BIOCHEMICAL STUDIES WITH 1-(ε-N-LYSYL)-1-DEOXY-D-FRUCTOSE

A Thesis submitted by

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a candidate for the Degree of

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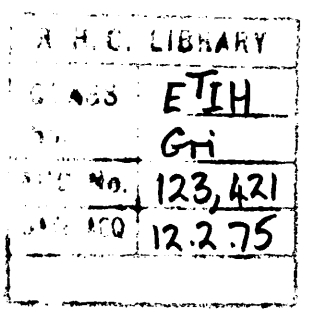
in

BIOCHEMISTRY



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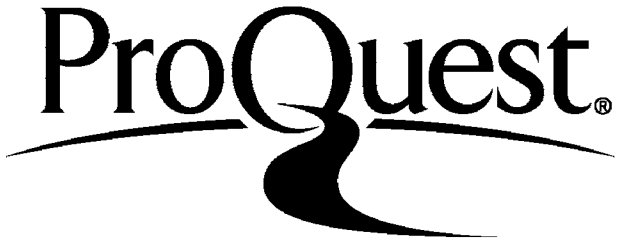

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Abstract

An investigation has been made of the biochemical properties of 1-(ε-N-lysyl)-1-deoxy-D-fructose (fructose-lysine) a derivative which can probably arise from dried milk powder by the action of intestinal enzymes. This compound was synthesised chemically, purified by column chromatography and subjected to a number of analytical procedures including electrometric titration, chromatography and electrophoresis and ^{13}C- and proton-nuclear magnetic resonance, all of which confirmed the authenticity and purity of the preparation. The molecule was unstable in both acid and alkali and at room temperature.

Simple fusions of sugars and lysine (HCl) were studied and methods devised for the preparation of 1-(ε-N-[U-^{14}C]lysyl)-1-deoxy-D-fructose and 1-(ε-N-lysyl)-1-deoxy-[U-^{14}C]D-fructose. A sample of 1-(ε-N-lysyl)-1-deoxy-D-lactulose (lactose-lysine), prepared by heating lactose with lysine HCl, was also obtained by a fusion procedure. This compound is also probably produced from dried milk powder in the alimentary tract.

Growth curves of E.coli B have been studied in the presence and absence of fructose-lysine in the medium. The bacterium was found to be impermeable to fructose-lysine but this compound was able to support colonial growth after the induction of an extracellular cell-bound enzyme(s) capable of the breakdown of fructose-lysine. During growth significant amounts of $^{14}CO_2$ were produced from fructose-lysine labelled with ^{14}C in the fructose moiety showing that the combined hexose was utilisable as a carbon source. The products of the breakdown of radioactive fructose-lysine were examined by chromatographic and electrophoretic techniques. Lysine was identified in the culture media and this suggested that hydrolysis of the C-N bond linking the amino acid to the sugar occurred. A stable compound derived from labelled fructose-lysine and produced apparently without cleavage of the C-N bond was also detected but not fully characterised. The possible existence of other intermediates has been postulated.

Rat liver slices, homogenates and mitochondrial preparations were shown to be capable of slowly metabolising fructose-lysine as judged by $^{14}CO_2$ formation from fructose-lysines labelled in either the fructose or the lysine moieties with ^{14}C and as shown by the appearance of degradation

products in the reaction mixtures. The sugar-amino acid C-N bond was not thought to be cleaved by liver enzymes.

The possible role of fructose-lysine as an enzyme inhibitor was examined using enzyme systems *in vitro* and the molecule was observed to serve as a weak inhibitor of the amino acid-metabolising enzymes, lysine decarboxylase and L-amino acid oxidase, and of some glycosidases. Some preliminary kinetic studies suggest that inhibition was of the competitive type. Fructose-lysine served as a substrate for oxidation by L-amino acid oxidase Lactose-lysine was hydrolysed by β-galactosidase to fructose-lysine and galactose and also acted as an inhibitor of lactose hydrolysis by this enzyme.

The fate of the sugar amino derivatives from milk powder is discussed in relation to their possible effects on digestion and general metabolism of infants.

Contents

Abbreviations

Abbreviations throughout this thesis follow the conventions recommended by The Biochemical Journal (73) with the following in addition:

FL or ε-FL	- 1-(ε-N-lysyl)-1-deoxy-D-fructose
LL	- 1-(ε-N-lysyl)-1-deoxy-D-lactulose
FL*	- 1-(ε-N[U-^{14}C]lysyl)-1-deoxy-D-fructose
*FL	- 1-(ε-N-lysyl)-1-deoxy-[U-^{14}C]D-fructose
I	- Inhibitor
PPO	- 2,5-Diphenyloxazole
POPOP	- 1,4-Di-2-(5-phenyloxazolyl)-benzene
Km	- Michaelis Constant
^{14}C-	- [U^{14}C]

I N T R O D U C T I O N

The object of this study was to investigate the biochemical properties of 1-(ε-N-lysyl)-1-deoxy-D-fructose, a compound which can be obtained by proteolysis of dried milk powder. No work on this compound or related sugar-amino acid derivatives possessing a C-N bond had previously been reported in the literature with the exception of some superficial dietary studies. It was thought that the results of this research might provide some insight into the reactions in vivo of this molecule in animal systems with particular reference to its metabolism in infants. Hence a general survey of the reactions of the compound both as an enzyme substrate and as a metabolic inhibitor of gut and liver and bacterial enzymes was of interest.

1-(ε-N-lysyl)-1-deoxy-D-fructose is an example of a sugar-amino acid complex derived from the corresponding glycosylamine. Glycosylamines result from the condensation of one molecule of a reducing sugar with one molecule of ammonia or of a primary or secondary aliphatic, alicyclic or aromatic amine. Ellis and Honeyman (1) have published a general review of glycosylamine chemistry. N-Substituted glycosylamines can, for example, be formed by the interaction of the non-protonated amino group of an amino acid, peptide or protein and the glycosidic hydroxyl group of a sugar. This reaction is facilitated by heating the compounds together, either in solution or as solids. Most glycosylamines of the sugar-amino acid type are unstable in aqueous solution and spontaneously isomerise, by means of the so-called 'Amadori rearrangement', to N-substituted 1-amino-1-deoxy-2-ketoses, also known as 'Amadori products'. The Amadori rearrangement has been reviewed by Hodge (2); several mechanisms for this reaction have been proposed. The reaction is catalysed by acid and thought to involve the cation of a Schiff-base (Fig. 1) and is essentially irreversible. Hence, the resulting 1-amino-1-deoxy-2-ketoses are fairly stable, especially when stored in the cold. The compounds are usually colourless and may be obtained as crystals which melt with decomposition. Most fructose-amino acid derivatives reduce ferricyanide in 0.1 N-alkali at room temperature (5). On heating with acid they decompose, rather than hydrolyse, with the result that the yield of free amino acid is low and the sugar moiety gives decomposition and rearrangement products. The glycosylamine formed by the reaction (see Fig. 1) between glucose and

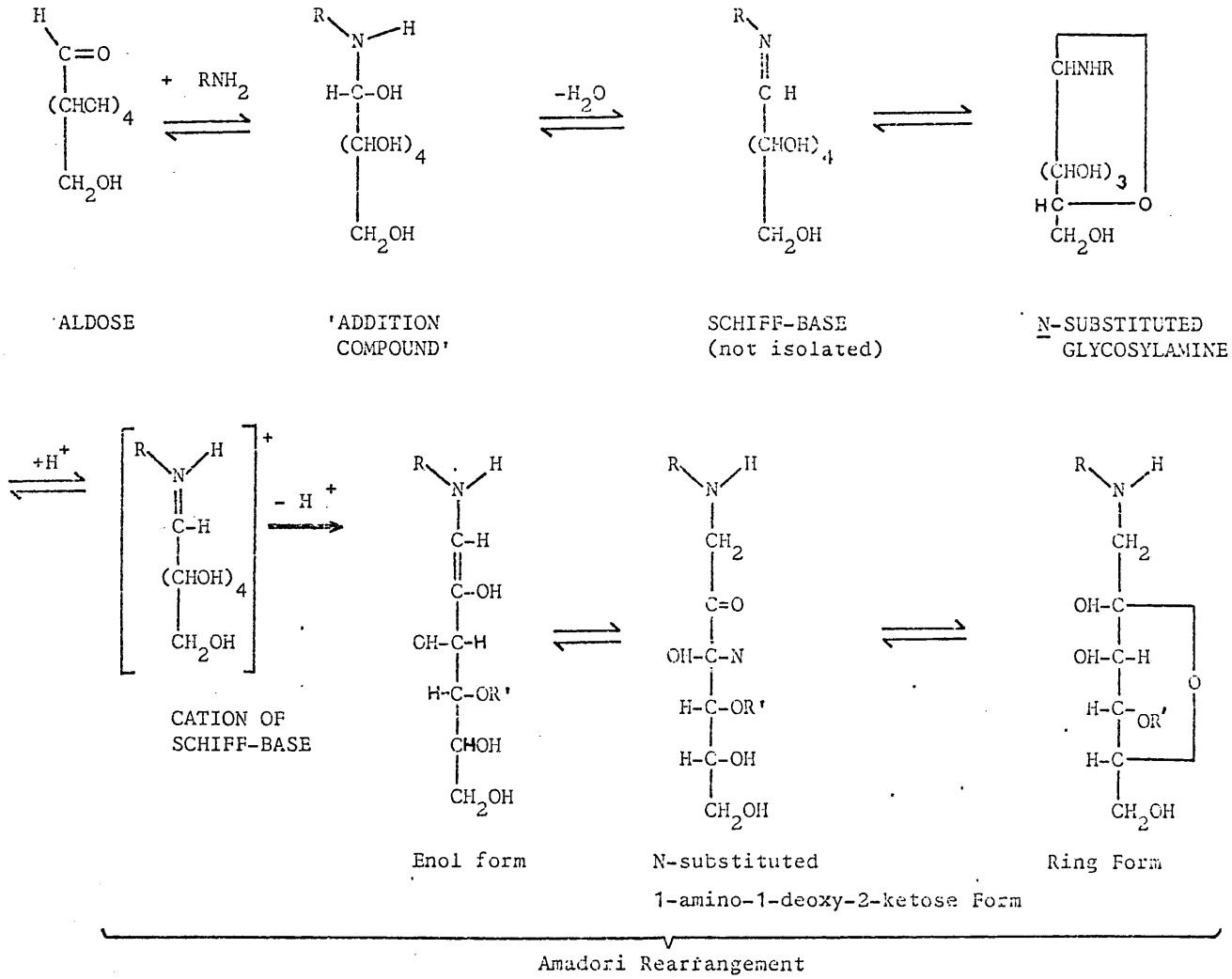
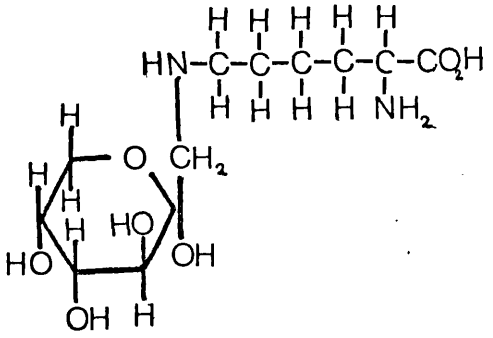


Fig. 1. The formation of N-substituted 1-amino-1-deoxy-2-ketoses via N-substituted glycosylamines.

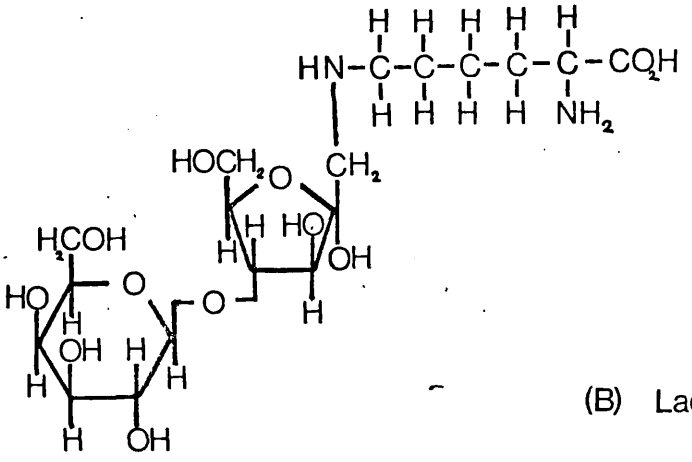
For fructose-lysine, R' = H

lactulose-lysine, R' = β-D-galactosyl

R = $(CH_2)_4 CH(NH_2)CO_2H$



(A) Fructose–Lysine



(B) Lactulose–Lysine

Fig. 2. Howarth formulae for 1-(ε-N-lysyl)-1-deoxy-D-fructose (A) and 1-(ε-N-lysyl)-1-deoxy-D-lactulose (B)

the ε-amino group of lysine is unstable because the free carboxyl group of lysine provides the proton necessary for the rearrangement. The Amadori product in this case is 1-(ε-N-lysyl)-1-deoxy-D-fructose, the main subject of this study (see Fig.2). The nomenclature given in this instance follows the rules of the IUPAC-IUB Commission on Biochemical Nomenclature (40) but the compound may also be named as an amino acid derivative, ie N-ε-(1-deoxy-D-fructose-1)-lysine. The trivial name, 'fructose-lysine', is also used in the literature (3), and will be used hereafter in this thesis together with an abbreviated form "FL".

Some biochemical properties of a related compound, lactulose-lysine (LL) formed by the reaction of lactose with the ε-amino group of lysine which can also be derived from dried milk powder (see Fig.2) were also studied.

Fig. 2 depicts the most likely structures for FL and LL but, presumably, mutarotation between the α- and β- forms at C-2 is possible and the existance of an enol form has been suggested by several authors (1,2,4) (see Fig.1). Also in the literature (2) the sugar moiety of FL has been depicted in the furanoid form. The exact course of the Amadori rearrangement is unknown although several possible mechanisms have been proposed (2,4).

Amadori rearrangement products such as FL first became of interest with the discovery of the Maillard reaction in 1912 (6,9). Maillard observed that simple amino acids reacted on heating with certain sugars to form dark brown products. The reaction has also been called non-enzymic browning, and the brown products have been referred to as melanoidins, 'humins', aterins and orpheins. The Maillard reaction has been reviewed by Ellis (10); Hodge (11) has reviewed the chemistry of browning reactions in model systems. The first step in the browning reaction involves the formation of glycosylamines as previously described (see Fig.1); occasionally an acid catalyst eg. HCl or NH_4Cl may be necessary, especially if the amine is a weak base. Excess acid catalyses the Amadori rearrangement. Subsequent browning, caused by the formation of unsaturated coloured polymers of varying composition, occurs by a complex series of reactions, involving both N-substituted glycosylamines and/or their Amadori

rearrangement products, including sugar dehydration (acid catalysed), amino acid degradation, sugar fragmentation (alkali catalysed), aldol condensations, aldehyde-amine polymerisation and the formation of heterocyclic nitrogen compounds. Reaction mixtures normally change through yellow and brown to a deep red, the extent of colour formation being a rough guide to the progress of the reaction. Maillard reactions in the case of Amadori compounds, are thought to begin with the enol form of the sugar moiety. Some details of the Maillard reactions of FL are given in Fig.3. Glucose most readily undergoes non-enzymic browning in the presence of lysine: this amino acid is regenerated during the reaction giving the effect of true catalysis. The course of the Maillard reaction varies with temperature, moisture content and pH. When compounds such as FL are synthesized many Maillard products are also produced which hinders purification.

The Maillard reaction continues to be a subject of much investigation in the field of food technology (eg. 13-16). The formation of melanoidins and related compounds is thought to be mainly responsible for 'off-flavours' (17,18) in stored foods; the reaction is also thought to be a cause of the loss of nutritive value of proteins during the heating of foods and such changes are often accompanied by yellowing or browning. It is well known that during the manufacture of roller-dried milk powders the Maillard reaction can take place between lactose and the ε-amino groups of the protein lysine residues; these represent about 90% of the total free amino groups in casein. Hence LL has been recovered (along with numerous other derivatives of the Maillard reaction) from the degraded product obtained by heating lactose and casein together in the solid phase (18). Lysine availability in the proteins of heated foods has been closely studied by food technologists (19-24) and it has been estimated that up to 60% of casein lysine can be glycosylated in badly scorched milk powders (3,24-26).

Recently, interest in the specific compounds FL and LL has arisen from observations made at the Institute of Child Health, London, with infants fed with Half-Cream National Dried (HCND) milk. It was found in some cases that, with infants up to 10 days old who showed symptons of diarrhoea due to diseases of the intestine (eg. cystic fibrosis or, especially, coeliac disease), feeding on diets containing reformulated

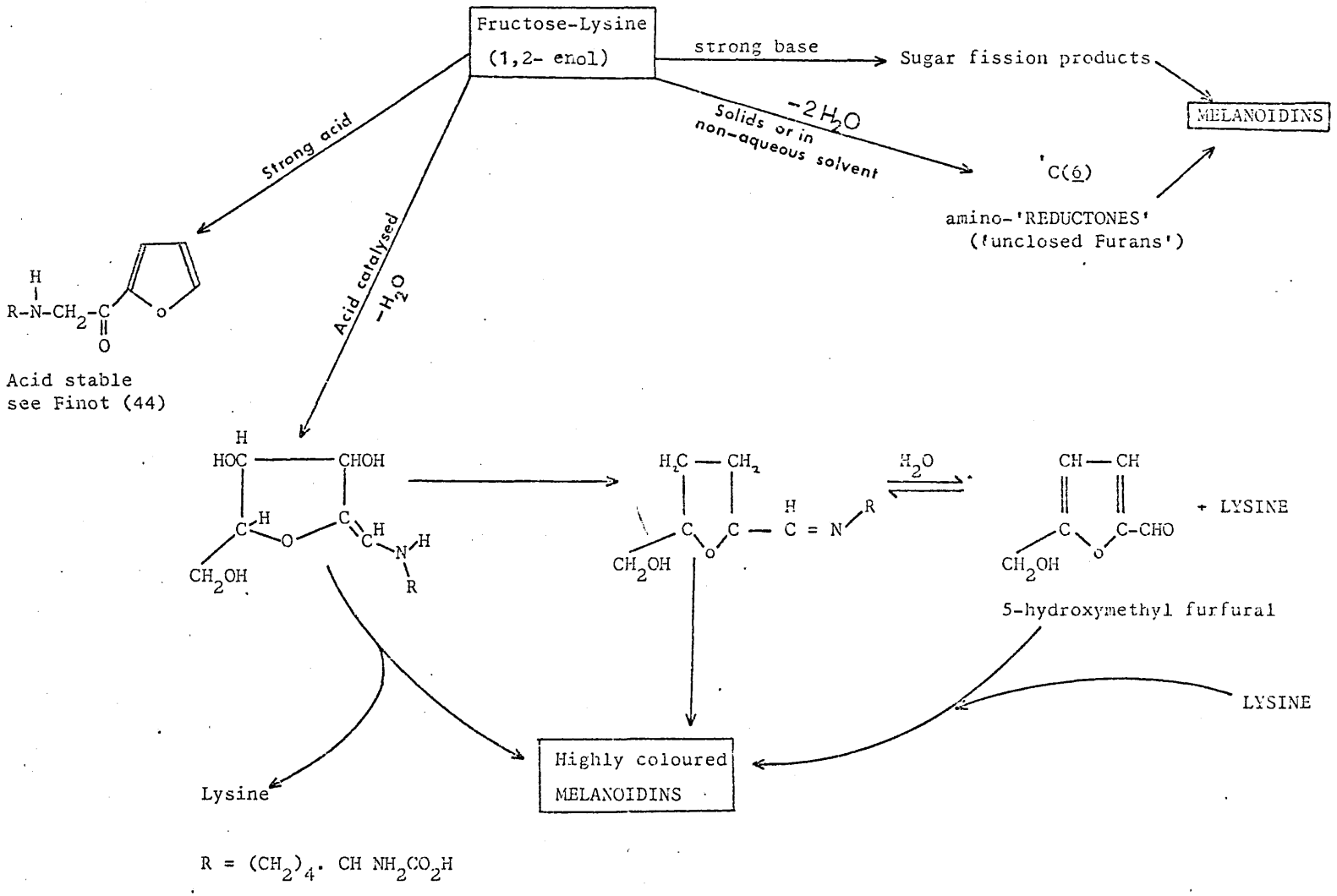


Fig. 3. Some initial Maillard reactions of FL.

HCND milk powder frequently worsened the condition. Their condition often improved when the dried-milk was removed from the diet and was replaced by chicken or rabbit protein (mixed with vegetable oil and suitable carbohydrate). In another group of infants (0-10 days old), being fed on HCND milk from whom faecal extracts were being taken and analysed for amino acids and peptides (Ernser and Seakins, 27-31). the presence of compounds thought to be FL and LL was noted. Thus there was circumstantial evidence to suggest that infants fed dried-milk diets were receiving quantities of LL and FLderived from the milk protein after digestion and that these compounds could possibly be linked with the worsening of the diarrhoea (Hagan (20) observed that rats fed fructose-glycine had diarrhoea during the entire feeding period). Glucose exists in minor amounts in bovine milk and hence only very small amounts of FL would be expected to be formed from the Maillard reaction during processing. However, FL was often observed to be more prevalent than LL in the faecal extracts of 0-10 day infants (30) and, since lactose 'browns' casein in the same way as glucose (32), faecal FL was thought to have been mainly derived from LL by the removal of galactosyl moieties by gut or intestinal microfloral enzymes (30). Thus, since FL appeared to be the main faecal product resulting from the breakdown of heat-damaged milk protein and a published method for the synthesis of this compound was available; our researches were mainly directed towards FL rather than the 'parent' compound, LL.

The manufacture of the HCND milk powder is carefully controlled and accidentally scorched batches are never used for baby-foods. Thus the formation of FL and LL probably occurs when the reformulated milk is sterilised and when it is again rewarmed before use. The Maillard reaction could also occur if the powders are stored for long periods (41); however, HCND packages are, for this reason, marked with 'shelf-lives'. Thus, although lysine is an essential amino acid, the small amounts made unavailable by Maillard condensations are probably not significant from a dietary point of view. Other foreign compounds which could aggravate the diarrhoea may of course be formed during the milk reformulation process; lactulose is one such example and, it should be noted that this sugar is commonly used as a laxative for adults.

