct from that which occurs as part of the reaction mechanism (Janski *et al.*, 1979; Linn & Srere, 1979). As yet it has not been possible to find any regulatory significance of this modification of the enzyme (Ranganathan *et al.*, 1980; Guy *et al.*, 1981).

We have investigated the properties of ATP citrate lyase in rat tissues, and in the present paper provide evidence that the enzyme is not homogeneous but exists as several forms separable by column chromatography. This heterogeneity appears to be independent of the possible mitochondrial association reported for this enzyme (Ranganathan *et al.*, 1980; Janski & Cornell, 1980*a*,*b*).

Materials and methods

Materials

Malate dehydrogenase, substrates, cofactors, M_r-standard proteins, mercaptoethanol and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Sephadex A-25, Sepharose 2B, Sepharose 6B and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). $NaH^{14}CO_3$ (56 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.).

Animals

Adult female Wistar rats were obtained from the colony maintained at 21°C in the Department of Biochemistry, Royal Holloway College, on PRD

* To whom all correspondence should be sent.

diet (Labsure, Poole, Dorset, U.K.). Normal fed rats were allowed food and water *ad libitum*. For most experiments on liver extracts, animals were allowed water only for 48h, and then re-fed for 72h on the above diet with 5% (w/v) sucrose added to the drinking water. This procedure serves to induce the synthesis of liver ATP citrate lyase (Suzuki *et al.*, 1967).

Assays of enzyme activities

ATP citrate lyase activity was determined by a coupled spectrophotometric assay (Srere, 1959) by using a procedure modified from that of Szutowicz *et al.* (1974). The assay mixture contained 50mM-Tris/HCl buffer, pH7.8, 10mM-$MgCl_2$, 100mM-KCl, 5mM-ATP, 0.2mM-CoA, 20mM-citrate, 0.15mM-NADH, 10mM-2-mercaptoethanol, 2 units of malate dehydrogenase and enzyme sample in a final volume of 1.0ml. Assays were preincubated for 7min at 37°C, and the reaction was started by the addition of CoA. The oxidation of NADH was monitored continuously at 340nm in a Beckman 25 or 3600 spectrophotometer. Acetyl-CoA carboxylase (Inoue & Lowenstein, 1975), citrate synthase (Ochoa, 1955), fatty acid synthetase (Lynen, 1969), lactate dehydrogenase (Stolzenbach, 1966) and rotenone-insensitive NADH–cytochrome *c* reductase (Smoly *et al.*, 1971) activities were assayed by established methods. For all enzymes, 1 unit of activity is defined as the amount of enzyme required to transform 1μmol of substrate/min at 37°C.

Column chromatography

Ion-exchange chromatography was performed by the procedure of Hoffmann *et al.* (1979). Tissues were homogenized in 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 1mM-dithiothreitol and 0.25M-sucrose, in a Potter–Elvehjem homogenizer [liver 1:4 (w/v), brain 1:2 (w/v)]. Homogenates were centrifuged at 45000*g* for 30min at 4°C, and the supernatants obtained were dialysed against 30vol. of homogenizing buffer for 2h. Dialysed supernatants were applied to DEAE-Sephadex A-25 columns (see Figure legends for dimensions) previously equilibrated with elution buffer, which was as above but without sucrose. After 2 bed volumes, a linear gradient of KCl (0–0.4M) was applied in the elution buffer. Gel-filtration chromatography was performed in a similar way, except that, since omission of the dialysis did not appear to influence the resulting elution profile, supernatants were normally applied without dialysis. Samples (liver 5ml, brain 10ml) were applied to Sepharose columns (2.2cm × 25cm), which were then eluted with buffer without sucrose, and 2.5ml fractions were collected. The void volumes of the columns were determined by the elution of Blue Dextran 2000. For both types of chromatography, fractions were collected into tubes containing 0.125vol. of 1M-Tris/HCl buffer, pH8.0, containing 10mM-dithiothreitol.