A variety of causes may produce diarrhoea in infants; sometimes,

during infection, the intestinal mucosa becomes 'flattened' and disaccharidase activities, particularly lactase (33), are depressed. Consequently in this condition with heavy dietary loadings (eg. one milk-feed) undigested lactose is likely to pass through the small intestine and may then cause diarrhoea by inducing an osmotic water loss. Also, the lactose could then be fermented by the bacterial flora of the large bowel, resulting in the formation of volatile acids and CO_2 which would also contribute towards the diarrhoea. Lactulose, which is not degraded by human digestive enzymes also reaches the colon and is fermented. FL and LL could, in theory, also worsen the diarrhoea by encouraging fermentation in the large intestine and by inducing an osmotic water loss. With regard to the former, Borodina et al. (34) found that N-D-fructosyl glycine stimulated the growth of Lactobacillus casei and Erbersdobler et al. (35) using heat-damaged protein containing high levels of FL, claimed that crude preparations of adult rat gut-microorganisms deaminated FL. Ersser et al. (30) suggested that the unusually large amounts of lysine which they detected in faecal specimens (which also contained FL and LL) from infants on a HCND milk diet could have been a result of partial hydrolysis of FL or LL by the gut bacteria.

FL and LL might also directly affect the growth pattern of colonic bacteria utilizing other substrates and, if this disturbed the microfloral equilibrium, which in the newborn is in the process of development and in a delicate state of balance, diarrhoea symptoms might well result. FL and LL could also function as enzyme inhibitors: as they possess both amino acid and carbohydrate moieties they could well interfere with carbohydrate- and protein-digesting enzymes in the gut (eg. disaccharidases) and hence allow increasing amounts of undigested materials to pass into the large bowel.

The fact that large amounts of LL and FL were found in infant faecal specimens suggests that these compounds may not be actively absorbed by the small intestine into the circulatory system. FL (mol. wt. 308) and LL (mol. wt. 470) are above the accepted maximum size (mol. wt. 200-250) for intestinal absorption to occur readily. However, small amounts of FL and LL were also detected in the urine by Ersser and Seakins (30) and this may have resulted from passage of the compounds

through the wall of the intestine into the blood-stream by passive diffusion followed by excretion in the kidneys. Valle-Riestra and Barnes (36) conducted feeding experiments with rats using ^{14}C-L-lysine-labelled egg albumen heated with glucose which also indicated that labelled Maillard products were in the urine. Erbersdobler (22) was also able to detect radioactivity in the portal vein of adult male rats after incorporation of ^{14}C-lysine-labelled FL into jejunum segments. The recovery of FL and LL in the urine after feeding heat damaged milk protein also indicates that the α-peptide linkages immediately adjacent to the lysine residues with blocked ε-amino groups may be hydrolysed in the intestine. Bjamason and Carpenter (37) made similar observations based on their studies with ε-*N*-acylated lysine units of proteins. Valle-Riestra and Barnes (36) indicated that the intestinal flora might participate in this peptide digestion.

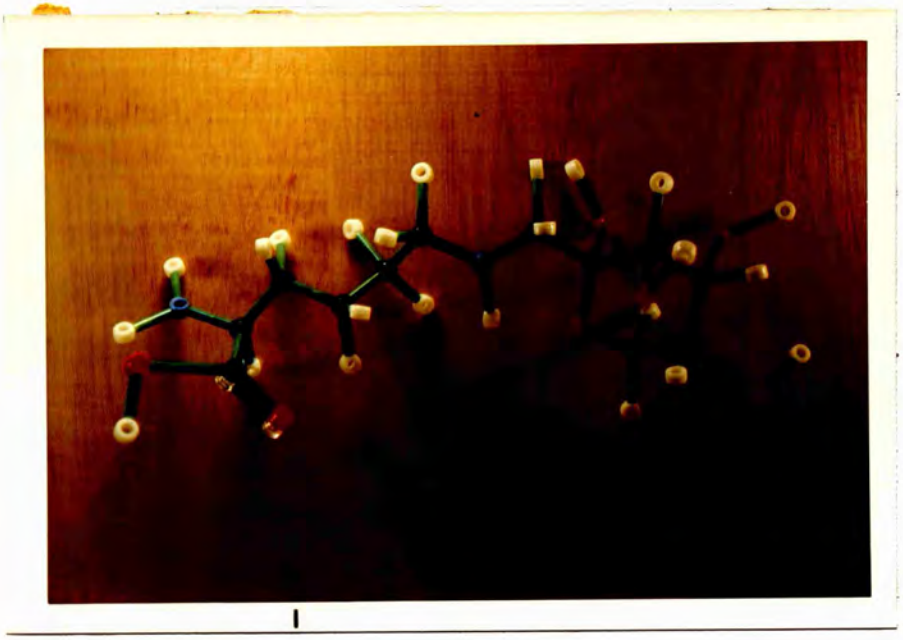
It is not known whether some of the FL or LL molecules absorbed into the blood stream are subsequently detoxified or otherwise metabolised in the liver or other body organs, or whether all are excreted in the urine unchanged. Early beliefs that some fructose-amino acids (other than FL) acted as intermediates in protein synthesis in rabbit reticulocytes (5,48) are now recognised as being erroneous (20).

A number of authors have noted the loss of nutritive value of foods after heating (20,21,23,24,26,36,38,42,43) and have employed feeding experiments with rats as a means of evaluation. Bujard *et al.* (3) estimated the percentage of lysine *eg.* in heated milk powders that had been inactivated by the Maillard reaction, by calculating the amount of free lysine which could not be released by complete hydrolysis *in vitro* (with pepsin and pancreatin *cf* the use of papain by Lea and Hannan (39) or by measurement with lysine decarboxylase). Since lysine is essential for the normal growth of rats they were also able to show (24), by means of a bioassay, that the percentage of biologically unavailable lysine in heat-damaged dried milk powder, was in close agreement with the above values obtained by the hydrolytic method. Van den Breul (26) also showed, using a rat bioassay, that chemical loss of lysine in milk could be directly correlated with the loss of biological activity; an improvement could be effected by supplementing the diets

with lysine. The usual conclusion drawn from feeding experiments with rats is that lysine, which is inactivated by the Maillard reaction, is not utilised; it therefore seemed unlikely that compounds such as FL could be metabolised.

When this study was initiated there was little evidence to suggest that FL or LL could act as enzyme substrates or affect the rate of enzyme-catalysed reactions. Indeed the feeding studies suggested that the sugar-lysine bond was relatively stable. Nevertheless the clinical observations on infant diarrhoea was partly interpretable in terms of bacterial metabolism of these compounds and possibly that cleavage by bacterial or human enzymes with the release of lysine could also occur.

The aim of this research project was, therefore, to carry out biochemical studies with LL and, in particular, FL on a broad front in an endeavour to discover whether these compounds could be metabolized and/or could interfere with normal human and bacterial metabolism. It was hoped that this would clarify possible relationships between heat-damaged milks and infant diarrhoea.



Fructose-Lysine

Lactulose-Lysine

Plate 1 **Molecular Models of FL and LL**

Black - C

Blue - N

Red - O

White - H

RESULTS AND DISCUSSION

Section I

Preparation and Properties of Fructose-lysine

Preparation. N-substituted 1-amino-1-deoxy-2-ketoses are easily synthesised by the classical method of heating amino acids and reducing sugars together. However, in some initial heating experiments in an attempt to prepare FL (Fig. 1) from glucose and lysine, literature reports were confirmed and many other compounds, some with very similar properties to FL, were observed to be produced. This problem arises because any conditions employed for the formation of sugar-amino acid complexes, such as FL, are also likely to catalyse their degradation and to allow isomer formation. It was intended to use the fusion method to prepare FL, and in particular, small amounts of ^{14}C-labelled forms because of the simplicity of the procedure. However, it was first essential to obtain an authentic specimen of FL by an unequivocal synthetic method in order to be able to clearly identify FL in mixtures of compounds resulting from fusion reactions. It was also necessary to prepare relatively large quantities of unlabelled FL for metabolic studies.

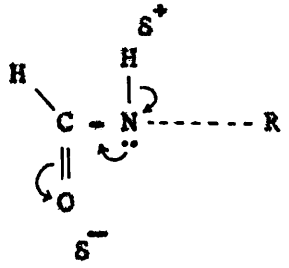
A chemical synthesis of FL in good yield had previously been reported by Finot and Mauron (1969, (12)) and, although they had described the compound as a 'fructosyl' derivative (incorrectly implying the presence of a glycosidic linkage) the method described was specific for the required ε-isomer of FL. The procedure (see Fig.4) involved blocking the α-amino group of L-lysine with a formyl group, reacting the acylated amino acid with glucose and then purifying the α-formyl fructose-lysine (α-fFL), so formed, by cation-exchange chromatography. The product was then deformylated with acid and again purified with an ion-exchange column. Finot and Mauron recommended the Hofmann procedure (47) for the preparation of α-formyl lysine via α-lysine formate from L-lysine monohydrochloride. None of the lysine derivatives used in this synthesis were commercially available other than the starting material, L-lysine HCl.

When the synthetic method was attempted as described in the publication by Finot and Mauron (12) many difficulties were encountered. For example, the isolation of α-formyl lysine proved to be a problem as the compound did not readily crystallise. The melting point of 187-188°C recorded by Hofmann could not be obtained but three successive re-

crystallisations gave needles with a sharp melting point of 180°C which were homogeneous and distinguishable from the authentic ε-isomer. Hence the derivative was assumed to be the required α-isomer.

The isolation by precipitation of α-fFL from column eluates was attempted using methyl ethyl ketone (MEK), as described by Finot and Mauron (12). The expected white precipitate appeared but prolonged stirring, as recommended, caused the compound either to redissolve completely or to collect on the sides of the beaker as a viscous oil. The α-fFL obtained by precipitation with acetone was hygroscopic and 'melted' at approximately 105°C, with decomposition, (cf 106-109.5°C reported by Finot and Mauron (12)).

The deformylation process using 2N-HCl at 100° as described by Finot and Mauron (12) (who claimed an 80% yield of purified FL from this reaction) was very inefficient and, in fact, only 10-20% as judged by thin layer chromatography of the α-formyl fructose-lysine appeared to have been deformylated. Deformylation might be expected to be difficult since the C-N bond between a formyl group and a secondary amino group has partial double bond character and hence is very stable;



Longer periods of heating the formyl derivative with 2N-HCl and treatments with 6N-HCl, 2N-NaOH and 3N ethanolic HCl were tried. The optimum conditions appeared to be with 2N-HCl heated (at 100°C) for 40 min when deformylation was approximately two thirds complete. Stronger acid or longer heating times did not increase the yield of FL but increased the number of other degradation compounds observed on chromatograms. 2N-NaOH gave rise to several decomposition products but did not effect deformylation. Since the C-N bond by which the formyl group is attached to the amino group has some resemblance to a peptide link, the cleavage of this bond was attempted with peptidase and chymotrypsin but without success. Porcine acylase, which hydrolyses N-formyl lysine (37) was also inactive. The absence of lysine from chromatograms of the above digests suggested that the C-N bond

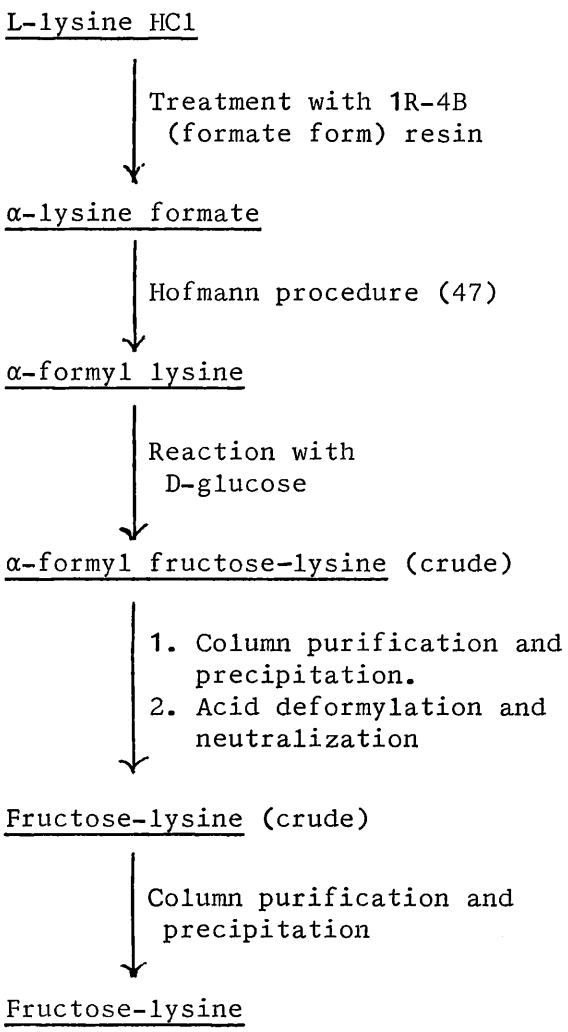


Fig. 1.1 Flow diagram summarizing the synthesis of FL as described by Finot and Mauron (12)

between fructose and lysine was, as expected, also not hydrolysed by any of these enzymes.

As with the α-fFL, Finot and Mauron (12) recommended the use of MEK to precipitate FL from column fractions. Again, acetone was found to be a better solvent for this purpose. In particular, it was very difficult to remove last traces of MEK from FL and it was thought that this might produce biochemical abnormalities in subsequent studies.

During the synthesis of FL, the compound itself and the various intermediates were subjected to long periods of time at room temperature, eg during column elutions, and at 35°C for repeated rotary evaporations; such conditions were conducive to slow self-decomposition. In addition, where pyridine acetate and formate buffers were removed by rotory evaporation the resulting concentrated syrups were strongly acid, which also facilitated decomposition. Hence a modified method for the preparation of FL from α-formyl fructose-lysine was developed where the time factor was reduced to a minimum and deacidification was quickly effected where required with N-N-dioctylmethylamine. Also the step requiring the use of an ion exchange column for the removal of the acid used during deformylation was also replaced by a stage employing N-N-dioctylmethylamine. This modified procedure for the synthesis of FL is described in the 'Materials and Methods' section (p.122).

Properties of fructose-lysine. The FL was isolated as a white, hygroscopic powder in 2.0-2.5% yield (cf a yield of 0.1-0.3% obtained using the original Finot and Mauron method (12)). Attempts to crystallise it from methanol or ethanol/water mixtures were unsuccessful and a concentrated FL solution (aqueous) left for 2½ years in the cold did not crystallise. The correct empirical formula for the sample used in subsequent biochemical experiments was varified by elemental analysis (Found: C, 46.51; H, 7.79; N, 8.91; O, 36.51; Calculated for C,H,N,O : C, 46.72; H, 7.809; N, 9.09; O, 36.36%).

In view of the fact that FL was very hygroscopic and insoluble in most solvents other than water, Infra-red analysis was unsatisfactory. The compound was, however, analysed by Nuclear Magnetic Resonance spectrophotometry. The proton spectrum in deuterium oxide showed two groups of peaks with chemical shift values characteristic of fructose and lysine resonances but these gave little definite information. However the trace varified the absence of an anomeric linkage (i.e. the FL sample was not a glycoside) since

the two peaks characteristic of α- and β-anomeric protons which were expected at low field could not be seen. A ^{13}C spectrum for FL in deuterium oxide was also obtained and is shown in Fig. 1.2 Although it is difficult to make definite statements on structure on the basis of this one trace for a single set of conditions, it can be observed that it was at least characteristic of the type of spectrum expected for FL. The three peaks at highest field, i.e. a,b and c, probably represent the β,ǵ and aliphatic carbon atoms of the lysine side-chain (see Fig. 1.). The two carbon atoms either side of the ε-nitrogen in the expected structure have very similar environments and would be expected to produce peaks at relatively high field and these may be the doublet d,e. Peaks h,i and j probably represent C-3¯, C-4¯ and C-5¯ of the sugar ring; peak g is separate from these and is at higher field and may represent C-6¯. Peak f is probably indicative of the α-carbon of lysine. The ^{13}C-trace shows only 11 peaks and it is likely that the acidic carbon of lysine has been lost at low field or, because it has no proton, has a long relaxation time and cannot be detected by resonance. The 'anomeric' carbon may be peak k at low field and is, as expected, attenuated through lack of an attached proton. The fact that only one set of resonances for the sugar is present suggests that the FL sample is either predominantly in the furanose or in the pyranose form. In solution, an equilibrium between these forms would be expected with the 6-membered ring making the major contribution, cf other reducing derivatives of fructose.

Chromatography The FL sample was analysed by partition chromatography using several solvents; in all cases it appeared to be essentially homogeneous. The compound was also electrophoretically pure when examined in 1M-acetic acid and ammonium formate and in borate buffers. FL specimens made by the original Finot and Mauron method and the modified method were chromatographically and electrophoretically indistinguishable. The mobilities quoted by Finot and Mauron (12) using a two-dimensional chromatographic system were confirmed in this present study.

It was noted, however, that amino acids (including FL) showed anomalous behaviour in the first solvent used by Finot and Mauron in their two dimensional chromatographic system, i.e. methyl ethyl ketone/acetic acid/water (5:3:2, v/v). On both paper and thin layer plates, FL standards produced elongated spots (when located with ninhydrin) and the R_f value could be increased by up to 50% simply by the application of increasing

Peak	ppm
a	23.63
b	26.96
c	32.18
d	50.60
e	50.82
f	57.47
g	67.13
h	72.23
i	73.12
j	180.86

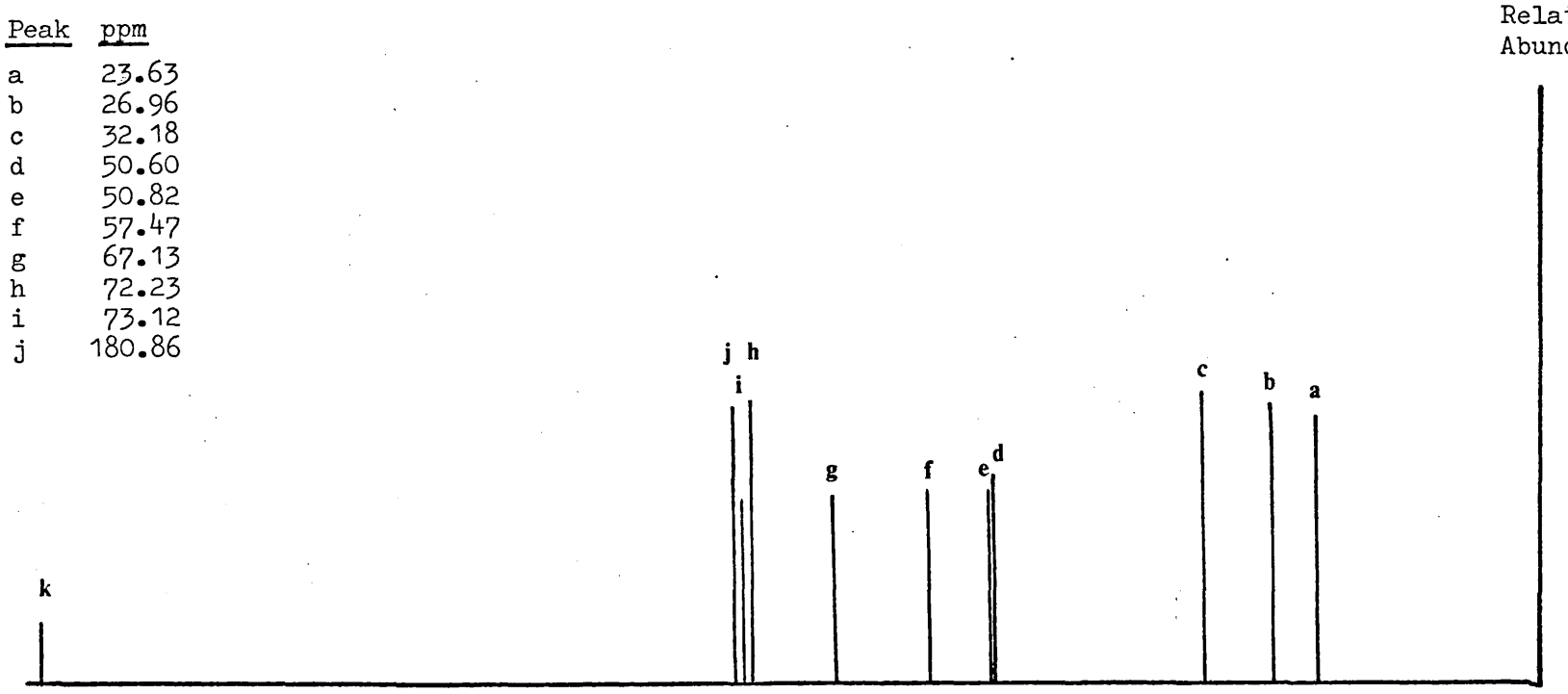
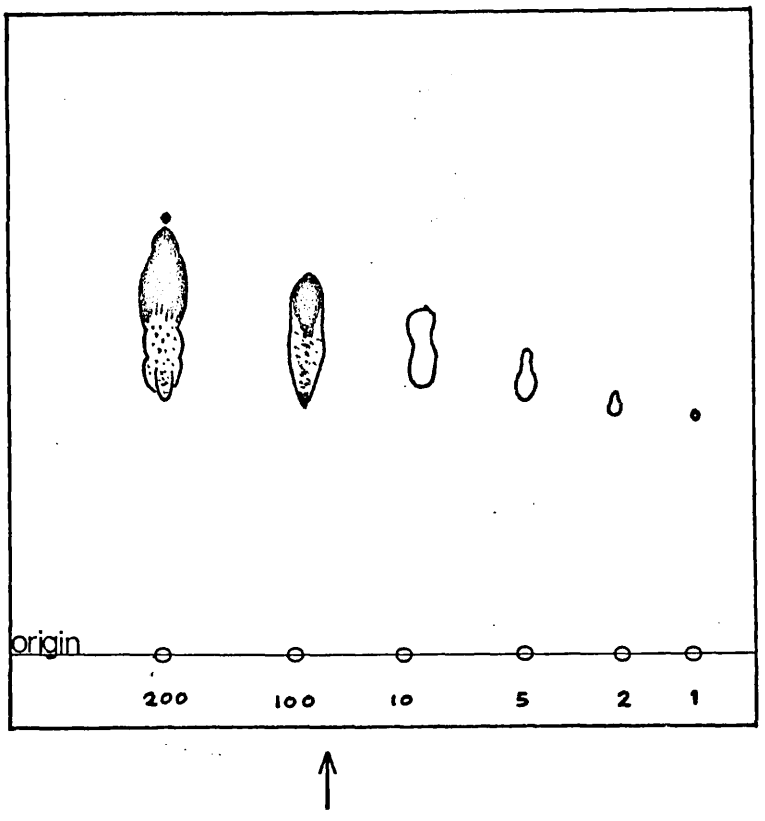


Fig. 1.2 ^{13}C-Nuclear Magnetic Resonance Spectrum of FL (22.63 MHz, in D_2O)

amounts of sample to the origin. Increasing sample loadings also caused progressive elongation of the spots, initially by forward tailing, followed by localisation of the concentration of colour at the leading end and at the retarded end of the spot (see Fig.1.3). Further sample loading increased the colour at the leading end and even greater loads resulted in up to 5 localised areas within the spot with the greatest concentration of colour occurring in the leading end. The opposite effect was observed, however, when the FL was applied as a component of mixtures also containing lysine. Here strong localisation of colour occurred at the retarded end of the spot. Spot elongation and colour localisation was also characteristic of lysine in this solvent and colour distribution was also affected by running this amino acid in the presence of FL. All these effects were the same no matter whether the chromatograms were developed once or twice in the solvent and they were especially pronounced when thin layer plates, rather than paper, were used (Fig.1.3). The solvent used by Finot and Mauron (12) for the second direction of separation on two-dimensional chromatograms, i.e. pyridine/acetic acid/ water (9:1:2, v/v), gave a good resolution of lysine, FL and free sugars and this was used in the present study for most unidirectional paper chromatographic analyses of mixtures containing FL.