Results

Ion-exchange chromatography

Anion-exchange chromatography on DEAE-Sephadex of liver extracts reveals two peaks of ATP citrate lyase activity (Fig. 1). The first peak is eluted without retention, whereas the second binds to the column, to be eluted by the salt gradient. For a series of five experiments the average recovery of activity in the first eluted peak was 14.2 ± 1.6% (mean ± S.E.M.). Our findings differ from those obtained by Hoffmann *et al.* (1979), who observed only a single peak of liver ATP citrate lyase activity, which was eluted with the salt gradient.

One possible interpretation of our observations would be that the non-retained peak is an artifact arising from overloading the ion-exchanger. If this were the case, decreasing the sample size should give rise to the loss of this first eluted peak. However, when the sample volume was decreased from 34ml to 8ml and with columns of similar bed volume (90ml), there was no such change in the elution profile (results not shown). Hence this possibility can be excluded.

DEAE-Sephadex chromatography was performed under the same conditions on liver from animals starved and re-fed as described in the

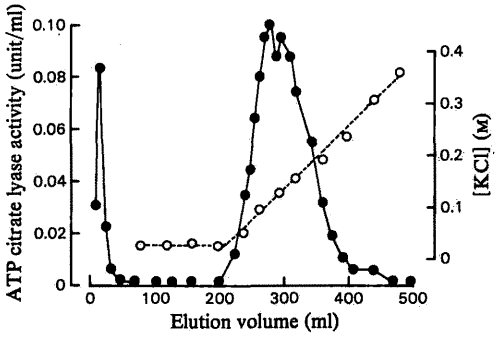

Fig. 1. *DEAE-Sephadex A-25 ion-exchange chromatography of ATP citrate lyase activity of crude extract from rat liver*

Tissue supernatant (34ml) from normal fed rats was applied to a 2.2cm × 22cm column of DEAE-Sephadex. ATP citrate lyase activity (●) was eluted with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA and 1mM-dithiothreitol, followed by a gradient of 0–0.4M-KCl (○). Fractions (8ml) were collected into 0.125vol. of 1M-Tris/HCl buffer, pH8.0, containing 10mM-dithiothreitol.

Materials and methods section. This dietary regimen gave rise to increases between 2- and 7-fold over the normal activity of 1.8 units/g wet wt. of liver. The same two-peak profile of ATP citrate lyase activity was obtained with no detectable change in the percentage of activity eluted as the non-retained peak ($17.7 \pm 3.6\%$, mean $\pm$ S.E.M., $n = 7$). Since the dietary manipulation did not alter the total protein content of the liver extracts, it was possible to compare the profiles for induced and normal fed rats, either by using the same total protein load but different ATP citrate lyase activities, or by using the same ATP citrate lyase activities but different amounts of total protein. This series of experiments (results not shown) produced the same proportion of non-retained activity throughout, consistent in each case with the above values. This is further evidence that the observed two-peak profile is not due to overloading of the ion-exchanger.

When 0.2 mM-phenylmethanesulphonyl fluoride was added to buffers used for homogenization, dialysis and chromatography, the two-peak ion-exchange profile obtained remained unchanged (results not shown). This proteolytic inhibitor protects liver acetyl-CoA carboxylase from proteolysis, which markedly affects the properties of that enzyme (Song & Kim, 1981). Thus neither of the observed ion-exchange peaks of liver ATP citrate lyase appears to be produced as an artifact by proteolytic degradation occurring during or after extraction.

When the dialysis period was extended to 24 h, both ion-exchange peaks were still observed. Similarly, extracts aged for 24 h at 5°C before the normal 2 h dialysis also contained both fractions of activity.

To test the possibility that the heterogeneity revealed here is due to mitochondrial-membrane binding, the chromatographic fractions were assayed for rotenone-insensitive cytochrome *c* reductase activity. Only a single peak of activity was found, and this was eluted with the salt gradient after the second peak of ATP citrate lyase activity (results not shown).

Ion-exchange chromatography of brain supernatants resulted in a similar two-peak elution profile of ATP citrate lyase activity. The only observable difference was that the first eluted peak contained a larger proportion of the total recovered activity, $39.9 \pm 7.4\%$ (mean $\pm$ S.E.M., $n = 3$). This result is significantly different from that for liver from both normal fed and induced rats at the $P = 0.05$ level for both Student's *t* and Mann–Whitney *U* tests.