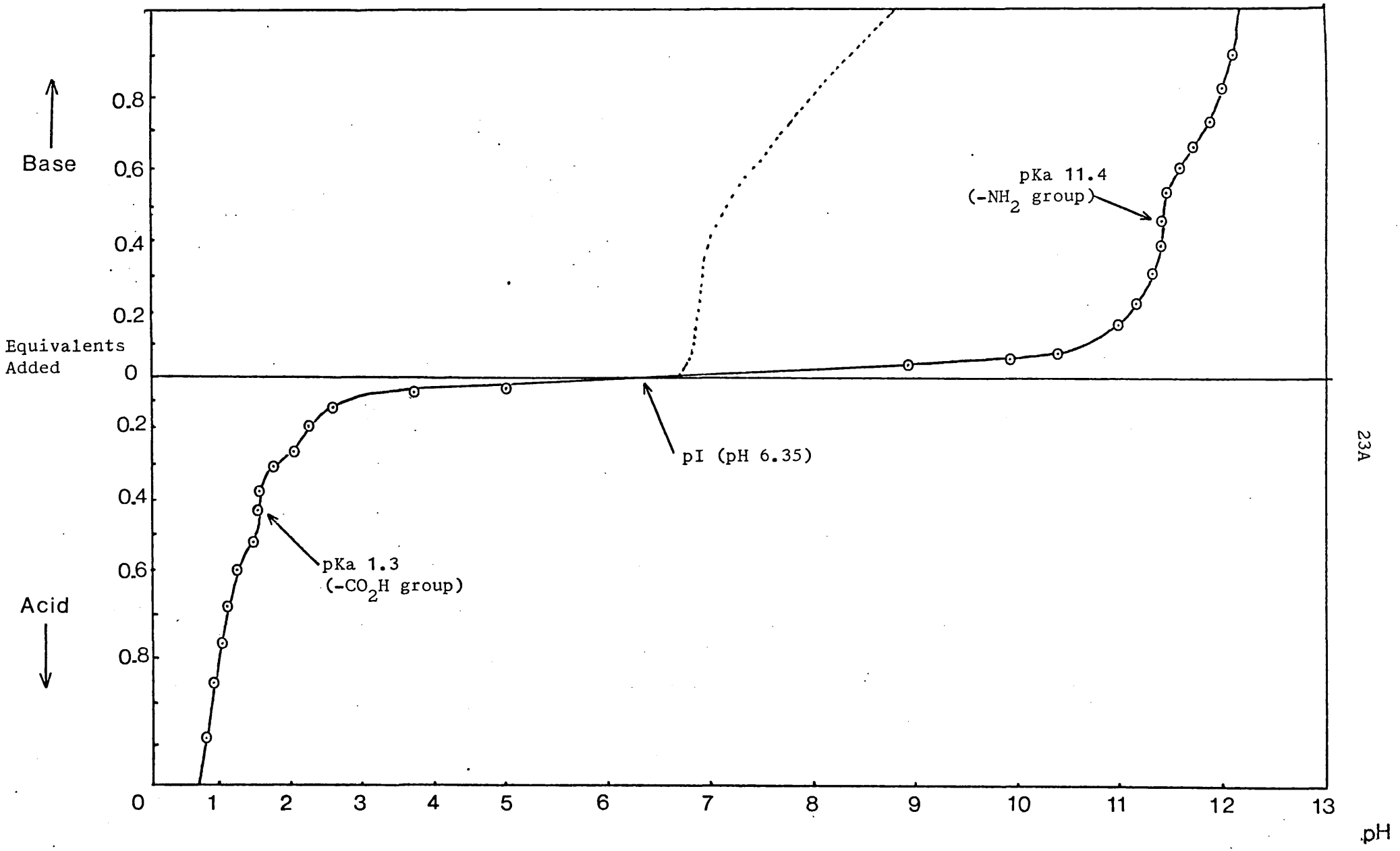
The titration curve obtained with FL was characteristic of a monobasic amino acid showing the presence of one titratable group with a pKa value of 11.4 (see Fig.1.4). The curve for the amino group was characteristically shifted towards neutral pH by the addition of formaldehyde showing that FL is able to form mono- or di- methylol derivatives. The inflexion in the curve corresponding to a pKa of 1.3 presumably reflects the presence of the α-carboxyl group and the pI value for the molecule was estimated to be 6.3.

Assay and Detection of FL FL in solution was assayed by its ability to reduce potassium ferricyanide in alkaline solution (the 'Borsook test'). The prussian-blue colour which developed on subsequent addition of the phosphoric acid reagent occurred as a fine colloid which may explain why, in the original paper (49), the authors supplemented their reagent with a 'gum ghatti infusion' which took several days to prepare; presumably it was intended that the increased viscosity would prevent the colloid from settling. It was found, however, using a reagent not containing gum ghatti, that flocculation and settling of the coloured colloid began only after standing



Figures represent relative concentrations of FL applied

Fig. 1.3 Thin layer chromatography of pure FL in methyl ethyl ketone/acetic acid/water (5:3:2, v/v)



23A

Fig. 1.4 Titration curve of FL in water (O—O) and in presence of formaldehyde (------)

for 30 min and an absorbance reading could be taken at any time during this period. The presence of protein or other materials in the sample assayed sometimes accelerated the rate of flocculation. However, an accurate reading of absorbance could be obtained by redispersing the colloid with an 'automix'. The absorbance reading for the blue colour was stable for 1hr. The test is highly sensitive for N-substituted 1-amino-1-deoxy-2-ketoses (e.g. formyl derivatives and melanoidins). Fructose, glucose, lactose, galactose, lysine and lysine hydrochloride gave negative Borsook reactions at the concentrations used throughout this investigation. FL did not, as expected, give a positive reaction with the Elson-Morgan reagent for amino sugars.

Ninhydrin solution was found to be the most sensitive reagent for locating FL on paper or thin-layer chromatograms; the modified ninhydrin reagent, as described by Moffat and Lytle (50), which gives characteristic colours with different amino acids, was mostly used and this reagent gave purple spots with FL and also with lysine. Triphenyl tetrazolium chloride in chloroform or acetone was less sensitive requiring 6-10 times as much sample for detection. Some commonly used carbohydrate-detecting reagents were examined in the hope of finding one which was specific for, or highly sensitive to FL; e.g. aniline oxalate, urea in HCl, periodate/permanganate, p-aminobenzoic acid, naphthoresorcinol, p-anisidine hydrochloride. All of these gave only weak or negative reactions with FL. Various treatments with reagents containing p-dimethylaminobenzaldehyde and o-dinitrobenzene (which reacts with Amadori products in solution (11)) also resulted in weak reactions. The Borsook test was modified as a spray reagent but was found to be insensitive and a poor locating agent for FL. Silver nitrate/NaOH reagent was sensitive to FL on paper chromatograms and a silver nitrate/ammonia spray detected FL on thin-layer plates but the latter produced a dark background colour.

Reactions with Acids Hydrolysis of FL with acid (2N-HCl or -H_2SO_4, 3hr, 100°C) resulted in browning of the solution and chromatographic analysis of the neutralised hydrolysate revealed, as expected, the presence of some lysine (31). FL samples were also shaken with 0.1M-H_2SO_4 (37°; 30 min) to assess the possible effect of acidification of FL in Warburg-respirometer experiments. In this case chromatographic examination of neutralised samples showed the presence of FL and material which remained near the origin of the chromatogram. This spot could also be produced by adding

sodium sulphate to solutions of FL and was, therefore, assumed to be fructose-lysine sulphate. Lysine itself also produced a compound, which was thought to be a sulphate, when treated with sodium sulphate. Samples of ^{14}C-labelled FL (see later) and lysine also formed sulphates and the R_{FL} values of these derivatives are shown in Fig. 1.5. FL shaken with 0.1 M-HCl (37°; 6 hr) was essentially unchanged, yielding only trace quantities of lysine on chromatograms, and this would suggest that the $C_{(1)}$-N bond of FL *in vivo* would be resistant to hydrolysis by gastric HCl.

Stability Solutions of FL left at room temperature began to turn yellow after approximately 40 hr and one of the first degradation products which could be detected on chromatograms was lysine. Hence suitable controls were devised in all experiments to ensure that FL had not significantly decomposed. The stock FL solution was only removed from deep-freeze for short periods to allow removal of aliquots for experimentation. The purity of the stock solution was checked at regular intervals over the entire period of the project by chromatography on thin-layer plates. Since 5-hydroxymethylfurfuraldehyde is one of the earliest decomposition products (see Fig. 3) and is highly coloured, this provided a useful visual marker of incipient decomposition of FL in solution; slight yellowness could be detected by eye before other products (*e.g.* lysine) appeared on chromatograms. The 5-hydroxymethylfurfuraldehyde could also be estimated quantitatively by calculating the molar extinction value at 270nm.

FL was unstable in alkali; when a solution (1mM) of FL was heated at 37°C in a solution adjusted to pH 10.5, with NaOH the compound decomposed almost completely in 30 hr with the formation of lysine and a number of other compounds including brown melanoidins. When the FL powder, as originally isolated, was dissolved in water, it gave a solution of pH 6.4. The stock solution was, therefore, stored at this pH to protect the sugar moiety which is probably unstable at alkaline pH. It was noted during chromatography of samples containing FL that had been allowed to brown, that one fraction of the pigment remained on the origin and the other moved with the solvent front. Alkali-degraded FL showed particularly large amounts of the immobile pigment.

Preliminary investigation of Maillard Fusion Reactions as a source of ketose lysines.

The Finot and Mauron method (12) for preparing FL was time consuming

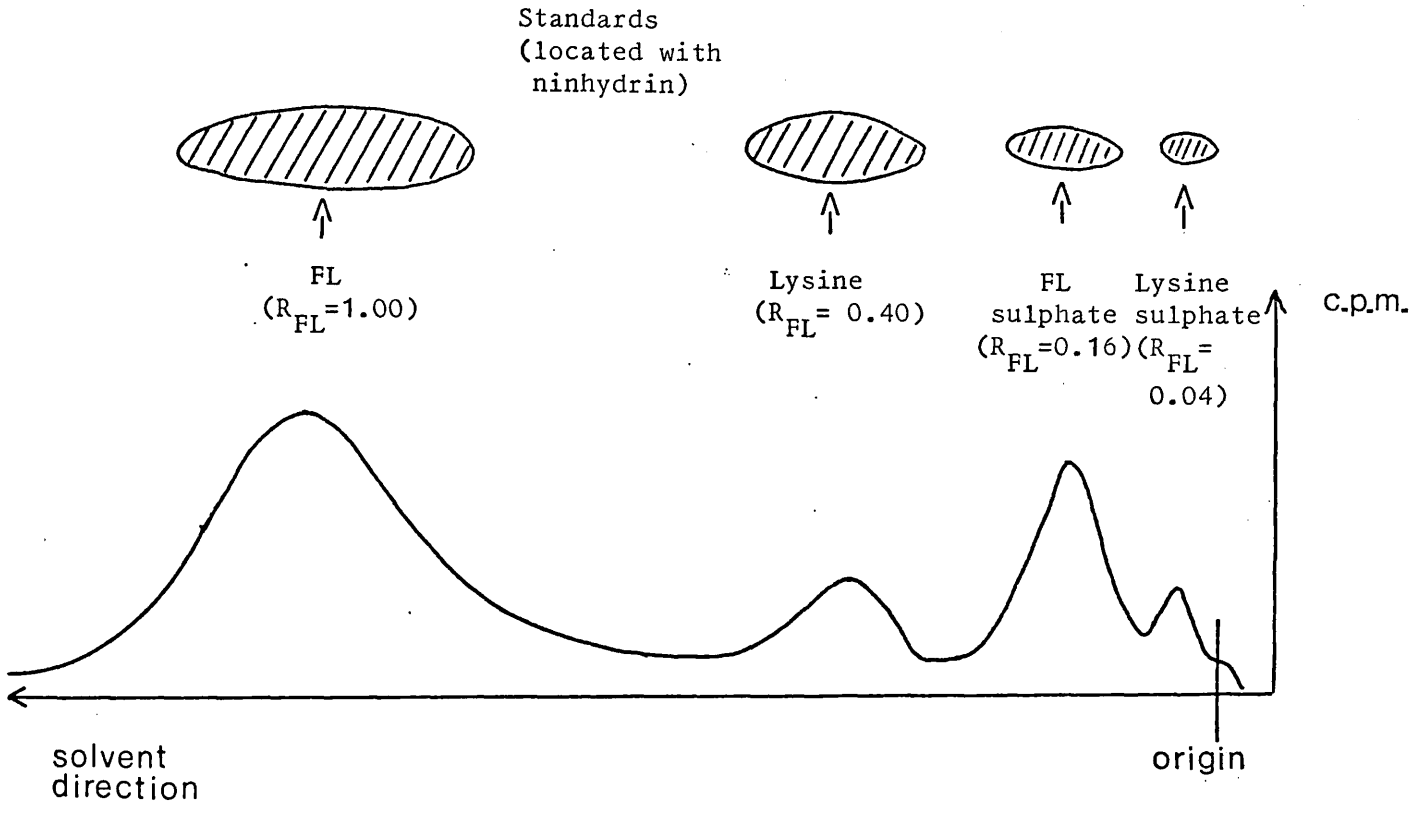


Fig. 1.5 Paper chromatography (Pyridine/Acetic acid/H_2O; 9:1:2, v/v) of an aqueous mixture of ^{14}C-FL, ^{14}C-lysine and Na_2SO_4. The chromatogram was scanned for radioactivity with a strip counter

due to the large number of stages and multiple column fractionations: the overall yield from lysine HCl and glucose was also very low. It was hoped that the time factor for preparing FL would be considerably reduced and the yield increased by a one-stage synthesis involving the direct reaction of lysine or lysine hydrochloride with glucose. This 'direct fusion' method was ultimately intended for use in synthesising radioactively-labelled FL samples with the minimum wastage of expensive starting materials. Contrary to the title, the Finot and Mauron paper (12) does not describe the preparation of LL; α-formyl-LL was prepared but the conditions employed by the authors for deformylation also completely hydrolysed the glycosidic bond. Hence, a method for the preparation of LL was also required.

With the intention of finding, empiracly, the optimum conditions for the maximum yield of N-substituted-1-amino-1-deoxy-2-ketose intermediates of the Maillard condensation, a variety of reaction conditions were investigated. Reference was made to the original papers where FL was amongst the compounds formed by the Maillard process (e.g. (45) (46)) and the general principles mentioned in the review by Ellis (10) were observed; i.e. that increased temperature, concentration of reactants and pH tend to accelerate the Maillard reaction. The various conditions tried are described in the 'Materials and Methods' section pp. 123-125

All the fusions carried out gave different mixtures of products and usually from 5-20 different spots were detectable, with ninhydrin, on thin layer plates that had been developed in two dimensions. In the reaction mixtures the major components found were usually the reactants and the ε-isomer of the corresponding ketose lysine product. The predominance of the ε-isomer in the mixtures was expected as the ε-amino group of lysine is more reactive than the α-amino group (10,11,43). Approximate % conversions were obtained by inspection of chromatograms by eye. Solid phase condensates using approximately equimolar quantities of reactants gave up to 20% conversion of lysine to the Amadori products and this was improved to about 30% when the sugar was used in excess. Fructose as well as glucose appeared to yield FL with lysine but the former sugar was less reactive. Liquid fusion methods generally gave better yields and the maximum conversion of lysine (or its hydrochloride) to the Amadori product was about 50% and this again occurred when the sugar was present in excess. If the amino acid was used in excess, the mixture browned very rapidly, indicating serious

sugar degradation, and only minor quantities of the Amadori products appeared. Increased temperature always accelerated the reactions; e.g. two-dimensional chromatography showed that a glucose/lysine mixture heated for 2 hr at 100° had reached a similar stage in the production of Maillard products as the same mixture heated for 12hr at 70°C. The number of products and the degree of browning increased with the heating time. The percentage of the required Amadori product appearing in the reaction mixtures tended towards a maximum any further increase in the time of heating beyond this point caused further conversion of the remaining starting materials but this was offset by an increase in the number of degradation products. Ultraviolet light could be substituted for heat but the reaction was much slower; the irradiated solution exhibited strong fluorescence from approximately 10hr onwards. The presence of NH_4Cl or alkali also catalysed the reactions between glucose or lactose and L-lysine; L-lysine hydrochloride was more reactive than the free base. Some water appeared to be necessary for maximum reaction rate; reactions in the solid phase or in methanol solution were much slower.

Preparation of ^{14}C-labelled FL Erbersdobler (22) prepared FL labelled in the lysine moiety with ^{14}C but did not give experimental details. For the preparation of 1-(ε-N-[U-^{14}C] lysyl)-1-deoxy-D-fructose (FL*) the molar ratio of sugar to [U-^{14}C]L-lysine hydrochloride was 40:1 in order to effect efficient conversion of the labelled amino acid into FL. On the other hand, for the preparation of 1-(ε-N-lysyl)-1-deoxy-[U-^{14}C]D-fructose (*FL) the molar proportions of [U-^{14}C]-D-glucose to lysine hydrochloride were 2:1 which were chosen so as to achieve the best conversion of radioactive sugar into FL by striking a balance between sugar wastage, through using it in excess, and sugar degradation by using too great a proportion of the base. A pilot experiment showed that the optimum conversion to FL in the case of both labelled compounds, occurred after 100 min heating at 100° (pH 9.0). The progress of the reactions could be followed by measuring the pH decrease and the increase in Borsook-positive substances; typical curves are shown in Fig. 1.6.

The labelled compounds obtained using these reaction conditions were separated by preparative paper chromatography and eluted with water. The fractions corresponding to labelled FL were tested for homogeneity by high voltage electrophoresis; both *FL and FL* showed identical mobilities to that of the FL standard. The ^{14}C-FL derivatives were detected on the electro-

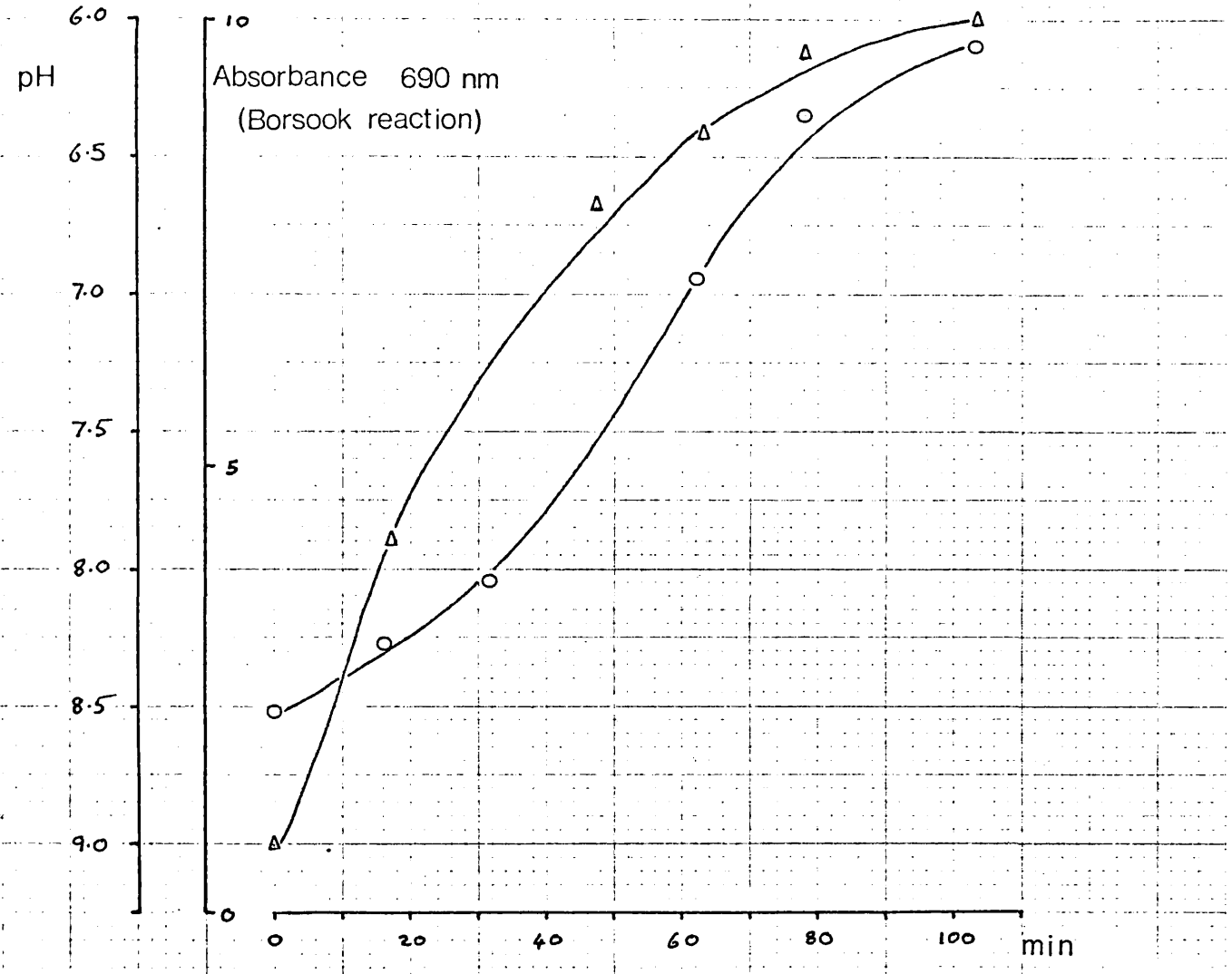


Fig. 1.6 Changes in pH and concentrations of Borsook-positive compounds occurring during the heating of D-glucose and L-lysine HCL in aqueous solution at 100°

Δ pH, —Δ——Δ— ; Borsook colour, -o——o—

phoretograms by scanning with a strip counter. The two labelled FL samples in both acid and borate buffers appeared as 'doublets', although the corresponding FL standards, when visualised with ninhydrin, appeared as an elongated single spot (see Fig.1.7).