Gel-filtration chromatography

Further studies of the heterogeneity of ATP citrate lyase were performed by gel-filtration chromatography on Sepharose 6B. As shown in Fig. 2,

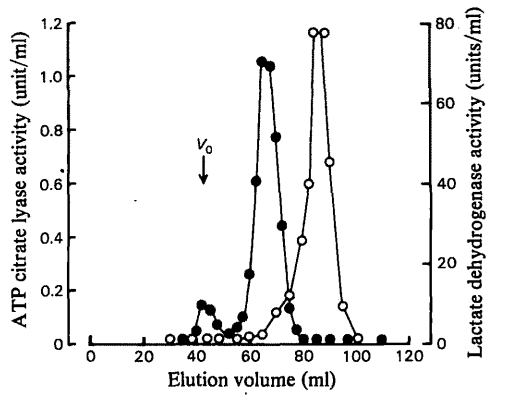

Fig. 2. *Gel filtration of rat liver extract on Sepharose 6B*
Supernatant (5 ml) from liver of a starved and re-fed rat was applied to a 2.2 cm × 25 cm column of Sepharose 6B, which was eluted with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 1 mM-dithiothreitol. ●, ATP citrate lyase activity; ○, lactate dehydrogenase activity. The arrow indicates the void volume (V_0).

there is a minor peak of activity eluted close to the void volume, followed by a major peak containing 90% of the recovered activity. Liver lactate dehydrogenase, used here as a cytoplasmic marker enzyme, shows no equivalent peak of activity at the void volume. This indicates that the high-M_r fraction of ATP citrate lyase is not due to entrapment of cytoplasm by membrane fragments present in the sample.

In a series of nine Sepharose 6B gel-filtration experiments, only one failed to show the presence of ATP citrate lyase activity at the void volume. In all other analyses, approx. 10% of the total recovered activity was present as the void-volume peak regardless of whether the animal had been normal fed, starved or starved and re-fed. In contrast with these results for liver, chromatography of brain extracts on Sepharose 6B always produced only a single peak of activity, corresponding to the major peak of the liver enzyme. This suggests that the high-M_r activity is not a methodological artifact, but arises either from the effect of some component of the liver extract, or from some specialized property of the liver enzyme itself.

The method of Andrews (1965) was used to estimate the M_r of the second-eluted and major peak of liver ATP citrate lyase obtained by gel filtration (Fig. 3). The result, M_r 4.1×10^5, closely agrees with the value, M_r 4.4×10^5, previously obtained by sedimentation equilibrium (Singh *et al.*, 1976). This is the first report of the use of gel-filtration chromatography in the estimation of the M_r of ATP citrate lyase, and it establishes that the major

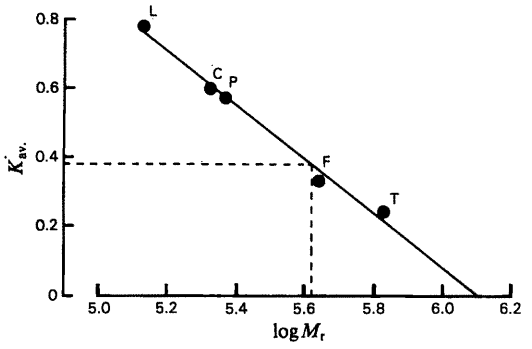

Fig. 3. *Estimation of M_r for the major peak of liver ATP citrate lyase activity by gel filtration on Sepharose 6B*
Calibration standards (●): L, lactate dehydrogenase (M_r 134000); C, catalase (M_r 210000); P, pyruvate kinase (M_r 237000); F, ferritin (M_r 440000); T, thyroglobulin (M_r 669000). The continuous line was plotted by linear-regression analysis. Broken lines indicate the observed $K_{av.}$ value for the major ATP citrate lyase peak of liver (see Fig. 2) and the estimated log M_r value.