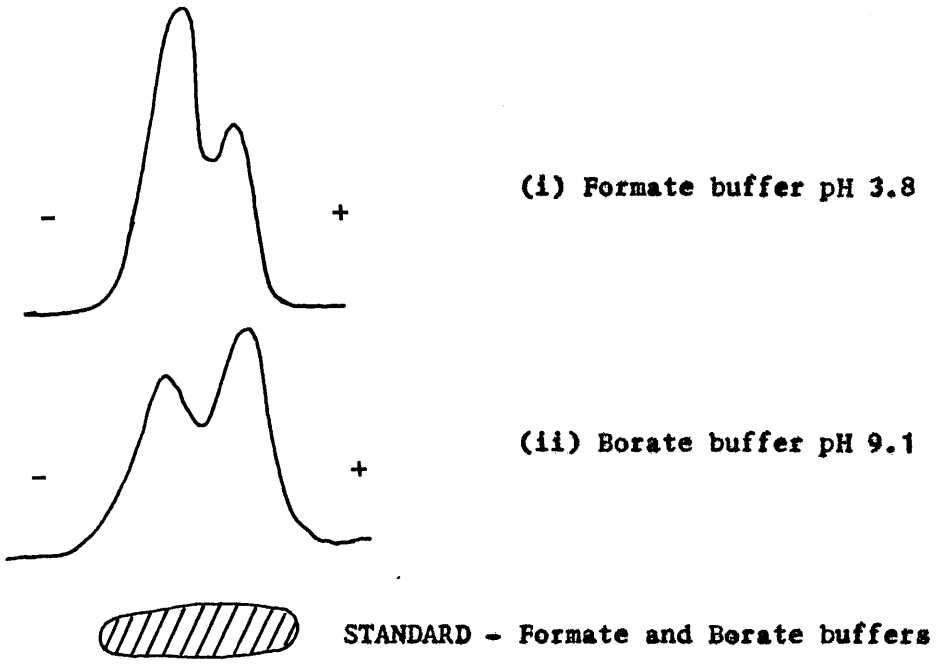


Fig. 1.7 Electrophoretic patterns of *FL and FL* detected by strip counter.

This phenomenon appeared to be a charge effect since, if either of the two peaks forming the doublet was isolated and then re-subjected to electrophoresis a doublet was again observed.

Paper chromatographic examination of the radioactive FL samples using methyl ethyl ketone, acetic acid, water; (5:3:2, v/v) as a solvent yielded a single radioactive peak, corresponding to the FL standard, in the case of FL*. The peak obtained in this solvent with *FL, however, appeared as a 'doublet'. It is unlikely that the doublet represents the α- and ε- isomers of *FL because these two compounds have been reported to have quite different R_f values (Finot and Mauron (12)). It is more likely that the 'doublet' is a 'solvent effect'. In view of the observations on the chromatography of FL in this solvent which have already been discussed (see p. 20) the possible consequences of contamination of the *FL sample by the α-derivative (1-(α-N-lysyl)-1-deoxy-[U-^{14}C]D-fructose) were taken into consideration when using *FL for metabolic studies.

The ^{14}C-FL derivatives appeared to be more labile than the unlabelled FL, presumably because of self-decomposition induced by radiation. For example, after 6 months' storage at -20°, up to 10% of the label in the FL* was present as free lysine. Hence, the radioactive FL samples used for E.coli (section 2) and liver experiments (section 3) were always freshly prepared. Both the labelled FL samples were capable of forming sulphate derivatives as was the case with unlabelled FL (see Fig. 1.5).

Preliminary examination of preparative methods for ketose lysine derivatives Although all reaction mixtures containing FL and LL were amenable to separation by paper chromatography, only relatively small amounts of the mixtures could be applied to the paper. In addition, the starting materials (especially sugars) were present in excess and therefore several chromatographic separations were necessary for their removal. Hence, some other methods of separating Maillard reaction mixtures in high yield were attempted. In particular it was hoped that better methods for the separation of ϵ- and α- ketose lysine derivatives could be developed, on the basis of the differing charges of the free amino groups. This was repeatedly attempted using columns of CM-52 ion exchange cellulose, in the pyridinium form, with Maillard reaction mixtures but the results were inconsistent.

A column of Sephadex G10 was successfully used to separate FL from melanoidins and other high molecular weight materials present in crude reaction mixtures. However, since the capacity of Sephadex is low the resolution between FL and the low molecular weight starting materials, glucose and lysine, was poor, hence these compounds always contaminated the FL fraction.

The use of activated charcoal columns for separating FL from reaction mixtures was also investigated. It was assumed that, of the reactants, the carbohydrates would readily pass through such columns whereas lysine would be retarded due to the presence of ionised groups; on this basis ketose-lysine derivatives would exhibit an intermediate affinity for carbon. Attempts to separate FL from reaction mixtures were made using a variety of elution techniques, based on previous literature, and column dimensions were also varied. In Fig. 1.8 the resolution of a Maillard reaction mixture containing FL is shown. Coloured by-products were irreversibly adsorbed and it can be seen that the expected order of elution of glucose and lysine occurred. However FL was eluted in a very diffuse band and the procedure was considered to be unsuitable for obtaining high yields of this product.

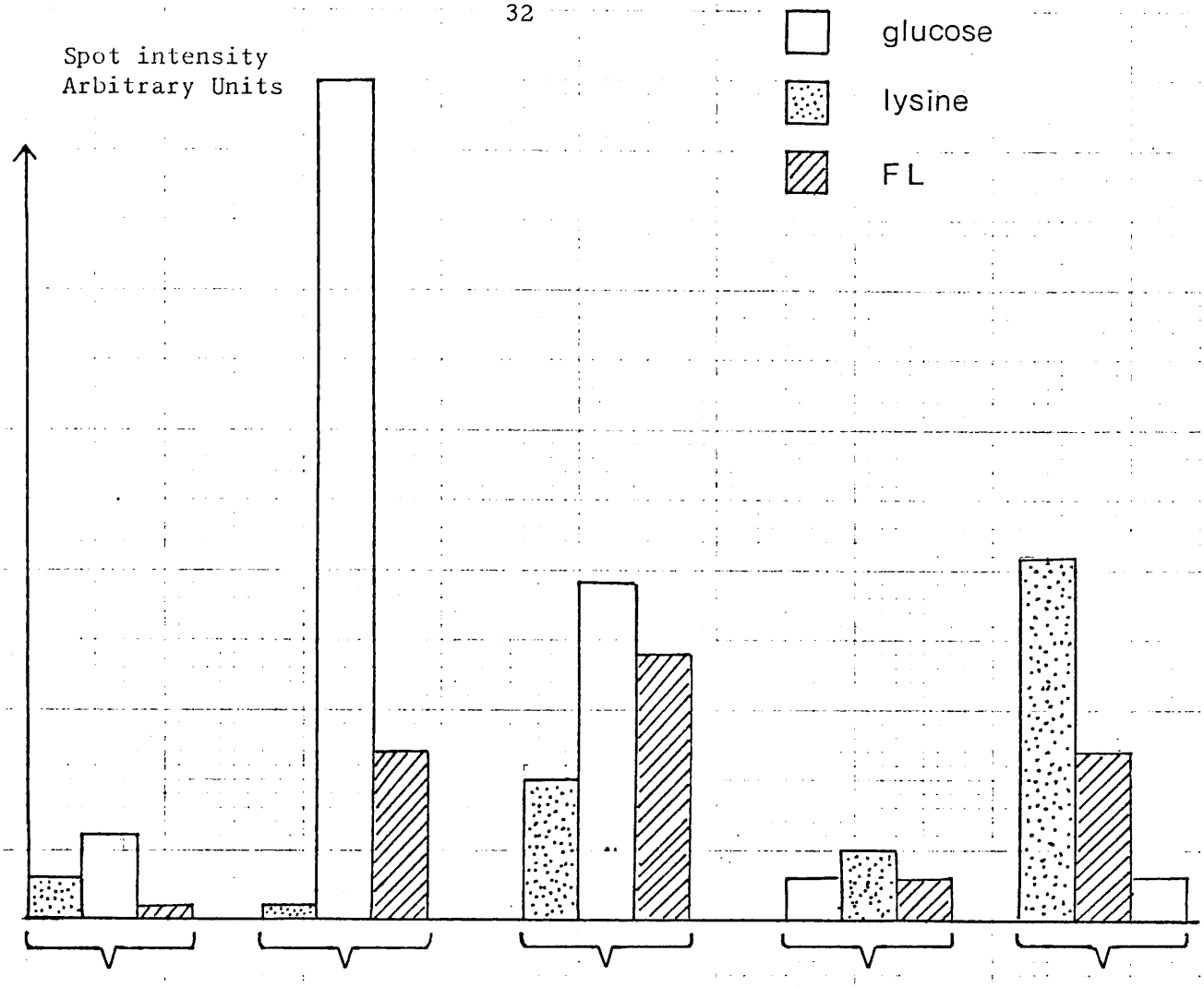


Fig. 1.8 The resolution of a glucose/lysine reaction mixture on a charcoal column. Fractions were assayed by evaporation of the eluates, separation on thin layer plates and visual quantitisation of the components.

Preparation of Lactulose-lysine Lactulose lysine (LL) was prepared by heating a mixture of lactose and lysine hydrochloride at 100°C for 1.5 hr (pH 9): the product was isolated by preparative paper chromatography.

As in the case of FL, LL also exhibited anomalous chromatographic behaviour. When using methyl ethyl ketone/acetic acid/water solvent (5:3:2, v/v), the LL appeared as three contiguous spots, the leading one giving the most dense colour with ninhydrin spray reagent. This phenomenon was assumed to be a solvent effect since, if the material in the leading spot was isolated and rechromatographed, it still gave rise to three spots.

A sample of the LL was hydrolysed with 2N-HCl (100°C for 3 hr). Amongst the products in the deacidified hydrolysate which were identified by two dimensional chromatography were lysine and ε-FL; α-FL could not be detected which suggested that the LL sample was not contaminated with the α-N-lysyl isomer. β-D-galactose was also detected in the hydrolysates using galactose dehydrogenase and following the reduction of NAD spectrophotometrically. Attempted hydrolysis with 0.1N-HCl (at 37° for 6hr) i.e. imitating the most stringent gastric conditions of acidity likely to occur in humans, yielded no detectable galactose as judged by the highly sensitive galactose dehydrogenase method. Thus ingested free LL would probably leave the stomach unchanged; in milk powders the LL reaching the stomach would still be part of the protein and in this structure the glycosidic bond may be even more stable to acid hydrolysis. Lysine was only detectable in trace quantities when LL was treated with 0.1N-HCl (37° for 6hr) showing that the $C_{(1)}$-N bond was also resistant to dilute acid.

The LL sample was stored in aqueous solution (pH 6.0) at -20°C. LL appeared to decompose more readily than FL, as judged by the greater rate of browning of solutions of LL left at room temperature.

S E C T I O N 2

Studies with Eschericia Coli B.

As previously mentioned, it is possible that FL may affect the intestinal microflora of the new born and hence lead to imbalance which could subsequently cause intestinal malfunction. Since FL is thought to be poorly absorbed by the intestine and has been detected in high concentrations in the faeces of some 0-10 day old babies (Seakins 27-30) its possible effects could be quite significant.

FL could have a direct bacteriocidal effect on some bacteria. For example, if FL were to act as a potent non-competitive inhibitor of an enzyme-catalysed reaction necessary for growth, it would rapidly cause death of the colony. Similarly, if it were to cause destabilisation of the bacterial cell wall it might facilitate cell lysis. Sometimes the incorporation of abnormal sugars into the cell wall (e.g. 2-deoxyglucose, Beily 1971 (66)) causes inhibition of successful cell division; FL, which resembles some of the amino sugars of the bacterial cell wall, might act in this way.

FL might act less drastically than this and merely alter the growth-rate of the bacterium and hence the colony. This effect would be observed if FL acts as a competitive inhibitor of enzymes involved in intra- and extra- cellular substrate utilisation. For example, the compound might compete with extracellular enzymes which are secreted for breakdown of polymeric substrates. Competition at this level is, however, relatively unlikely as polysaccharidases and polypeptidases generally have low K_m values and are not limited by low molecular weight compounds, e.g. free sugars or free amino acids. Extracellular enzymes metabolising low molecular weight substrates (e.g. disaccharides) are uncommon in bacteria as these substrates are readily absorbed into the cell through permease action.

FL could interfere with permeation of substrates. Since permeases are proteins catalysing substrate transport through the cell wall and membrane, FL could retard this process by acting as a competitive inhibitor. Permease inhibition would, however, need to be substantial before it affected growth rate since permeases normally provide the cell with substrates at several hundred times the rate at which they are utilised. Fl could also affect passive or facilitated diffusion of solutes by physically altering cell wall characteristics.

FL may itself be utilised by bacteria as a substrate for growth. As FL is a novel substrate, it is possible that hydrolysis would occur at the C-N bond linking the amino acid and the sugar, thereby releasing L-lysine and hexose. Such enzymes ('FL-ases') could either be constitutive or require induction by the FL. The occurrence of either an intracellular or an extracellular 'FL-ase' is feasible but, since FL is of low molecular weight, it is more likely to induce a permease than an extracellular enzyme. If, however, the C-N bond is resistant to cleavage, FL could conceivably be degraded by some rather non specific enzymes which normally react with fructose or lysine. Possible reactions envisaged are: decarboxylation or deamination of the lysine moiety, splitting of the lysyl carbon chain or cleavage of C-C bonds in the fructose moiety. Such reactions would reduce steric hinderance and presumably facilitate cleavage of the C-N bond.

The inner cell membrane of bacteria is essentially impermeable to solutes. FL would, therefore, be expected to enter the cell only in small amounts, by simple diffusion. Rapid absorption of FL from the external medium would require the presence of a constitutive or induced 'fructose-lysine permease' to effect active transport.

Most bacterial carbohydrate-permeases are stereospecific. Thus, for example, although E.coli posesses a fructose permease, it is unlikely that it will catalyse FL accumulation. Similarly, E. coli has an inducible lysine permease which is highly specific and unlikely to transport lysine derivatives. Fl, if it enters the cell, probably does so by inducing its own specific permease.

E. coli was chosen as a representative of the gut flora most suitable for experimentation. Background literature on methods for growth media preparation, cell disintegration, etc. and on the absorption and utilisation of solutes was available. E. coli grows readily in an aqueous minimal medium containing inorganic salts and a simple carbon source such as glucose. Thus, since the growth conditions are then controlled and defined, the direct effects of adding a novel substrate, such as FL, should be easily observed against the standard pattern. Lactobacillus bifidus, although a more prevalent member of the 0-10 day old infant gut flora, is comparatively difficult to grow in culture. A suitable, simple, defined medium could not be found in the literature. Intestinal disorders are more often associated

with unusual coliform growth than with Lactobacilliform growth which tends to exert a stabilising effect, (Bullen 1971 (67)).

Part I - Growth Curve Experiments

The following experiments were carried out to determine whether FL, present in various concentrations similar to that of the glucose in the medium (i.e. approx. 10^{-2}M), would affect the standard growth curve.

Experiments were carried out under both aerobic and anaerobic conditions since, in the gut, local conditions probably vary widely between these two states. Oxygen tension sometimes influences the type of sugar permease evolved. For example, a phosphoenolpyruvate-requiring type of active transport (group translocation), which involves sugar phosphorylation is possible under anaerobic conditions.

FL was added to standard cultures (see Materials and Methods p.128) at concentrations between 10^{-2}M and 10^{-3}M; in order to conserve the limited amount of FL available higher concentrations were not used.

The effect of FL on the anaerobic utilisation of glucose (Exp. 1).
FL was added to standard cultures to give concentrations of 10^{-3}M, 2×10^{-3}M, 5×10^{-3}M and 10^{-2}M.

In every case (10 separate experiments in duplicate) the growth curves obtained with added FL were similar to the control cultures containing only glucose (see Fig.2.1). However a slight depression of the growth rate (0-5%) and a slight increase of the stationary-phase value (0-5%) were produced by the FL. A control experiment showed that FL alone, in minimal medium did not show an absorbance at 420nm over the period of the experiment. These values of '0-5%' were not considered sufficiently significant to merit closer study.

It was noted that most of the growth curves obtained under anaerobic conditions showed a small initial, rapid growth phase characteristic of the end of a sigmoid curve lasting 15-20 min. prior to a steady anaerobic lag phase. This was probably due to utilisation of residual dissolved oxygen.

Once the maximum growth had been reached and during the subsequent dying phase, addition of either glucose or FL caused no further growth, (though dying rate was decreased for approximately 30 min.). This suggested that the cells had terminated their own growth, in the normal way, by producing various acids (the pH decreased from 7 to 4.5-5.0) and other toxic metabolites. All cultures gave an overall growth equivalent to approximately 0.55 absorbance units at 420nm. The average time taken to reach this value was 12 hr.

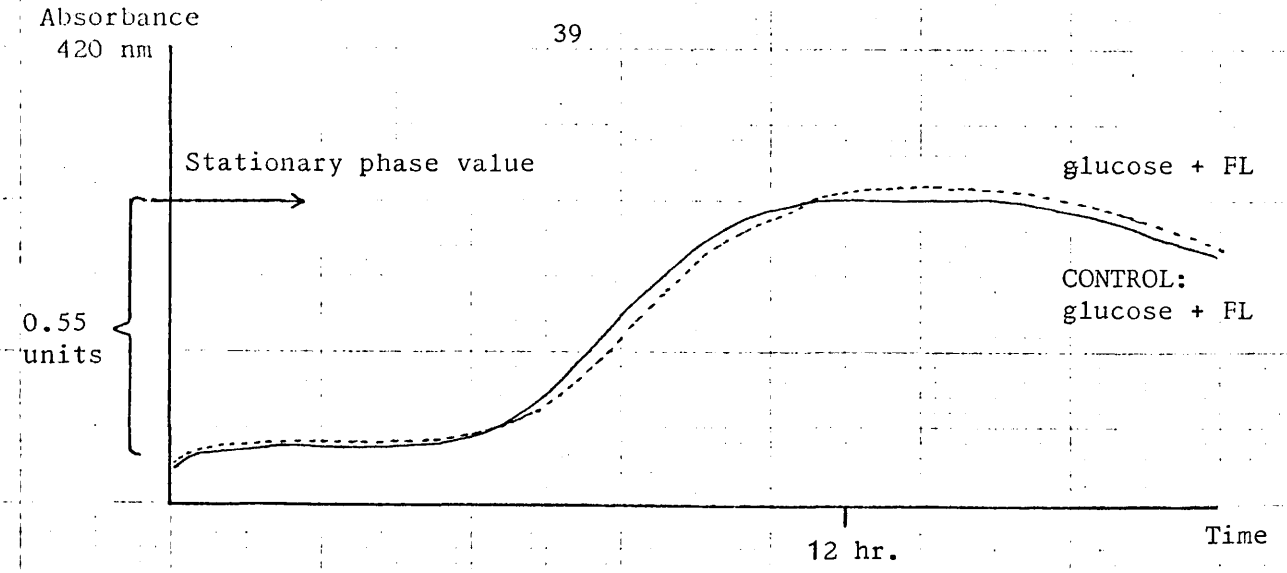


Fig. 2.1 E.coli growth curves (anaerobic, see text)

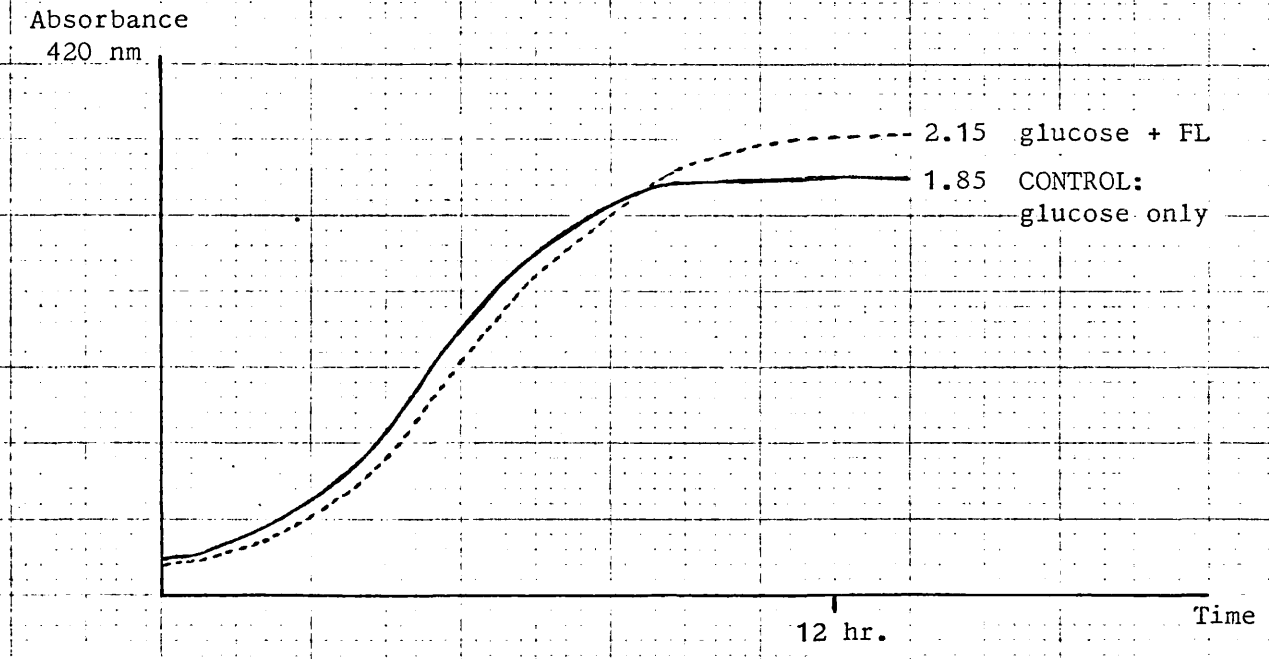


Fig. 2.2 E.coli growth curves (aerobic, see text)

It is apparent, therefore, that the growth of E.coli on glucose was not inhibited by the presence of FL under the conditions used.

The effect of FL on the aerobic utilisation of glucose (Exp. 2). The general method for obtaining growth curves was as described in 'Materials and Methods'. FL was added to standard cultures to give a final concentration of 2×10^{-3}M. Growth curves (see Fig. 2.2) of cells grown in the presence of FL showed a lag phase and log phase growth-rate similar to those grown on glucose alone. These growth curves were typically sigmoid. However, when FL was present, the growth curves (as compared with those for glucose only) indicated an increase in overall cell growth, observed as a 12-15% increase of the stationary phase absorbance value. A repeat of the experiment, where the FL concentration was reduced by half to 1×10^{-3}M, showed the elevation of the stationary-phase value to be also reduced by half. Thus growth-activation by FL is probably directly proportional to the concentration of FL.

Hence it was considered that FL was probably being utilised as a food source and that utilisation of carbon from FL was less efficient than carbon from glucose since the addition of FL (responsible for 15% extra growth) contributed a further 40% available organic carbon.

During the lag and log phases the FL curves followed the control curves (glucose only) which is to be expected as growth rate is determined by cell generation time (approximately 60 min. for E. coli under optimum conditions) which is not limited by the substrate concentration used in these experiments.

Each point on the curves was an average of two readings from duplicate cuvettes. The pattern described was observed in seven separate experiments. The only variations were in the length of the initial recovery phase which, in turn, depended on the age of the parent overnight-culture and the number of cells originally transferred to the cuvettes. The experiment was repeated using different overnight-cultures harvested at the following stages in their growth curves: (a), log phase; (b), late resting phase (flat portion); (c), dying phase. In these cases there were increased lag phases in the order (a) (b) (c) but all curves 'flattened out' at the same absorbance value. The presence of FL did not alter this effect on the lag phase and only influenced the final stationary-phase value.

All growth curves shown in this section, when presented on the same set of axes, are for inocula of identical 'age'.

The effect of heated FL on glucose utilisation by E.coli (Exp. 3). Kuwabara et al (68) in 1972 reported that a crude glucose-lysine mixture heated at 120°C for 40 min. caused inhibition of the growth of Staphylococcus aureus. They were of the opinion that this was due to the production of pre-melanoidins from glucosyl lysine.

A solution of FL (2×10^{-1}M) was autoclaved at 120°C for 5 min. in a sealed capillary tube. The solution became orange in colour with increased absorbance at 420nm, due to the formation of melanoidins and pre-melanoidins. The heated solution was then added to standard cultures to give a final FL concentration of 2×10^{-3}M. Growth curves in both aerobic and anaerobic conditions were obtained as previously described in 'Materials and Methods' (see Fig. 2.3 (aerobic) and Fig. 2.4 (anaerobic)). In both cases the presence of FL caused the lag phase to be significantly extended (approximately 2 hr.) and the log phase growth rate to be slightly depressed. In the case of anaerobic growth, the stationary-phase value was 90% of the control (glucose only) value. In aerobic growth the stationary-phase value was 115% of the control value (as per exp. (2) with unheated FL). Heated FL may not have affected glucose utilisation directly but may have affected the natural changes that occur during a lag phase e.g. swelling of cells due to water uptake may have been inhibited.

A thin-layer chromatographic separation of the autoclaved FL solution showed that approximately half of the compound had been broken down to yield an amino acid chromatographically identical to lysine. There was also a faint ninhydrin-positive spot near the origin of the chromatogram and traces of several compounds with Rf values greater than that of FL.

A further experiment was carried out in order to estimate the effect of heating FL at 37° i.e. the temperature of incubation used for growth curve experiments. Solutions of 2×10^{-3}M FL in sterile water were held under sterile conditions at 37° for 36 hr. and either shaken in a conical flask or left to stand in a sealed cuvette. These solutions were then inoculated with overnight cultures and growth curves plotted both under aerobic and anaerobic conditions. In both cases the growth patterns were identical to the controls where FL had not been heated. A thin-layer chromatographic separation showed only a trace of lysine in the original heated FL samples.

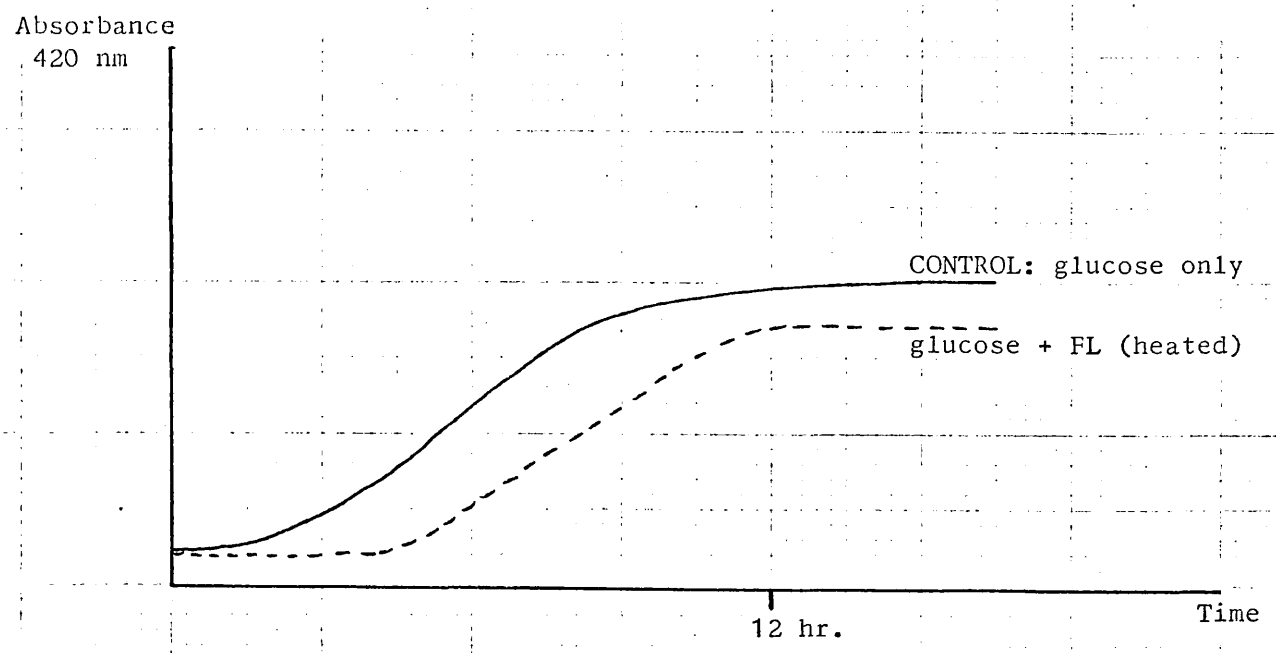


Fig. 2.4 E. coli growth curves (anaerobic, see text)

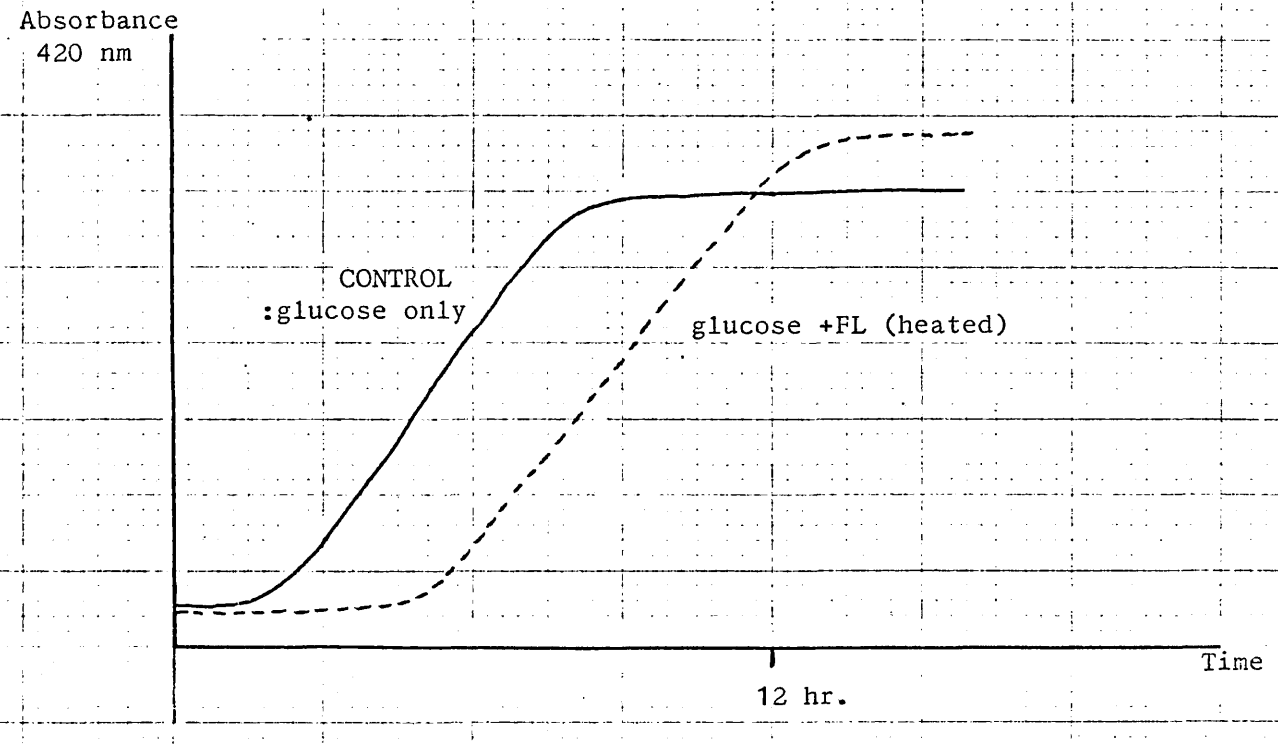


Fig. 2.3 E. coli growth curves (aerobic, see text)

The effect of lysine and fructose on aerobic utilisation of glucose (Exp. 4) This was examined in order to determine whether the components of the FL complex affected the aerobic growth curve in the same way as FL itself.

Sterilised fructose or lysine solutions were added to standard cultures to give a final concentration of 2×10^{-3}M. The resulting growth curves were essentially unaffected by the monosaccharide or the amino acid but fructose caused an elevation (27%) of the stationary-phase value (see Fig. 2.5).

Thus the fructosyl moiety of FL may be available for utilisation since fructose itself promotes a similar growth pattern to FL. The inhibition observed in Exp. 3 was, therefore, not due to lysine or fructose freed by the heat-catalysed, self-degradation of FL.

The effect of pre-exposure of E.coli to FL on glucose utilisation (Exp. 5) FL was then added to a standard culture, containing no glucose, to give a final concentration of 2.5×10^{-3}M. The cells showed no growth during a 3 hr. period. A sterile glucose solution was then added to give a final concentration of 2.5×10^{-3}M. The cells commenced growth immediately and the growth curve obtained closely followed the pattern of the control cells which had NOT been previously treated with FL. Growth was complete after 3 hr. (see Fig. 2.6).

This experiment gave the same result when repeated under aerobic conditions. Thus prolonged contact of E.coli cells with FL does not appear to impair subsequent utilisation of glucose.

Aerobic induction of a FL-utilising system in E.coli (Exp. 6). The results of Exp. 2 suggested that E.coli could utilise FL as a carbon source for growth in the presence of glucose. FL was added to standard cultures (2.5mM or 5.0mM) containing no glucose and growth curves measured. These showed (see Fig. 2.7) that no growth occurred during the first 8 hr. The cells then commenced growth and continued to complete a typical sigmoid curve. The actual rate of growth during the log phase was approximately the same as that for glucose-cultured cells. The cultures were set up in duplicate and the experiment repeated twice. A small initial increase of absorbance 420nm, observed in every culture over the first 3 hr., was probably caused by increased light refraction resulting from cell swelling rather than by cell division.

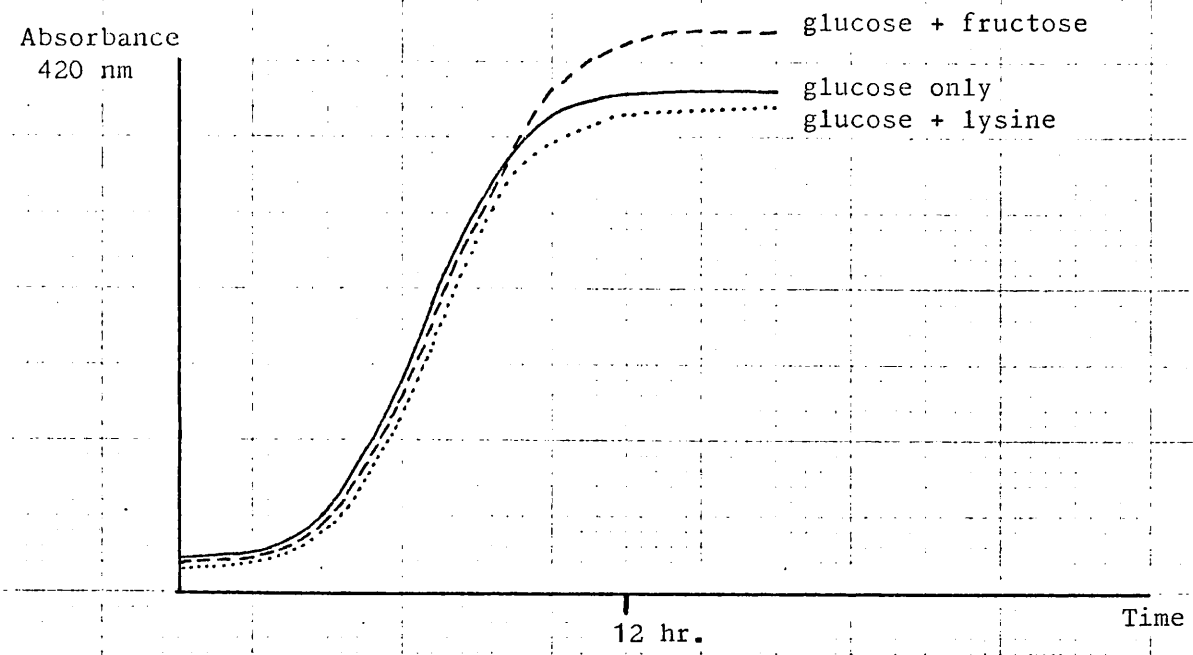


Fig. 2.5 E.coli growth curves (see text)

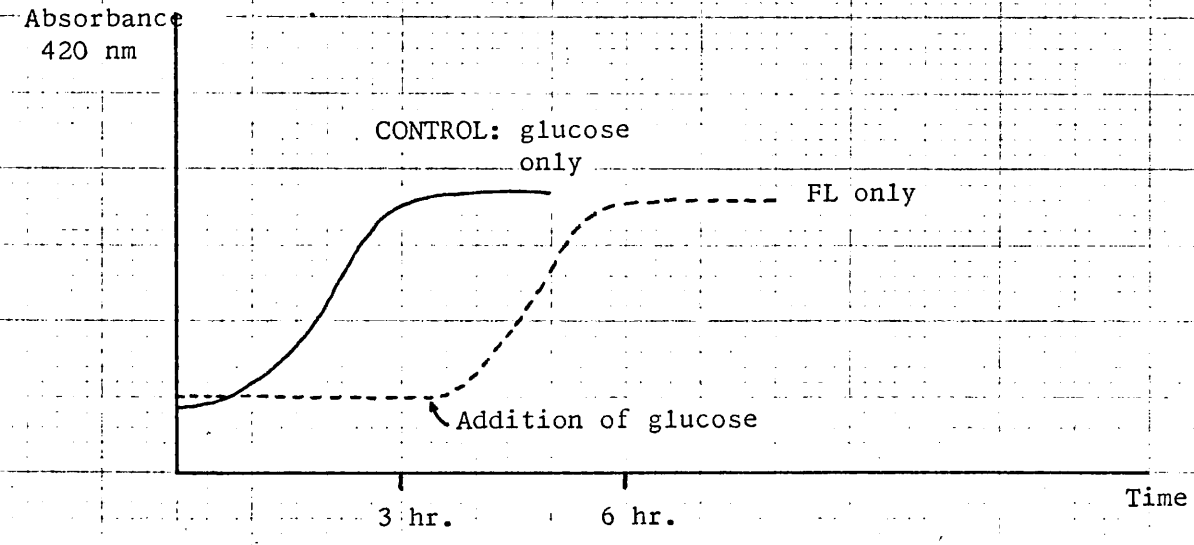


Fig. 2.6 E.coli growth curves (see text)

Thus E.coli was initially unable to utilise FL supplied as the only organic carbon source but was able to evolve an induced FL-utilising system.

Anaerobic induction of a FL-utilising system in E.coli (Exp. 7). Standard cultures were set up in the usual way but in the absence of the normal substrate, glucose: FL was again added to give a final concentration of 2.5×10^{-3}M. A typical resulting growth curve is shown in Fig. 2.8.

A preliminary small increase in absorbance was observed which was probably due to utilisation of endogenous glucose. After this, the cells remained at zero growth-rate for 10 hr. After this period growth commenced giving rise to an elongated sigmoid curve which reached a stationary-phase value after 24 hr. This result was repeated twice.

Thus, FL was able to induce a FL-utilising system under anaerobic conditions as was the case in air.

The permanent nature of the induced FL-utilising system was demonstrated using standard cultures set up under both aerobic and anaerobic conditions and grown in the absence of glucose. The aerobic and anaerobic cultures were innoculated with INDUCED cells from experiments 6 (aerobic) and 7 (anaerobic) respectively. FL was then added to each (final concentration 2.5×10^{-3}M).

In both cases immediate growth ensued and sigmoid curves, of similar shape to those of the sigmoid portion of curves obtained in experiments (6) and (7), were observed. However, slightly extended ($\frac{1}{2}$ hr) lag phases were observed in this case presumably due to deterioration of the inocula. Induced cells from both experiments (6) and (7) were also shown to utilise glucose in the normal way.

Thus E.coli does not lose FL-utilising ability over the equivalent length of time taken to 'acquire' it and this may well remain a permanent feature of the cells.

In a further study, which was essentially a repeat of the above procedure, the original aerobically grown cells (from Exp. 6) were subcultured anaerobically and the anaerobically induced cells (from Exp. 7) subcultured aerobically. Both types of subculture showed immediate commencement of growth and followed a similar pattern of growth to those shown in the final curves of experiments (6) and (7) as described above.