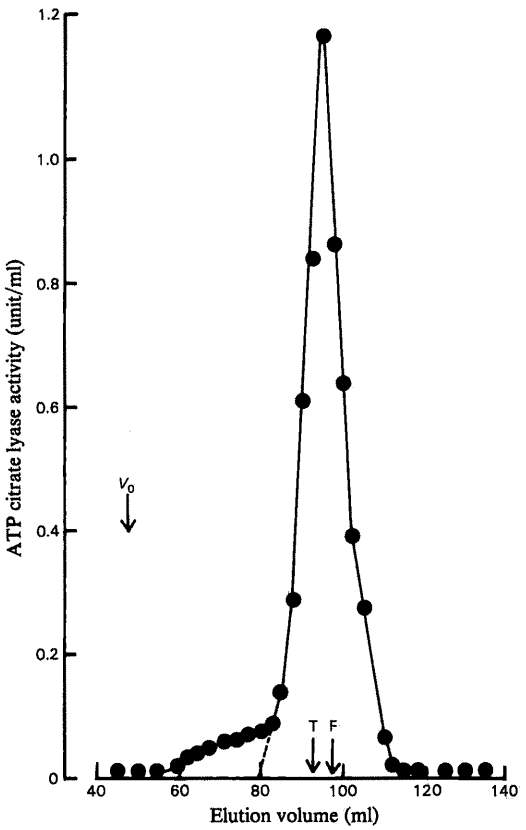

Fig. 4. *Gel filtration of rat liver extract on Sepharose 2B*
Liver supernatant (5 ml) from a starved and re-fed rat was applied to a 2.2 cm × 25 cm column of Sepharose 2B. ●, ATP citrate lyase activity. The arrows indicate the void volume (V_0) and the elution of the standard proteins thyroglobulin, T (M_r 669000), and ferritin, F (M_r 440000). The broken line represents the separation of the two components of the activity and is drawn according to the symmetry of the major component.

gel-filtration peak of the liver enzyme, and the only peak of the brain enzyme, correspond to the tetrameric enzyme molecule. The minor gel-filtration peak of the liver enzyme therefore represents some form of high-M_r association state, which, since it is eluted at the void volume of the column of Sepharose 6B, must be of minimum M_r 4×10^6.

The chromatographic properties of the liver enzyme were further investigated by gel filtration on Sepharose 2B (Fig. 4). Here the elution profile, which is typical of three experiments, shows a gradual increase in ATP citrate lyase activity from just after the void volume until a peak, comprising 90% of the activity and corresponding to the tetrameric M_r, is reached. The profile of the high-M_r enzyme activity suggests that it is not a single species but consists of a range of sizes around M_r 10^7.

Column fractions were assayed for acetyl-CoA carboxylase and fatty acid synthetase activities. Neither of these two lipogenic enzymes was present in the fractions containing the minor, void-volume, peak of ATP citrate lyase, but were found as single chromatographic peaks corresponding to their accepted M_r values.

Sepharose 2B gel-filtration chromatography was also performed on liver extracts homogenized in the absence of sucrose. The sucrose-free extracts contained a higher activity, compared with normally prepared extracts, of the mitochondrial-matrix enzyme citrate synthase (0.011 and 0.001 unit/ml respectively). However, this disruption of mitochondria in the absence of sucrose did not result in any appreciable increase in the proportion of the high-M_r ATP citrate lyase activity (results not shown).

Re-chromatography

To investigate the nature of the multiple forms of liver ATP citrate lyase, a series of re-chromatography experiments was performed in which the most active fractions of the various chromatographic peaks were subjected to a second chromatographic separation (Table 1). On re-chromatography of the first-eluted peak from DEAE-Sephadex, all the recovered activity was eluted only with

the salt gradient. These observations suggest that the basic, non-retained, form has changed into the more acidic and retained form.

This change in the elution characteristics is rapid, since it is seen to be complete even though the re-chromatography is performed immediately. Such behaviour is in contrast with the observation that crude liver supernatants, either dialysed or aged without dialysis for 24h before application to the ion-exchange, retain both chromatographic peaks of activity.

When the major gel-filtration peak was re-run on Sepharose 6B, the activity was recovered at the same elution volume, indicating that the enzyme tetramer is stable. However, re-chromatography of the high-M_r enzyme (void-volume peak) on Sepharose 6B showed that this associated form is unstable, releasing the tetrameric enzyme. The re-chromatography on DEAE-Sephadex of the peaks obtained by gel filtration also yielded only the acidic activity, with the exception of a small amount of activity eluted as the basic form when the major peak from Sepharose 6B was used. As indicated in Table 1, the percentage recovery of activity from the second column was variable, but this can be accounted for by the availability of only small amounts of enzyme during such experiments, and also by the instability of ATP citrate lyase. The enzyme requires thiol protection in the form of dithiothreitol, both in the elution buffer and in the buffer into which the chromatographic fractions are collected (Cottam & Srere, 1969).

Table 1. *Re-chromatography of various chromatographic fractions of rat liver ATP citrate lyase*

Re-chromatography was performed with 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA and 1mM-dithiothreitol, with a step gradient of 0.4M-KCl to elute retained activity from the DEAE-Sephadex columns. Experimental conditions for individual re-runs on DEAE-Sephadex were as follows. DEAE-Sephadex basic peak sample (6ml) applied to a 1.6cm × 9cm column, 1.6ml fractions being collected; this run is typical of three. Sepharose 6B peak samples (2ml) applied to 0.9cm × 4.5cm columns, 1ml fractions being collected. Re-chromatography on Sepharose 6B involved 4.0ml samples applied to 2.2cm × 25cm columns, 2.5ml fractions being collected.

		Re-chromatography on DEAE-Sephadex		
Source of sample	Units applied	Units recovered as basic peak	Units recovered as acidic peak	Recovery of activity (%)
DEAE-Sephadex basic peak	0.31	0.00	0.21	68
Sepharose 6B void-volume peak	0.15	0.00	0.15	100
Sepharose 6B major peak	1.20	0.013	0.63	54
		Re-chromatography on Sepharose 6B		
		Units recovered as void-volume peak	Units recovered as major peak	
Sepharose 6B major peak	3.1	0.00	2.6	84
Sepharose 6B void-volume peak	0.37	0.00	0.36	97

Table 2. *Effect of high-speed centrifugation on liver ATP citrate lyase elution profile on DEAE-Sephadex ion-exchange chromatography*

Livers from two starved and re-fed rats were homogenized as described in the Materials and methods section, and the homogenates were mixed. Samples were then centrifuged as indicated for 90min and the supernatants were dialysed against 50 vol. of 20mM-Tris/HCl buffer, pH8.0, containing 1mM-EDTA, 1mM-dithiothreitol and 0.25M-sucrose. Samples (1.5ml) were applied to 0.9cm × 4.5cm columns of DEAE-Sephadex. The columns were eluted with buffer without sucrose, and after 3 bed volumes a step gradient of 0.4M-KCl was applied. Fractions (1ml) were collected. The values shown are averages for duplicate columns, and the results are typical of three experiments.

		Activity recovered as basic peak		Activity recovered as acidic peak		Total recovered activity	
	Units applied	(units)	(% of applied activity)	(units)	(% of applied activity)	(units)	(% of applied activity)
Control supernatant (45000*g*)	1.2	0.235	19	0.89	73	1.125	92
High-speed supernatant (150000*g*)	0.93	0.46	49	0.53	57	0.99	106
	−0.27	+0.225		−0.36		−0.135	

Effect of high-speed centrifugation on elution profiles of the liver enzyme

A further series of chromatographic analyses was performed on liver supernatants prepared by centrifugation at 150000*g* for 90min, in place of the 45000*g* for 30min normally employed. Sepharose 6B gel-filtration chromatography of this high-speed supernatant (Fig. 5) revealed the presence of only the tetrameric enzyme. A control sample obtained from the same homogenate but centrifuged simultaneously at 45000*g* for 90min was found to contain both major and minor peaks of activity, and in proportions similar to those observed in Fig. 2 (results not shown). The effect of centrifugation on the ion-exchange profile is shown in Table 2. Both ion-exchange peaks are still present in the high-speed supernatant, even though the high-M_r component is absent.

Corresponding to the removal of the high-M_r peak there is a decrease in the total activity of the high-speed supernatant (Table 2). It is of some interest to determine the nature of this lost activity. There is in fact a loss of a similar magnitude from the acidic peak, suggesting that the high-M_r enzyme activity normally resides there.