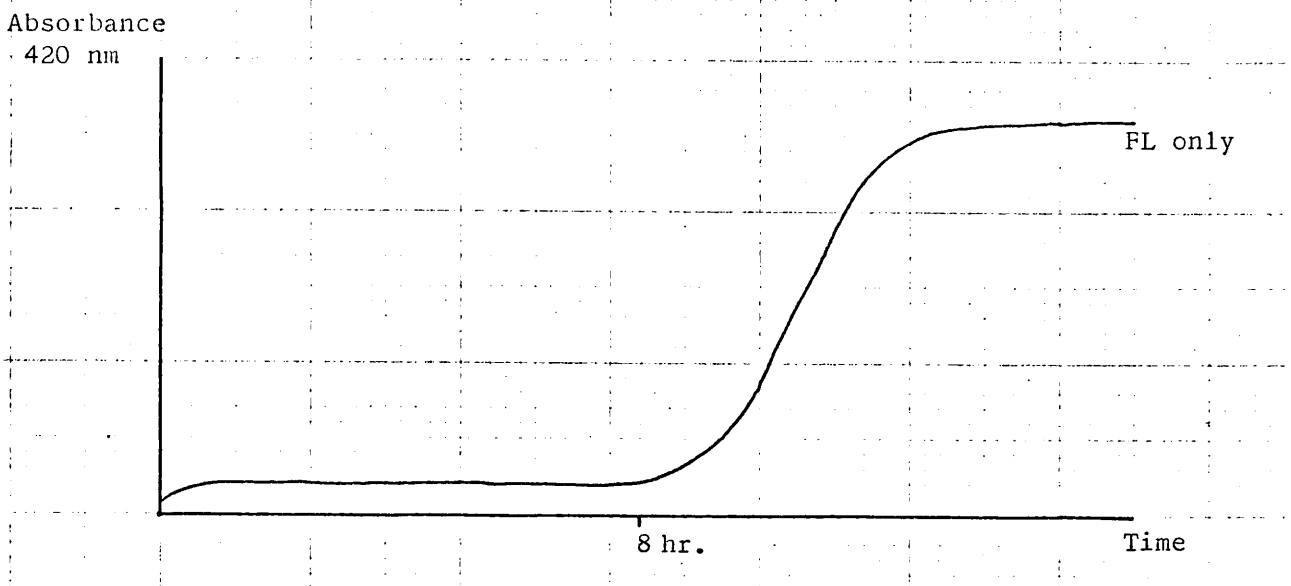


Fig. 2.7 E. coli growth curve (aerobic, see text)

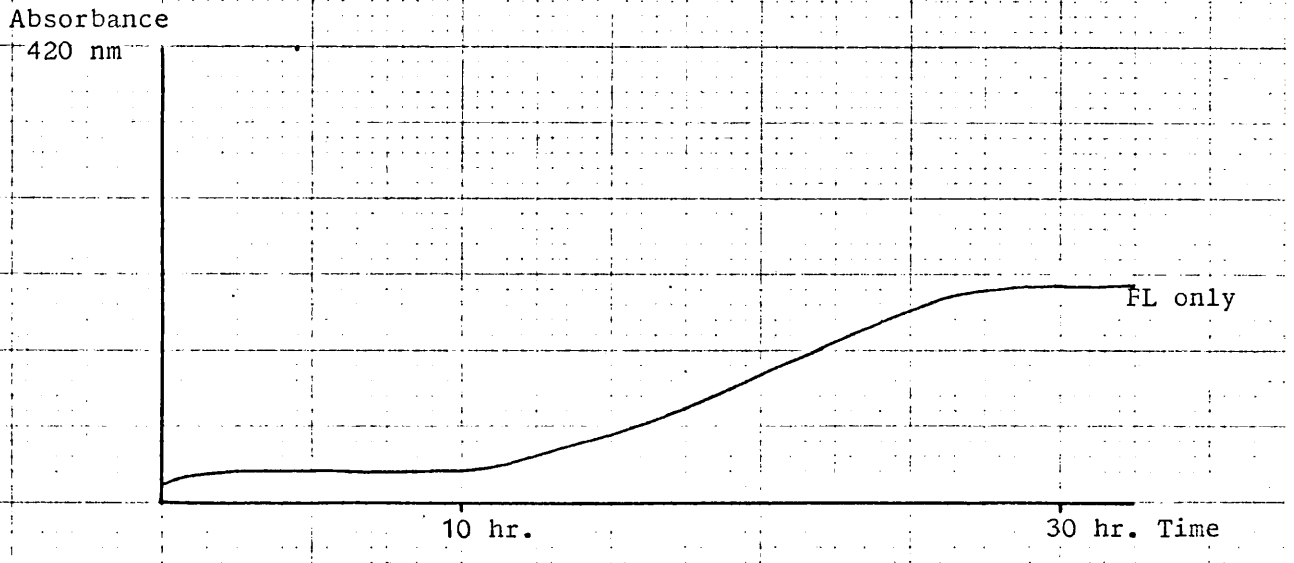


Fig. 2.8 E.coli growth curve (anaerobic, see text)

Subcultures of cells originally grown on glucose in the presence of FL, as in experiments (1) anaerobic and (2) aerobic, also commenced growth immediately when incubated at high or low O_2 tension in fresh media containing FL (2.5×10^{-3}M) as the only carbon source.

Summary This investigation has shown that FL has no inhibitory effect on the utilisation of glucose by E.coli but inhibition does occur if the compound is heated. Heat-degraded FL acts, as does FL, in causing an overall increase in cell growth. After suitable exposure to FL, a FL-utilising system is induced; for resting cultures this takes 8-10 hr. FL-utilisation can also apparently be induced by FL when the cells are growing aerobically on glucose. Cells growing anaerobically on glucose can also be induced by FL but in this case by the time induction occurs the cells have lost viability (Exp. 1) presumably due to an accumulation of antimetabolites. The results of the studies suggest that only the fructosyl part of FL may be used for growth: E.coli may split FL to fructose and lysine.

Since the FL-utilising system is permanent and results in the metabolism of FL, it could be: (i), an induced extracellular enzyme which splits FL to absorbable products; (ii), an induced permease transporting FL into the cell where constitutive enzymes, normally cryptic towards FL, immediately metabolise it, or (iii), assuming the natural presence of a FL permease, an induced intracellular enzyme or series of enzymes. These alternatives are summarised in Fig. 2.9. Combinations of (ii) and (i) or (ii) and (iii) are also possible. Uptake of solutes such as FL by simple diffusion is normally considered to be too slow in bacteria to allow rapid growth curves, of the type observed, to occur.

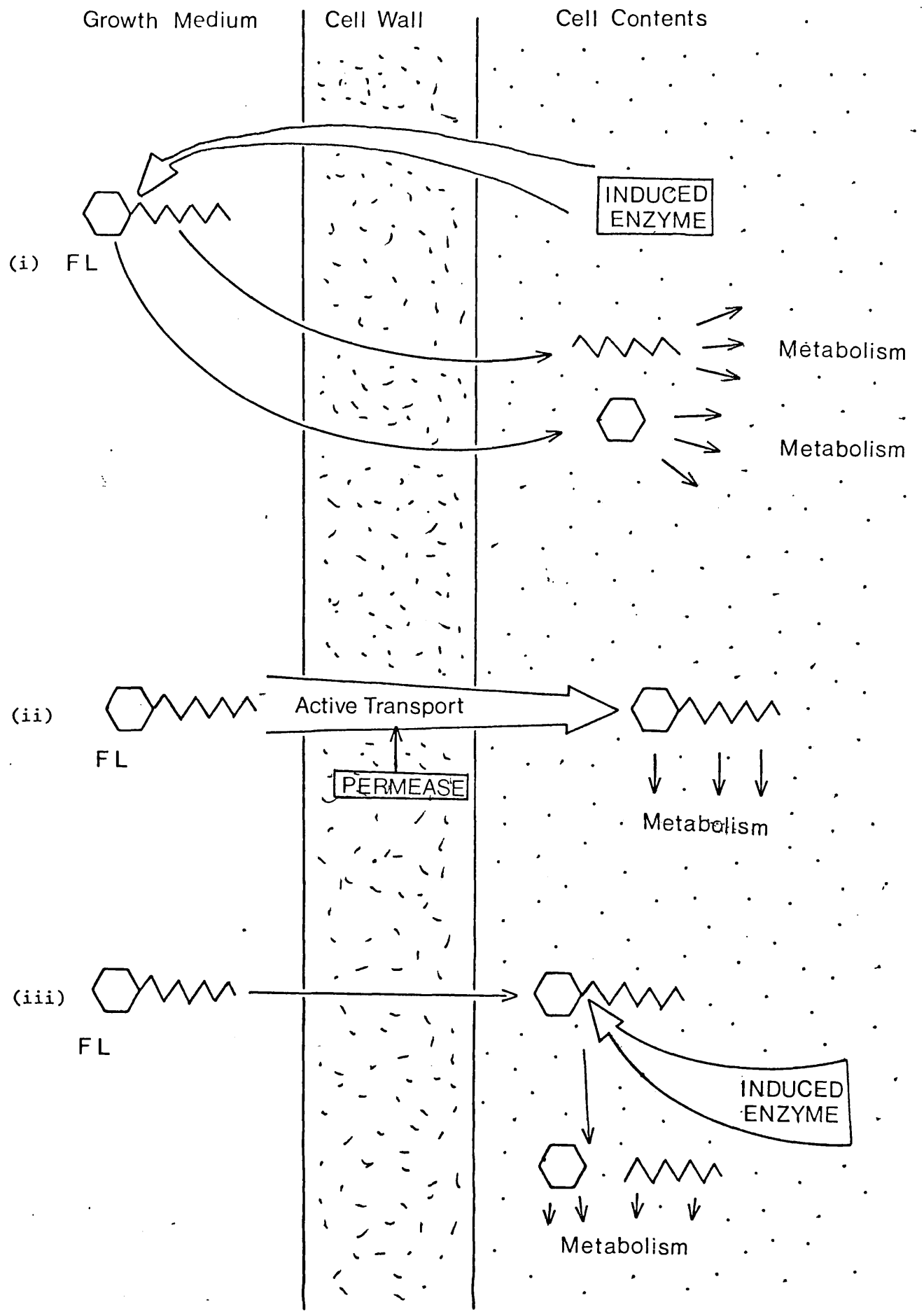


Fig. 2.9 Schematic representation of possible nature of the FL-utilising system

Part II - The FL-Utilising System

The following studies were designed to elucidate the nature and to determine the location of the induced FL-utilising system. Whole-cell preparations and various types of cell extract were investigated for their ability to degrade FL and cultures of growing cells were used to study the absorption of FL and its degradation products.

Investigation of the cell contents of non-induced E.coli for the presence of an 'FL-utilising system' (Exp. 8). This is part of the investigation was designed to show whether the FL-utilising system is a natural constituent of normal 'wild-type' cells. For a bacterium, crypticity of a constitutive enzyme towards its substrate is the safest criterion of the impermeability of the cells to that substrate. Consequently, if the presence of a FL-utilising system was shown to be constitutive, this would indicate that failure to utilise FL was due to permeability problems, i.e. that the induction, observed in previous experiments, was at the permease level.

Cell-free extracts were prepared by sonication of non-induced cells in minimal medium. FL was then added and aliquots of the mixture were taken at intervals and assayed for FL using the Borsook reaction; control solutions contained no FL (see Materials and Methods, p.133). The results obtained are shown in Fig. 2.10.

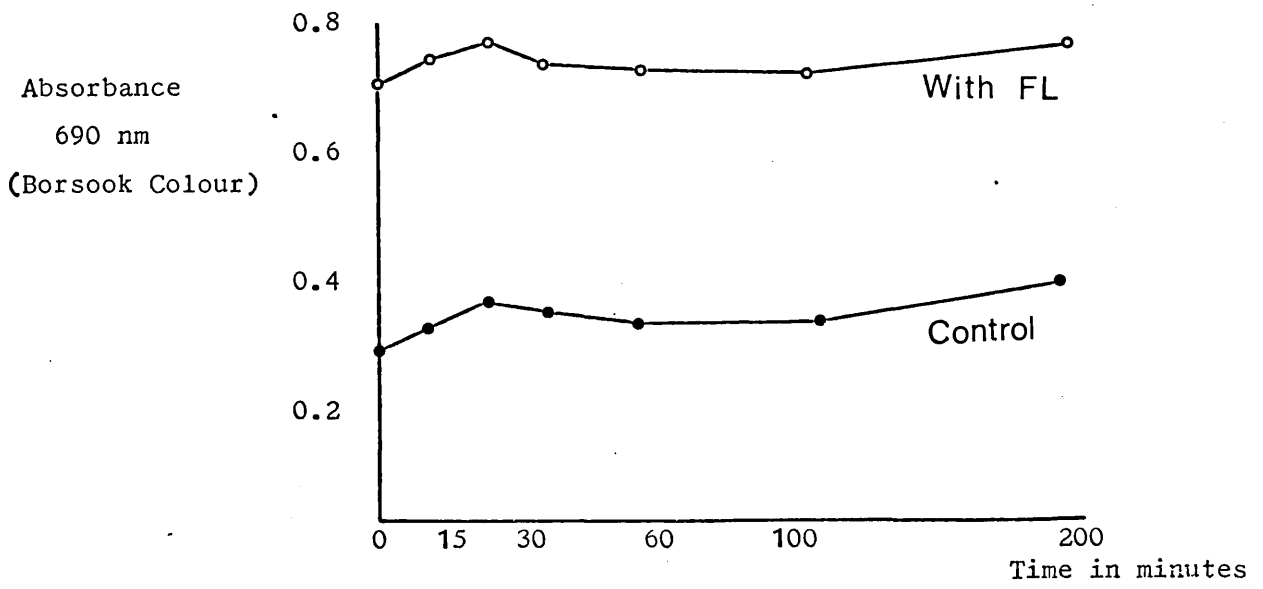


Fig. 2.10 The level of FL in digests containing a cell-free extract of E. coli. Each point is the average of two readings.

The values for the controls showed that the cell extract contained Borsook-positive substances which showed a small increase (0.1 absorbance units) over the incubation period. This curve exactly paralleled that which was obtained with the FL-containing extract and therefore there was no indication of disappearance of FL.

The experiment was repeated three times under different ionic conditions and pH values but in all cases the FL levels as estimated by the Borsook assay, remained constant. Hence E.coli cells do not appear to possess a constitutive FL-utilising system unless it is of a labile nature and is destroyed during extraction.

However, the utilisation of FL might involve a change in the structure of the compound which is not detectable by the Borsook reaction or which results in products which are themselves Borsook positive. Other experiments were therefore carried out where FL* (500,000 cpm) and *FL (500,000 cpm) were added to the cell extract in addition to unlabelled FL. After incubation (200 min), the mixtures and controls (without FL) were separated by paper chromatography and, where necessary, chromatograms were scanned for radioactivity (see Materials and Methods). No degradation products of FL could be detected by this procedure. Subsequent examination of the chromatograms revealed a large number of overlapping ninhydrin-positive areas. Patterns from all incubation mixtures were essentially similar (except for the absence of FL from controls), and this confirms that FL remains unchanged upon incubation with cell extracts.

It was considered that the activity of enzymes involved in Fl-utilisation could have been lost during the preparation of the cell extract despite the fact that the usual precautions were taken and extraction was effected in a minimum time using a simple salt solution. Hence, further modifications of the standard method of preparation were made in a further attempt to detect relevant enzyme activities. Firstly, the overnight culture was harvested in late log phase (i.e. the region of most rapid protein synthesis) and the centrifugation times were substantially reduced and secondly, a more concentrated cell extract was used as a source of possible enzymes. In both cases, however, no apparent changes in the level of FL could be detected.

Studies on the disappearance of FL during induced growth of E.coli (Exp. 9)
It has been shown that E.coli is capable of growth on FL, when present as the only available organic carbon source, and this implies that at some point during the resulting induced growth curve, FL must be degraded. This was further studied by attempting to measure the disappearance of FL from cultures. Wild-type cells were cultured in the presence of FL (2.5mM) and samples were removed at suitable intervals for Borsook assay of the FL level in the medium; growth curves over the whole period of the experiment were also constructed.

The results of this investigation are depicted in Fig. 2.11. A normal growth curve showing induction after 8 hr. was obtained. FL disappearance was seen to begin at approximately the same time as cell growth and continued slowly to apparent completion just prior to the stationary phase. The residual Borsook colour after 15 hr. was equivalent to an absorbance of 0.08 i.e. equal to that of control reading obtained with a supernatant solution of an 18 hr. culture grown on glucose and was presumably due to normal cell products. Thus all the FL provided appeared to have been utilised. This same result was obtained in each of two repeats of the experiment.

The apparent disappearance of FL could have been due to either: an induced permease removing it from the external medium or to a change in the structure of FL, occurring in the external medium, which rendered it Borsook-negative.

The shape of the Borsook-colour curve indicates that the former explanation of FL-disappearance is unlikely. Removal of a substrate by a permease is usually very rapid and may be completed in 2-3 min. after addition of substrate to a culture. If a 'fructose-lysine permease' had appeared at 8 hr., a sudden disappearance of FL would have been expected to follow. An inoculum (0.5ml) of five times the usual for growth experiments was used to ensure that the number of cells present would not limit such an effect and, as a further precaution, a control experiment was carried out to show that the environmental conditions in the above experiment were likely to be conducive to normal permeation of FL, and that the cells were sufficient in number to cause complete uptake of solutes (at 2.5mM concentration) if permease induction had made this suddenly possible. Thus FL-induced cells (as above) were taken at the onset of growth (i.e. after 8 hr.) and their ability (normally constitutive in E.coli) to accumulate glucose investigated and a

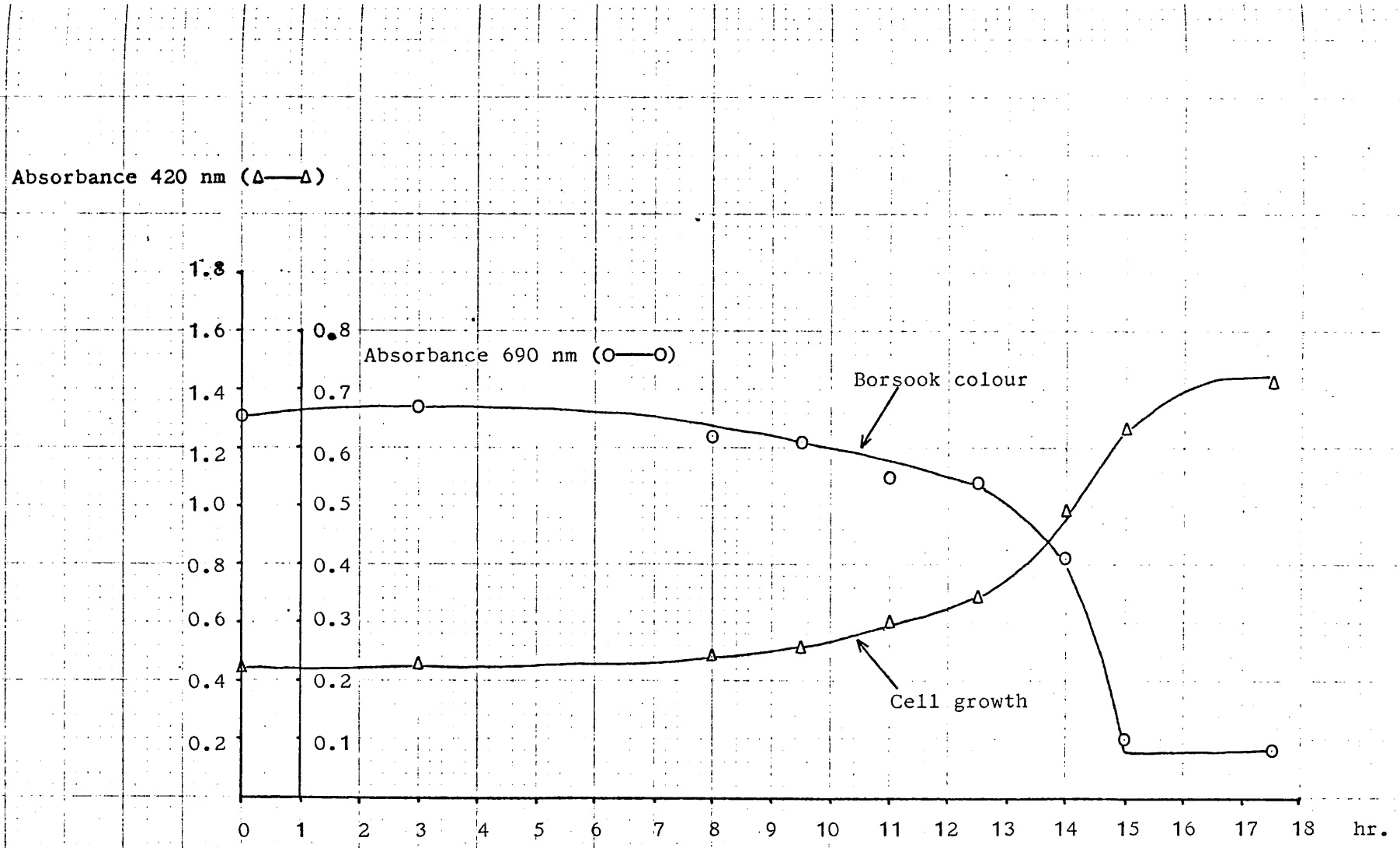


Fig. 2.11 Comparison of E.coli cell growth with apparent disappearance of FL.

normal rapid disappearance of glucose was observed as shown in Fig. 2.12.

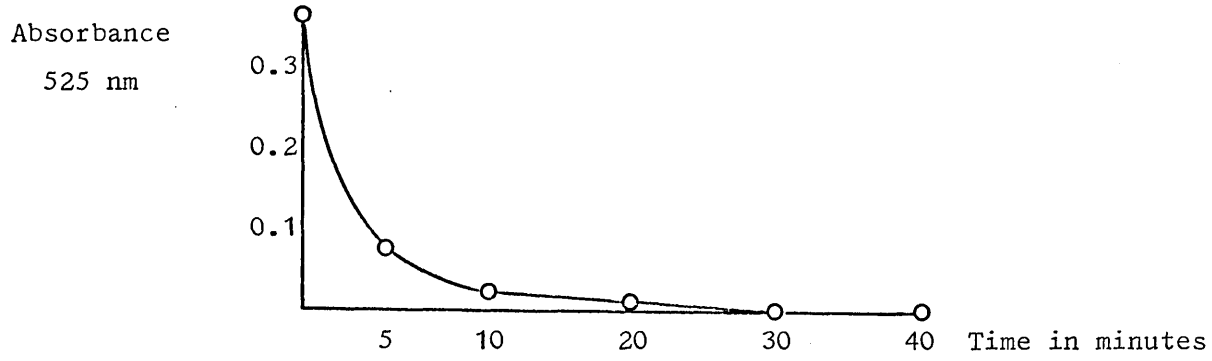


Fig. 2.12 Glucose uptake by induced E.coli cells

Hence the number of cells and the conditions appeared to have been favourable for permeation of FL. Since the graph in Fig. 2.12 is typical of those shown in the literature (e.g. (72)), it can also be inferred that FL, which was also present in the medium, does not significantly interfere with glucose permeation.

Thus it was considered to be unlikely that the disappearance of FL had been caused by a 'fructose-lysine permease'. However, some bacterial permeases do catalyse slow solute uptake and 'fructose-lysine permease', if it exists, could be such an example of an exceptionally inefficient permease. If, as previously suggested, (see p.48) a fructose-lysine metabolising system is not constitutive, the induction of such a system following permease-catalysed accumulation of FL should produce a further restraint of the onset of cell growth beyond 8 hr. of Fig.2.11 but this was not observed. However, passive diffusion of minute quantities of FL into the cell, during the 8 hr. before growth, might cause simultaneous induction of a permease and an intracellular 'FL-ase'.