However, an anomalous increase in activity is apparent in the basic peak. This increase was found in all experiments and, as shown in Table 2, cannot be accounted for by variations in the recovery of activity from the columns. Such an unexpected change in the activity of this peak could mask the loss of enzyme. Thus the possibility that some high-M_r enzyme activity is basic in nature cannot be excluded.

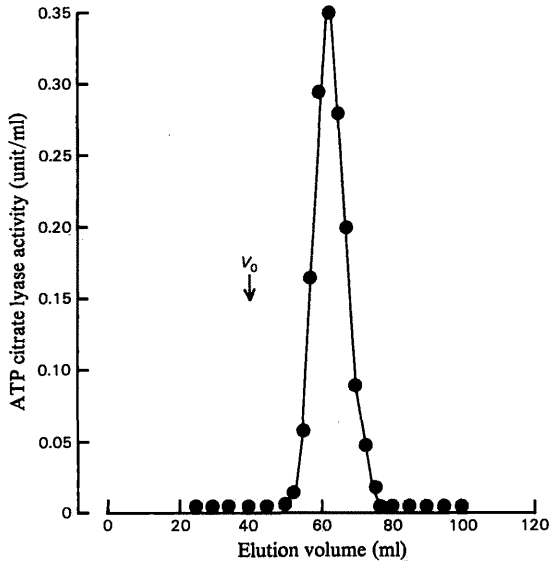

Fig. 5. *Effect of high-speed centrifugation of liver extracts on the elution of ATP citrate lyase activity from Sepharose 6B*
Liver homogenate from starved and re-fed rats was centrifuged at 150000*g* for 90min at 4°C. Supernatant (5ml) was used in a chromatographic separation performed under conditions identical with those described in Fig. 2 legend.

Effect of Mg^{2+} ions on the gel-filtration profile of the liver enzyme

The effect of additional salts on the Sepharose 6B gel-filtration profile of liver supernatants (45000*g*)

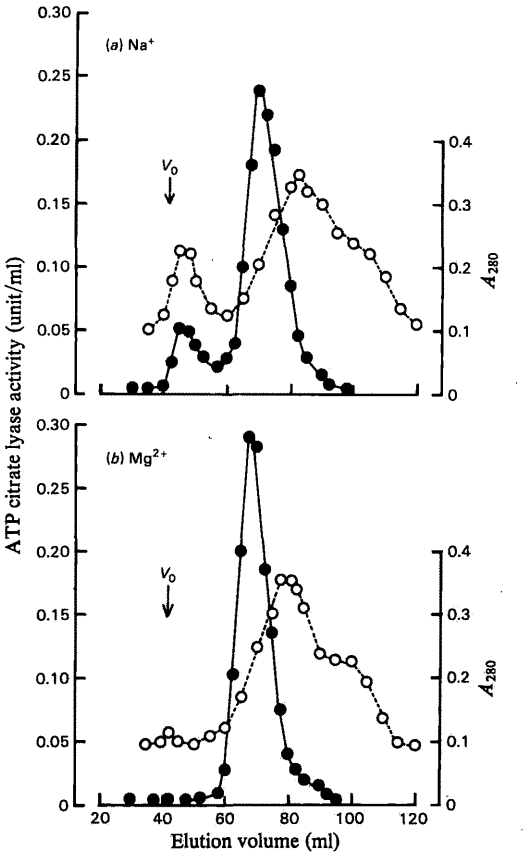

Fig. 6. *Effects of Mg^{2+} and Na^+ ions on the elution of rat liver ATP citrate lyase activity from Sepharose 6B*
Rat liver supernatants were prepared from starved and re-fed rats as described in the Materials and methods section except that all buffers contained either (*a*) 20mM-NaCl or (*b*) 10mM-$MgCl_2$. Supernatants (5ml) were applied to 2.2cm × 25cm columns of Sepharose 6B, which were eluted with buffers containing the respective added chloride salt. ●, ATP citrate lyase activity. The absorbance at 280nm (○) was measured at 1:9 (v/v) dilution of fractions in distilled water. The arrow indicates the void volume (V_0).

was tested by experiments in which either 10mM-$MgCl_2$ or 20mM-NaCl was present in both homogenization and elution buffers. Comparison of Fig. 6(*a*) with Fig. 2 shows that NaCl at this concentration is without apparent effect. However, $MgCl_2$ has caused the removal of the high-M_r peak of activity (Fig. 6*b*). It is also seen that loss has occurred of an A_{280} peak, which in Fig. 6(*a*) is co-eluted with this high-M_r enzyme. Since Mg^{2+} ions do not cause any inhibition of ATP citrate lyase under our assay conditions, and since no ATP citrate lyase activity can be detected in resuspended pellets from liver homogenized in the presence of Mg^{2+}, these observations suggest that the high-M_r component is being disrupted to release tetrameric enzyme.

Discussion

We present here evidence that ATP citrate lyase in crude liver extracts is heterogeneous in both size and ionic charge. Anion-exchange chromatography reveals the presence of a minor, basic, peak comprising 14% of the total recovered activity. The remaining activity binds to DEAE-Sephadex under the conditions employed and is eluted by a KCl gradient. Our demonstration of two ion-exchange peaks in crude tissue extracts contrasts with the results obtained by Hoffmann *et al.* (1979), who in their original use of this method found only a single, acidic, peak of liver ATP citrate lyase.

Gel-filtration chromatography also reveals ATP citrate lyase heterogeneity in crude liver extracts. Approx. 10% exists in an associated high-M_r state (M_r around 10^7), whereas the remaining activity exists as the tetrameric enzyme (M_r 4.1×10^5).

It may be seen that the minor peak of ion-exchange chromatography and the minor component on gel filtration are present in similar proportions, namely 10–15%. Moreover, on re-chromatography both these minor peaks change, since they are eluted as the major form of the activity. The simplest interpretation of the heterogeneity of the liver enzyme is therefore that there would be two forms, one basic and of high M_r, and the other acidic, tetrameric, enzyme. We published such an explanation in a preliminary account of this work (Corrigan & Rider, 1981). However, the observation reported in the present paper that both ion-exchange peaks remain when the high-M_r activity has been removed by high-speed centrifugation renders such a simple account untenable. In this experiment there is evidence for acidic tetrameric, basic tetrameric and acidic high-M_r forms of the enzyme. However, the existence of basic high-M_r enzyme activity cannot at present be excluded.

Our findings indicate that the heterogeneity of brain ATP citrate lyase is less complex than that found in liver. In brain extracts, both the ion-exchange peaks of activity are found, albeit in near-equal proportions. However, on gel filtration only tetrameric enzyme is found, the high-M_r material being absent. Thus brain appears to contain only two forms of the enzyme, acidic and basic, both of which are tetrameric. This demonstration that for the brain enzyme ion-exchange heterogeneity occurs in the absence of size heterogeneity supports our view that for the liver enzyme the two types of heterogeneity are independent of each other.

Prolonged dialysis or aging of crude liver extracts indicates that both ion-exchange forms persist in otherwise untreated supernatants. However, re-chromatography of the ion-exchange fractions establishes that the basic form of the liver enzyme changes rapidly in the partially purified condition to give the acidic peak. Similarly, re-chromatography of the high-M_r enzyme activity shows that this form is also unstable and disintegrates to the tetrameric state. These results suggest that the observed heterogeneity of ATP citrate lyase does not arise from the existence of true isoenzymes, but is due to alternative states in which the enzyme can exist.

Studies elsewhere on hepatocytes with the detergents digitonin and Kyro EOB have indicated that 25% of the ATP citrate lyase activity is either bound to mitochondrial membranes (Janski & Cornell, 1980*a*) or located in the mitochondrial matrix (Janski & Cornell, 1980*b*). Also, it has been shown that 10% of the activity of purified liver ATP citrate lyase binds to mitochondrial preparations (Ranganathan *et al.*, 1980). The integrity of the mitochondria in our preparations is preserved by the presence of iso-osmotic sucrose. Therefore any enzyme bound to mitochondria will have been removed from the supernatants by the centrifugation normally employed. Nonetheless, we have sought to determine whether either the ion-exchange or the gel-filtration heterogeneity could represent ATP citrate lyase attached to fragments of mitochondrial outer membrane stripped off during homogenization. Rotenone-insensitive cytochrome *c* reductase was employed during ion-exchange chromatography as a marker for the mitochondrial outer membrane. As neither of the peaks of ATP citrate lyase is co-eluted with this enzyme, the ion-exchange heterogeneity appears to be independent of any possible mitochondrial binding. Since rotenone-insensitive NADH–cytochrome *c* reductase is also present on liver microsomal membranes (Sottocasa *et al.*, 1967), the possibility of binding to microsomal membranes may be similarly excluded. Furthermore, the persistence of both ion-exchange peaks in supernatants prepared by centrifugation at 150000*g* for 90min (Table 2) and at 100000*g* for 60min (results not shown) also rules out the possibility that the ion-exchange heterogeneity is due to membrane binding.