The alternative explanation of FL disappearance from the medium is that is suffers extracellular degradation. From the results obtained it is possible that an extracellular 'FL-ase' was induced. The slow speed of FL breakdown could then have been a result of enzyme regulation where either the activity was low or the rate of synthesis was depressed. Also the speed with which the enzyme was able to leave the cell could have been rate limiting. The type of breakdown which could have occurred is cleavage of the sugar-AA bond which would produce Borsook-negative compounds.

It should be noted, for example, that fructose is not Borsook-positive

at the concentrations which would have been present in the medium if this hypothetical cleavage had taken place.

In a repeat of the experiment where wild-type cells were cultured in the presence of FL, a more detailed study of the region of the curve where induction had occurred was made. The results of this study are shown in Fig. 2.13 and Fig. 2.14. Fig. 2.13 shows the first part of a typical growth curve and Fig. 2.14 illustrates the corresponding changes in Borsook-positive compounds in the medium. The initial rise in the absorbance in Fig. 2.14 probably results from compounds leached from damaged cells. Then, after an equilibrium period lasting 4-5 hr. the growth of cells and the disappearance of FL commences simultaneously (between 8 and 9 hr.). Thus the action of the assumed extracellular 'FL-ase' probably results in the formation of degradation products of FL which can be rapidly utilised for growth.

Fig. 2.11 shows an extended pre-log phase, for both curves which are linear rather than exponential (as normally occurs with E. coli utilising glucose). This suggests that if 'FL-ase' is produced the activity and hence cell growth during this period are probably limited by a finite rate of enzyme-protein synthesis. Later, (after 12.5 hr; Fig. 2.11) if the increase in the absolute amount of 'FL-ase' becomes exponential, excess products of FL degradation would result and become available as substrates thereby allowing growth itself to continue exponentially (as observed).

On balance, at this point in the investigation it was believed that the results favoured induction of an extracellular 'FL-ase' rather than of a permease.

The Distribution of 'FL-ase' Activity

The presence of the proposed 'FL-ase' in the medium and in preparations of various parts of induced E.coli cells was next investigated. When the site of action of a bacterial enzyme is extracellular, activity will normally be found in the external medium and/or bound to the cell wall but rarely in the cell contents. An enzyme acting intracellularly will normally be detectable in the cell contents and sometimes attached to the cell membrane. The following cell fractions were therefore tested for the presence of 'FL-ase'.

(a) Activity of cell contents (Exp. 10) . A cell-free extract was prepared and incubated with FL in minimal medium as previously described (see Exp. 8).

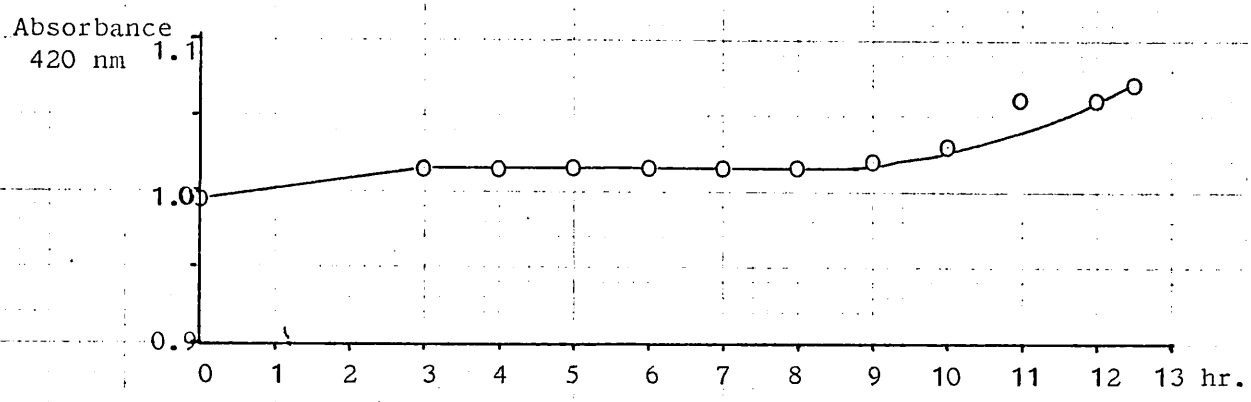


Fig. 2.13 Growth curve for non-induced E.coli cells in the presence of FL (2mM)

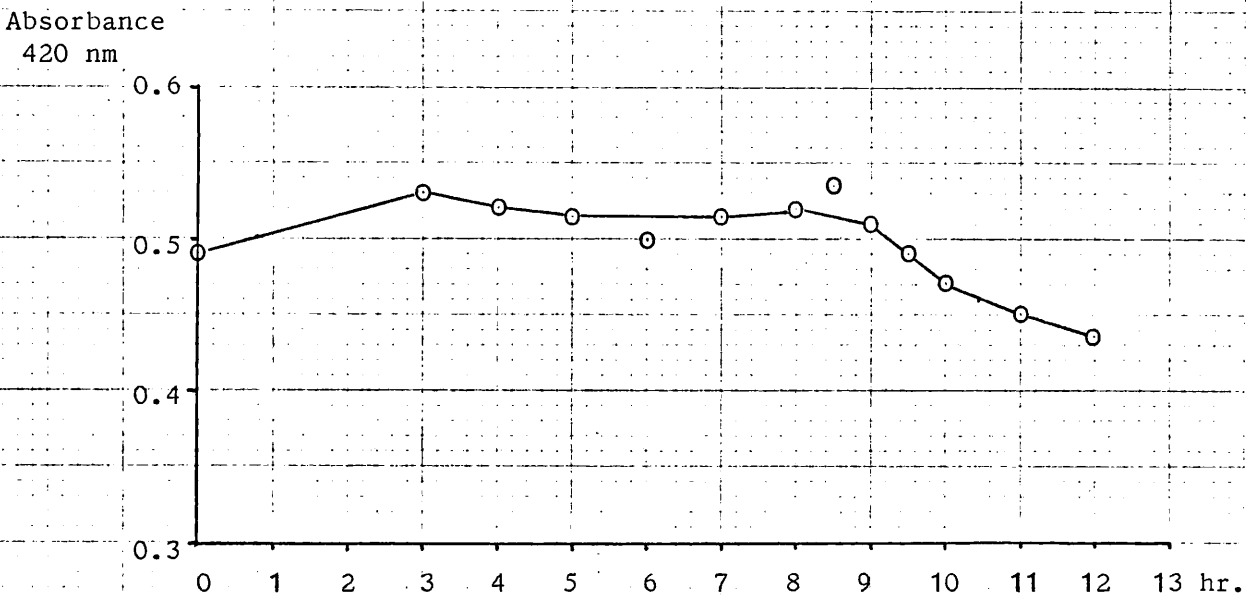


Fig. 2.14 The level of FL (Borsook test) in the medium of non-induced E.coli cells growing on FL as shown in Fig. 2.13

Samples were taken at intervals and the results are shown graphically in Fig. 2.15.

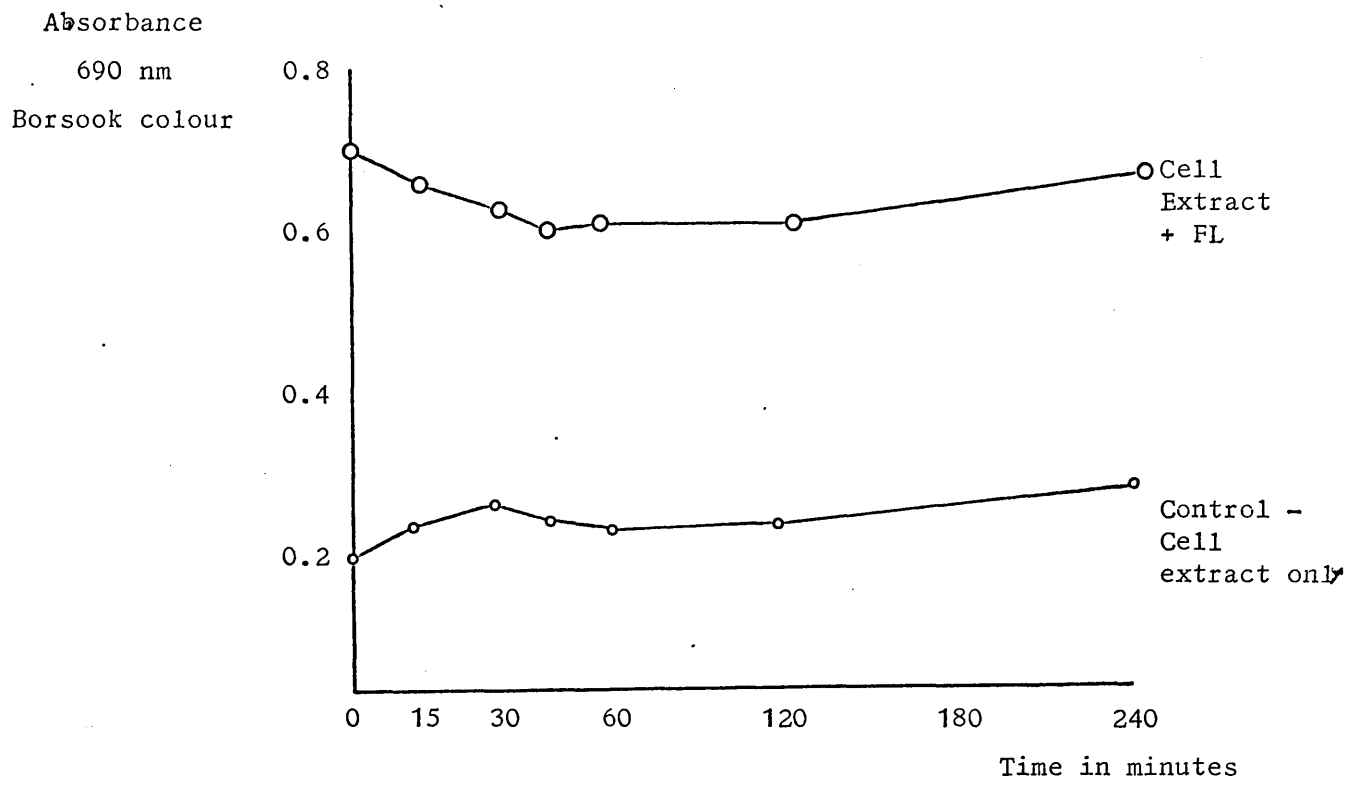


Fig. 2.15 The level of FL in digests containing a cell-free extract of induced E.coli cells.
Similar results were obtained in each of two repeats of the experiment.

The cell extracts containing FL showed a small initial decrease in Borsook-positive substances which was not shown by the control. This indicated that FL had disappeared from the medium and was probably degraded by an 'FL-ase'. The disappearance of FL might, however, have been due to non-enzymic degradation under the conditions of the experiment. Hence, as a further control the experiment was repeated using boiled cell-extract and in this case, after the addition of FL, no significant absorbance change was observed over the experimental period (4 hr.). The fact that initial absorbance decreases were never observed with FL and either non-induced cell extracts (i.e. Exp. 8) or heated induced cell-extracts suggests that the observed presence of low 'FL-ase' activity was real but was lost after 1 hr. At the time it was considered that the presence of activity in the cell contents of E.coli

could have been produced by release of a bound enzyme by sonication during preparation.

(b) Activity of external medium (Exp. 11). An induced culture was grown on FL (2.5×10^{-3}M) under standard aerobic conditions and on reaching the stationary phase a sample (5 ml) of the culture was taken and the cells isolated by centrifugation. These were then washed free of supernatant solution and resuspended and incubated in minimal medium (5 ml) containing FL (2mM). The supernatant solution after cell removal was sterilised by millipore filtration and also incubated with FL (2mM). Both incubation mixtures were assayed at intervals for FL and the results are shown in Fig. 2.16.

The graph clearly shows that although whole, washed cells caused a rapid decrease in the amount of FL in solution, there was no 'FL-ase' activity in the medium in which they had been growing. It was also found that there was still no free 'FL-ase' activity in the medium after the first 2 hr. of the experimental period (see Fig. 2.16). Thus an extracellular 'FL-ase' was not apparently released into the medium during normal induced growth.

There are two possible explanations of this result. The first is that an extracellular 'FL-ase' is induced but that it remains totally bound to the cell wall and secondly, that a slow-acting permease allows cellular accumulation of FL. In the latter case the FL could then be metabolised by the intracellular 'FL-ase' which the previous experiment showed could have been present at low levels.

(c) Activity of the cell wall (Exp. 12). In the following experiment a crude, cell-wall preparation was investigated for FL-ase activity. An induced culture was again grown in the presence of FL (2.5×10^{-3}M). The culture was harvested in stationary phase and the cell wall preparation obtained (Materials and Methods, p. 134)*

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*Footnote Methods are available for purifying cell walls by differential centrifugation but, in the interests of reducing the time between sonication and use of the preparation, hence conserving 'FL-ase' activity, these were not attempted. The cell wall preparation, as used, was presumably contaminated with cells with intact walls that had survived sonication and centrifugation. However, these cells were probably either very few in number or sufficiently damaged to prevent growth: an absorbance increase of only 0.02 units, corresponding to growth, was recorded over the 2 hr. period and also the Borsook curve was not characteristic of growing cells (cf Fig. 2.17).

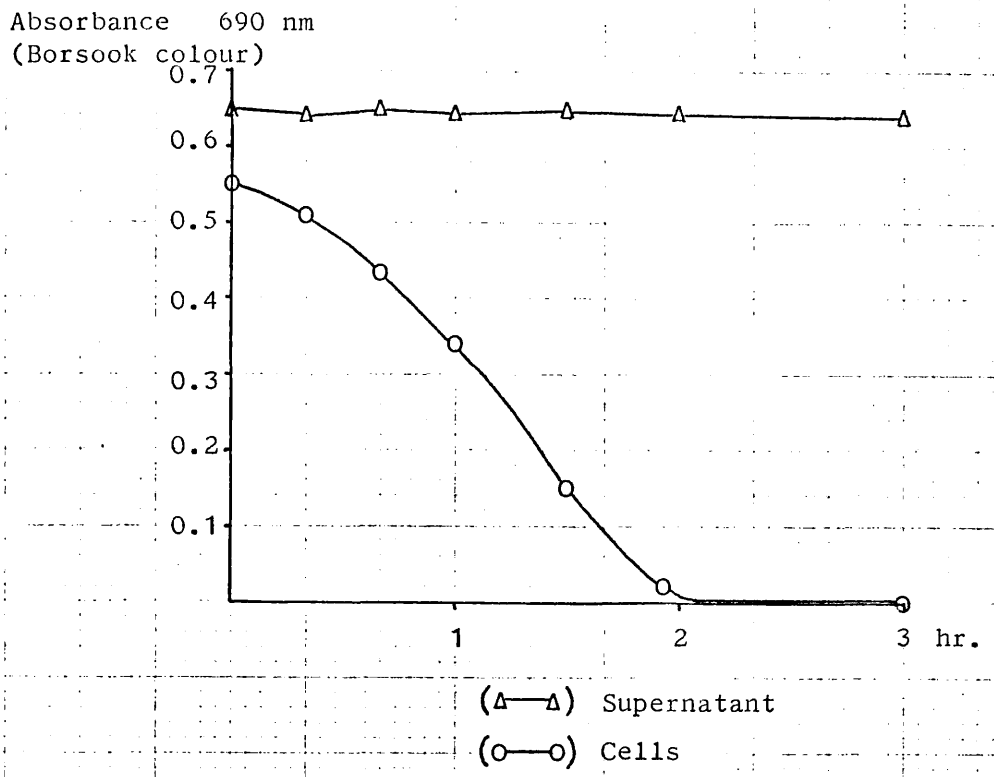


Fig. 2.16 The level of FL in the medium (Borsook test) when incubated with the supernatant or cell fraction of an induced E.coli culture; see text p.55

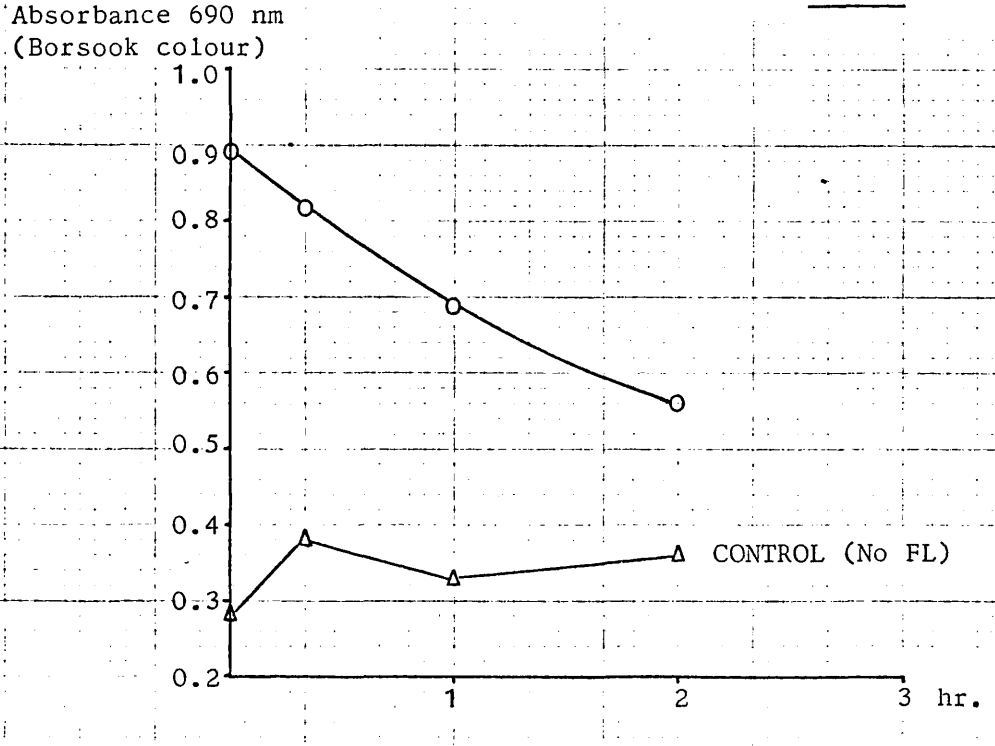


Fig. 2.17 The level of FL in the medium (Borsook test) when incubated with cell-wall preparation of E.coli; see text p.55

The cell wall preparation was washed with minimal medium, centrifuged and resuspended in minimal medium (12.5 ml). FL was added (2×10^{-3}M) and its disappearance monitored as in the preceding experiments. Fig. 2.17 shows that the preparation possesses a significant 'FL-ase' activity.

The net decrease in the level of FL was 0.42 absorbance units (0.37 and 0.39 in repeat experiments).

From the results of experiments 6,7,9 and 11 it was estimated that the rate of decrease of FL upon incubation with the cell wall preparation would be approximately three times that actually obtained (assuming all FL-ase activity of intact cells to be located on the cell wall). Thus it was probable that sonication inactivated the enzyme(s), perhaps by local heating effects. In addition, it is possible that only bound 'FL-ase' is active and that much of it became detached during sonication.

Attempted solubilisation of bound 'FL-ase' (Exp. 13). Attempts were made to solubilise 'FL-ase' from the cell surface by treatment with various ionic and non-ionic detergents following pretreatment with lysosyme. This type of procedure has been used for solubilising other membrane bound enzymes of E.coli (e.g. DNA polymerase; Knippers 1970 (69)). It was envisaged that, if successful, the procedure would provide the first step in the purification of the enzyme for further detailed studies of its biochemical properties and reaction constants.

Stationary phase cells from a stock culture of induced cells grown on FL (2.4 mM) were isolated by centrifugation and resuspended in minimal medium at pH 6.5. Cell samples were added to solutions of lysosyme, (150 mg/L) also in minimal medium, and after incubation for 45 min. (37°C), detergent was added and incubation continued for a further 45 min. The cells were again removed by centrifugation and the centrifugate sterilised by millipore filtration and incubated with FL (2mM). Aliquots were taken for Borsook assay at the start of the reaction and after 2 hr. The following changes in the level of FL were observed over the 2 hr. period:

TABLE 2.1 The solubilisation of 'FL-ase' activity with detergents

DETERGENT (Final Concentration = 1%)	Change in absorbance at 690 nm in 2 hr.
Cell wall (crude) fraction with no pretreatment	- 0.27
Sodium Deoxycholate	- 0.015
SDS	- 0.060
BRIJ 58	- 0.005
TRITON X - 100	zero
NONIDET	+ 0.020
Water (control)	- 0.01
Water ≠	- 0.03

≠ lysosyme at double concentration during pretreatment.

The Borsook assay was shown to be unaffected by the presence of the detergents (1% w/v) used in this experiment.

Only SDS (Sodium lauryl sulphate) the detergent producing the greatest disintegration of the walls in these experiments appeared to liberate significant amounts of 'FL-ase' activity. It was expected that a preparation with higher 'FL-ase' activity would have been produced by SDS but presumably the solubilization of the 'FL-ase' destroyed much of the activity. It should be noted that the activity of the crude cell wall fraction was five times that of the SDS-treated fraction.

In the case of the other detergent pretreatments, 'FL-ase' was either not solubilised or lost activity.

In an experiment where the cells were preincubated with lysosyme for 15 min. and then SDS added and incubation continued for a further 30 min. there was a slight improvement in the recovery of 'FL-ase' (a decrease in absorbance of 0.09/2hr.), hence showing the lability of 'FL-ase' in the detergent. SDS did not inhibit FL utilisation by whole, induced cells over 45 min. but BRIJ 58 reduced the amount utilised by half. This latter inhibition ceased when the cells were then isolated from the detergent and resuspended in

minimal medium containing FL. A sample containing only FL and lysosyme in minimal medium showed that lysosyme itself did not degrade FL.

In view of the poor yields of solubilised 'FL-ase' this line of approach was discontinued.