Gel-filtration chromatography indicates a component of very high M_r, and therefore particular attention to the possibility of mitochondrial binding must be paid here. The fragmentation of mitochondria caused by the removal of sucrose from the homogenizing medium should result in greater yields of mitochondrial membranes in our supernatants. However, no increase in the proportion of high-M_r ATP citrate lyase was detected. Moreover, in subcellular-fractionation studies we have been unable to detect ATP citrate lyase in mitochondrial fractions (results not shown), although this could be due to the instability under the conditions employed of the putative mitochondrial association. Further doubt is cast on the possibility that the high-M_r enzyme activity is bound to mitochondrial membranes by our observations on the effect of Mg^{2+} ions. Janski & Cornell (1980*a*) have shown that 10mM-Mg^{2+} stabilized the association of ATP citrate lyase with mitochondrial membranes in a manner similar to its effect on the binding of hexokinase, another ATP-utilizing enzyme, to the mitochondrial outer membrane (Rose & Warms, 1967). In the present work, however, we find that 10mM-Mg^{2+} appears to disrupt our high-M_r form, liberating the tetrameric enzyme. Finally, binding to membranes, whether mitochondrial or microsomal, is unlikely, since the high-M_r fraction was present in unchanged proportions when the routine centrifugation conditions were replaced by 100000*g* for 60min (results not shown).

It could be that the high-M_r enzyme activity is due to self-association of ATP citrate lyase. Previously, traces of octameric enzyme have been detected in purified preparations of the mammary-gland enzyme, which is otherwise tetrameric (Guy *et al.*, 1981). The possibility that ATP citrate lyase exists in liver as a highly polymerized form in addition to the tetramer thus requires further investigation.

An alternative explanation is that the high-M_r form involves the association of ATP citrate lyase with other cell constituents. The enzyme catalysing the next step in lipogenesis, acetyl-CoA carboxylase, undergoes reversible polymerization in the presence of citrate as part of its regulation, and linear chains of similar size to the high-M_r ATP citrate lyase are produced (Lane *et al.*, 1974). Under the chromatographic conditions employed in the present work, acetyl-CoA carboxylase does not polymerize and therefore is not co-eluted with the associated form of ATP citrate lyase.

In the context of the possible association of ATP citrate lyase with other proteins, it is noteworthy that acetyl-CoA carboxylase has been found to bind to a specific kinase (Lent & Kim, 1982). This protein has been shown to be self-associating as well as capable of binding to the carboxylase. One might speculate that our observations could be explained by the existence of a similar protein binding to ATP citrate lyase.

We have shown in the present work that liver ATP citrate lyase exists in crude extracts in at least three alternative states separable by column chromatography. We conclude that this heterogeneity is not due to binding to mitochondria, nor to co-polymerization with the other lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthetase. Currently, there is considerable interest in the possible involvement of ATP citrate lyase in the short-term regulation of lipogenesis. In particular, although the enzyme is subject to complex reversible phosphorylation, apparently under hormonal influence (Guy *et al.*, 1980; Alexander *et al.*, 1981; Ramakrishna & Benjamin, 1981; Ramakrishna *et al.*, 1981), no progress in finding the regulatory significance of this modification has yet been made (Janski *et al.*, 1979; Ranganathan *et al.*, 1980; Guy *et al.*, 1981). Future work on the regulatory importance of this enzyme should take into account the evidence given in the present paper that ATP citrate lyase appears to exist in various states, some of which are unstable on partial purification.

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