The utilisation of radioactive FL by E.coli. (Exp. 14). In further experiments attempts were made to follow the disappearance of FL from media during the growth of FL induced E.coli cells. Cells isolated from a stock culture (5 ml) and re-suspended in fresh minimal medium (5 ml) were incubated with 2mM FL labelled with ^{14}C in the lysine moiety (FL*; 100,000 c.p.m.). FL disappearance in the medium was monitored (after cell removal) by the Borsook assay and the FL* levels were estimated by counting radioactivity in samples of medium taken at intervals and absorbed on to filter papers. The results are illustrated in Fig. 2.18.

The graph clearly shows that although FL in the external medium was rapidly disappearing, it was not due to accumulation by the cell because all the radio-activity remained in the external medium. Thus an enzyme must have been present which was degrading FL extracellularly.

Previous experiments had indicated that the 'FL-ase' was not free in the culture media of induced stock cultures or those used in the above experiment. Neither was protein apparent in the medium in the latter case. Thus, it is likely that the extracellular 'FL-ase' is cell-bound, i.e. attached either to the cell wall or to the exterior of the cytoplasmic membrane.

Since the cells used were harvested from a stock culture in log phase of growth on FL, the addition of further FL should have allowed the continuation of this logarithmic growth. Turbidity measurements which were made at zero time and after 4 hr. confirmed that further growth had occurred. The E.coli cells must, therefore, have utilised as well as degraded the FL in the medium. Since the experiment showed that the lysine moiety of FL* did not enter the cell, it follows that only the hexose part of FL was used as a carbon source during growth. The extracellular 'FL-ase' may have cleaved the hexose carbon chain whilst the sugar was still bonded to the lysine moiety or alternatively, first removed the mono-saccharide by degrading the C-N bond thus releasing free sugar. Further studies (Exp. 15) were made using fructose-lysine universally labelled in the fructose residue (*FL) to ascertain whether radicactivity originally present in the sugar moiety of FL could be accumulated by E.coli cells during the disappearance of FL in or from the external medium. The method employed was essentially the same as for the previous experiment with FL* and the results obtained are shown in Fig. 2.19.

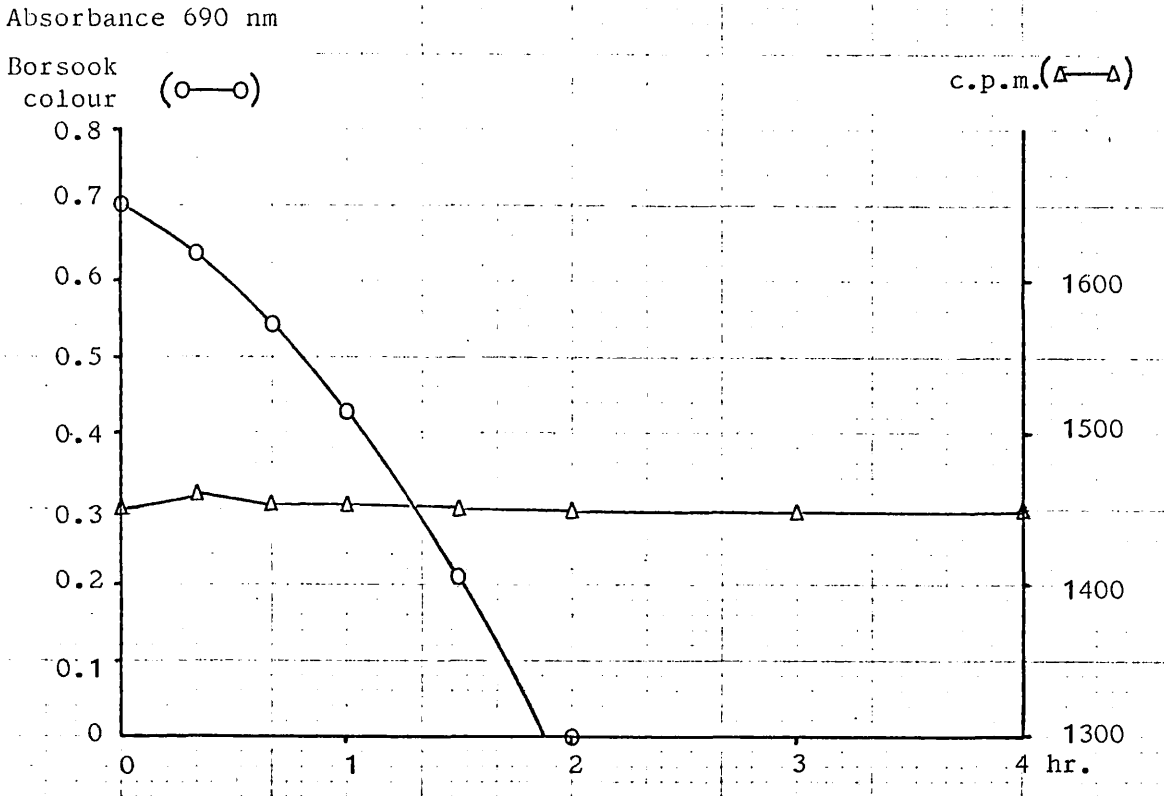


Fig. 2.18 The level of FL* (Borsook test and radioactivity) in the medium of an induced cell culture of E.coli; see text p.59

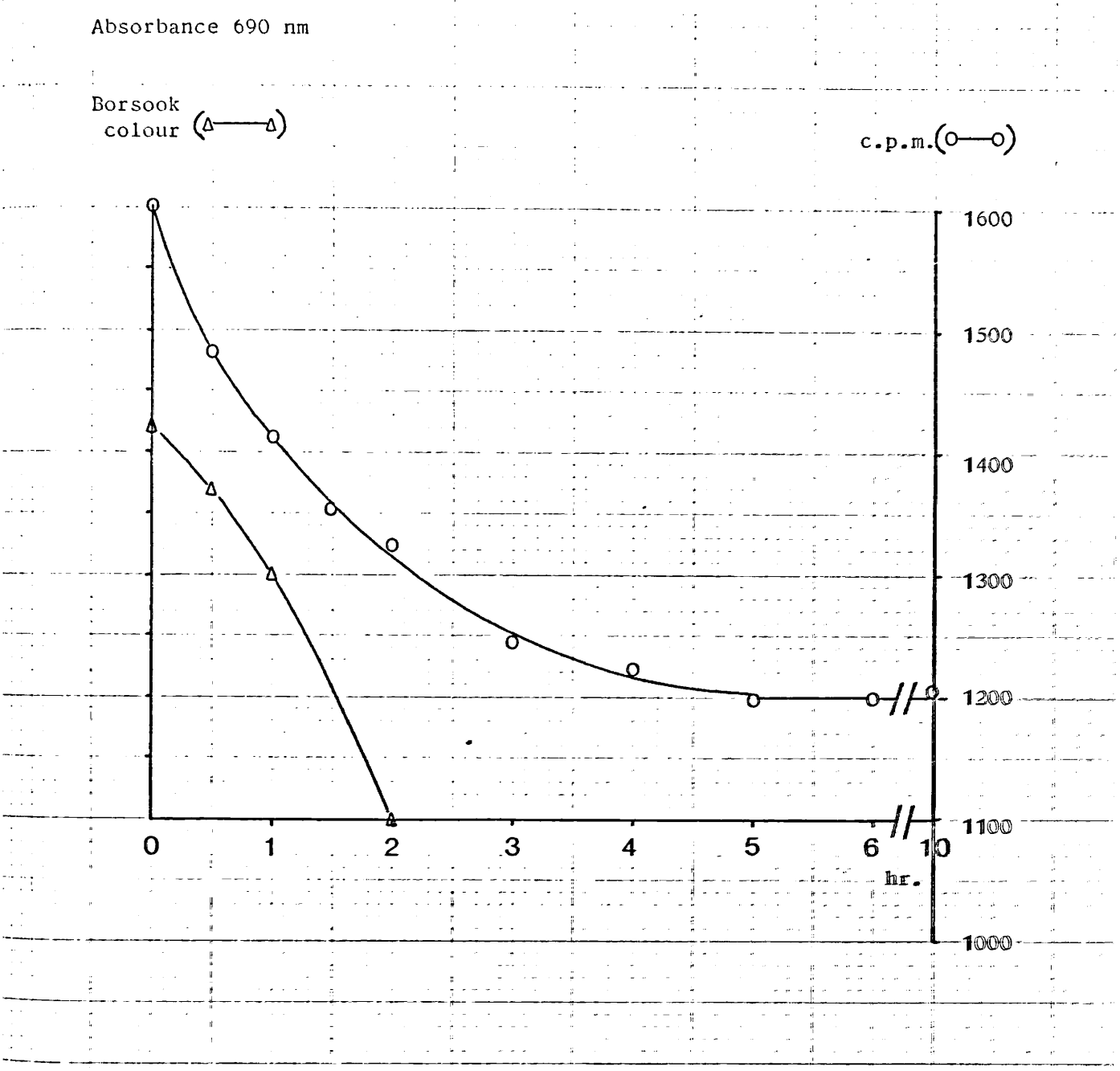


Fig. 2.19 The level of *FL (Borsook test and radioactivity) in the medium of an induced cell culture of E.coli;

see text p.59

It is apparent from this graph that the disappearance of radioactivity from the medium occurs concomitantly with the usual disappearance of FL, as estimated by Borsook assay. The same effect was observed in each of two repeats of this experiment. Thus, it may be concluded that degradation of the FL molecule by extracellular 'FL-ase' is accompanied by partial removal of the fructose moiety (either in an intact or a degraded form) from the medium.

The disappearance of radioactivity from the medium occurred at a slower rate than the disappearance of Borsook colour. This implied that the breakdown of FL was more rapid than the absorption, by the cells, of the products of this breakdown. Also, the disappearance of the radioactivity was incomplete and, in fact, only 25% of the total number of radioactive counts provided as *FL were removed from the medium. Further, detailed studies on the nature and products of 'FL-ase' action are discussed in Part IV (see pp.69-83).

As the cells were incubated in sealed centrifuge tubes without shaking, conditions were essentially anaerobic. Hence the experiment was repeated with cells growing under aerobic conditions. In this case the results were essentially similar to those depicted in Fig. 2.19 except that the rate of disappearance of FL, as measured by both Borsook assay and loss of radioactivity, was increased. The disappearance of radioactivity (again 25% of the total) was complete after 3½ hr. This effect was probably a result of the increased growth rate of cells due to aerobic conditions. Also, constant shaking of the culture may have facilitated contact of the FL with the bound 'FL-ase'.

Although the studies with *FL and FL* show that radioactivity disappears from the medium it is possible that it is not accumulated by the cells. The radioactivity might, for example, be lost from the medium as free $^{14}CO_2$. It was, therefore, necessary to show that the radioactivity disappearing from the medium could be subsequently detectable in the cell contents. The cell culture (1.5 ml) remaining after 5 hr. (see Fig. 2.19) should theoretically have contained a total of 7,500 cpm. Measurement of radioactivity of the cell contents revealed a 40% recovery (2988 cpm) of this estimated total. Since the sonication and radioactive counting stages involved in examination of the cells both had efficiencies of 65-75%, a recovery of between 40% to 55% was expected.

In conclusion it appears that the apparent loss of radioactive *FL from the medium was a result of firstly, degradation of the compound by extracellular 'FL-ase' followed by absorption of some of the labelled fructose moiety or a derivative of this moiety by the cells.

Summary

The distribution of FL-metabolising activity in various types of cell cultures and cell extracts is summarised in Table 2.2 which shows the approximate relative rates of FL-disappearance:

TABLE 2.2 Disappearance of absorbance units Borsook test colour/ml. medium/hr. (corrected for standard number of bacterial cells)

SAMPLE	VALUE
Non-induced cells	zero
Cell-free extract of non-induced cells	zero
Induced cells in log phase	1.00
Cell-free extract of induced cells	0.05
Cell-wall preparation (crude) of induced cells	0.30
Isolated induced cells - further enrichment with FL	1.00
Culture medium alone, from induced cells	zero

Studies with radioactively labelled FL showed that carbon, present in cultures of E.coli as FL, does not permeate the cell until the FL has been broken down. Thus the evidence provided by the experiments in Part II suggests that FL induces a totally cell bound, extracellular 'FL-ase' which may be synthesised intracellularly or, alternatively, in the cell wall or cytoplasmic membrane. No conclusive evidence for the presence of a 'FL-permease' which could act as part of an alternative utilisation system was found. This discovery was not expected since exoenzymes are uncommon in gram-negative bacteria (e.g. E.coli) and the occurrence of exoenzymes capable of metabolising low molecular weight compounds (e.g. FL) is uncommon in all bacteria.

Part III - Respiration Studies with ^{14}C-labelled FL

Experiments were carried out to determine whether FL labelled in the fructose or lysine moieties gave rise to $^{14}CO_2$ when used as a substrate for normal induced cultures of E.coli.

Induced cells were grown in Warburg flasks with KOH papers in the centre well to absorb the $^{14}CO_2$ (see 'Materials and Methods' p.118). The cultures, in minimal medium, containing labelled FL (usually 1.25×10^{-3}M) showed normal sigmoid growth curves which rose to a stationary phase value of 0.6 absorbance$_{420}$ units in 10-12 hr. Culture samples and KOH papers were taken in the middle (usually after 5 hr.) and at the end (usually after 12 hr.) of the logarithmic phase of growth and at other selected times (sample KOH papers were replaced with fresh KOH papers). Since the lengths of the lag phases in different experiments were found to show a variation, oxygen uptake was measured at intervals (see 'Materials and Methods' p.118) to monitor the onset and progress of growth which allowed the appropriate times for taking samples to be estimated without interrupting incubation. This also allowed the growth rates of cultures in different flasks and in different sets of experiments to be easily compared. A typical respiration curve is presented in Fig. 2.20. The absorbance at 420nm of the culture samples was measured to check the extent of growth, prior to storage of the samples in deep freeze; these were used for further investigations (see Part IV). The amounts of $^{14}CO_2$ detected on the KOH papers are shown in Table 2.3 for *FL and Table 2.4 for FL* and a summary of the data for the free CO_2 is given in Table 2.5.

It is clear that up to 24 hr. there is a continuous production of $^{14}CO_2$ from both labelled FL derivatives. However, the standard deviations of the average values calculated also increased with time. This may, in part, have been a result of fluctuations of the pH values of the flask contents which occurred during incubation and which affected metabolism and/or the ratio of dissolved and free $^{14}CO_2$; losses of CO_2 during retrieval of the KOH papers from the flasks may also have been contributory. The values for the dissolved $^{14}CO_2$ (see Table 2.3) recovered by treatment of *FL digests with acid clearly show that the amount of $^{14}CO_2$ in solution decreased with time. This may suggest that some of the CO_2 in solution, resulting from FL metabolism by respiratory processes was being re-utilised

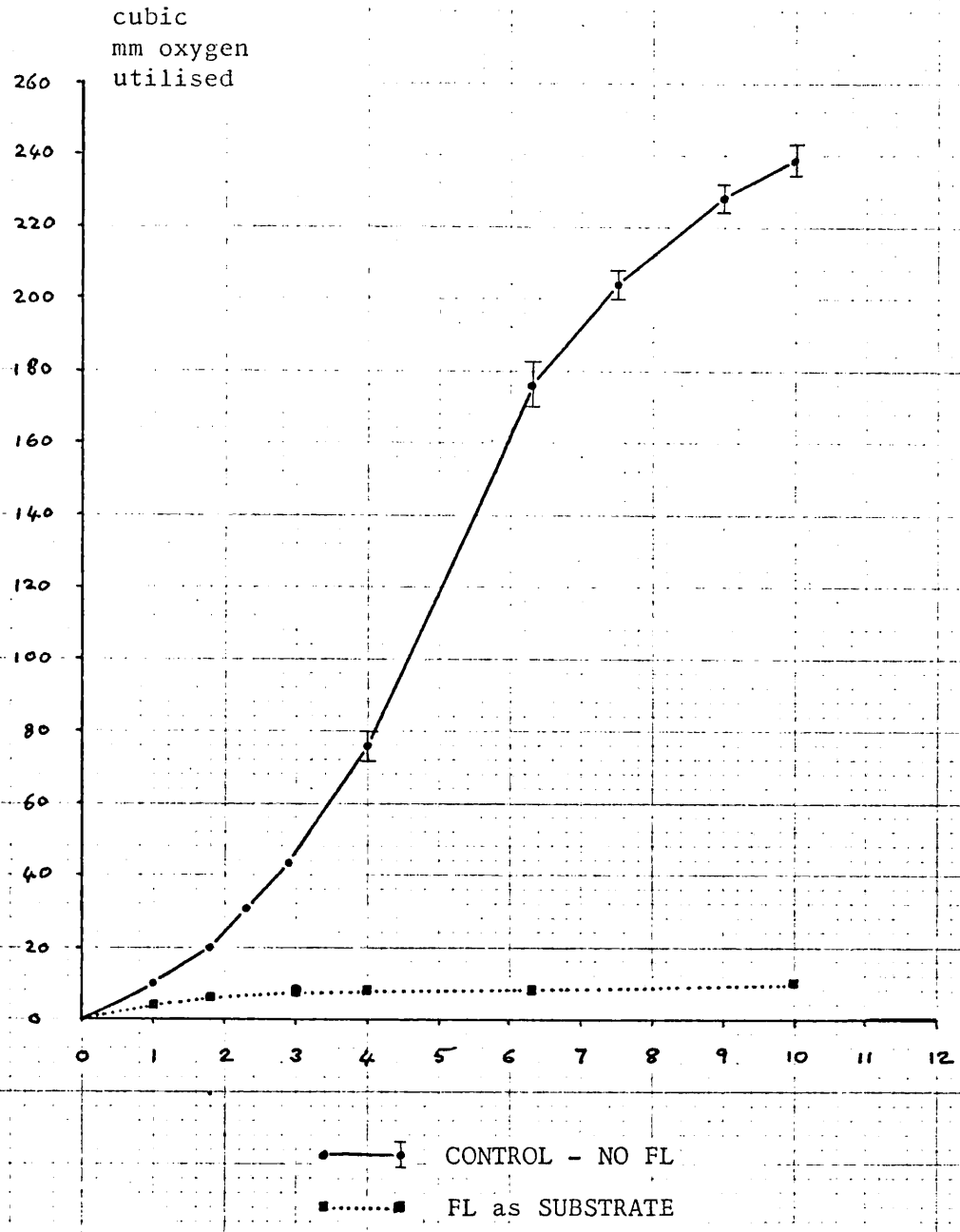


Fig. 2.20 Respiration Curve for FL using induced E.coli cells in experiment 17 (see Tables 2.3 & 2.4)

Each point on the curve for 'FL as substrate' is the average of five flasks.

TABLE 2.3 $^{14}CO_2$ production from *FL by induced E.coli cells

Experiment number	Flask number	c.p.m. (*FL) added	Time (hr) at which KOH paper was removed ##	pH check on completion of incubation	$^{14}CO_2$ (% c.p.m.#) recovered on KOH paper - 'A'	$^{14}CO_2$ (% c.p.m.#) liberated on acidification of the medium on completion of the incubation - 'B'	Total % c.p.m.# ('A' + 'B')
16	(1)	10^5	5	7.6	3.2	0.5	3.7
	(2)	10^5	5	7.6	2.3	-	-
			24	7.4	13.3	1.5	14.8
17	(1)	2.5×10^6	5	7.0	2.8	6.6	9.4
	(2)	2.5×10^6	5	7.0	2.7	-	-
			12	7.5	6.9	5.0	11.9
	(3)	2.5×10^6	5	7.0	2.7	-	-
			12	7.5	6.9	-	-
			24	7.0	8.6	3.7	12.3
18	(1)	2.5×10^6	6	7.0	2.6	4.8	7.4
	(2)	2.5×10^6	6	7.0	2.5	-	-
			12	7.0	4.0	3.6	7.6
	(3)	2.5×10^6	6	7.0	2.9	-	-
			12			-	-
			24	7.0	4.5	0.7	5.2
19		5×10^6	5	6.5	2.1	1.2	3.8

Results expressed as % of c.p.m. originally added as labelled FL.

The values given for each flask are the total recorded c.p.m. after start of incubation (e.g. Flask (3) of Experiment 17; the 24 hr. value is the sum of the KOH papers collected at 5, 12 and 24 hr.).

TABLE 2.4 $^{14}CO_2$ production from FL* by induced E.coli cells

Experiment number	Flask number	c.p.m. (FL*) added	Time (hr) at which KOH paper was removed /	pH check on completion of incubation	Free $^{14}CO_2$ (% c.p.m./) recovered on KOH paper - 'A'	$^{14}CO_2$ (% c.p.m./) liberated on acidification of the medium on completion of the incubation - 'B'	Total % c.p.m. / ('A' + 'B')
16	(3)	3×10^5	5	7.8	0.2	0.2	0.4
	(4)	3×10^5	5	7.6	0.1	-	-
			24	7.4	1.4	0.5	1.9
17	(4)	2×10^6	5	7.0	0.3	-	-
			12	7.4	1.0	3.8	4.8
	(5)	2×10^6	5	7.0	0.2	-	-
			12	7.4	0.75	-	-
			24	7.0	1.4	2.3	3.7
18	(4)	2×10^6	6	7.0	0.3	0.1	0.4
	(5)	2×10^6	24 //	7.0	0.7	2.5	3.2

/ see explanation for Table 2.3

// the values for this flask represent the period 12-24 hr.

TABLE 2.5 Summary of the data presented in Tables 2.3 and 2.4

Compound	Time (hr.)	Total % conversion to free $^{14}CO_2$	
		Ranges	Average Values
*FL	5 or 6	2.1 - 3.2	2.6
	12	4.0 - 6.9	5.1
	24	4.5 - 13.3	8.8
FL*	5 or 6	0.1 - 0.3	0.2
	12	0.75 - 1.0	0.9
	24	1.4 - 1.4	1.4

by the bacterium: CO_2 can be fixed by microorganisms for example by malic enzyme. The fact that $^{14}CO_2$ was evolved between 12 and 24 hr. (i.e. after colonial growth had ceased and FL was no longer detectable) shows that the products remaining after FL-utilisation were then, also, further metabolised and, since little or no oxygen uptake was observed during this period, that anaerobic respiration may have been involved.

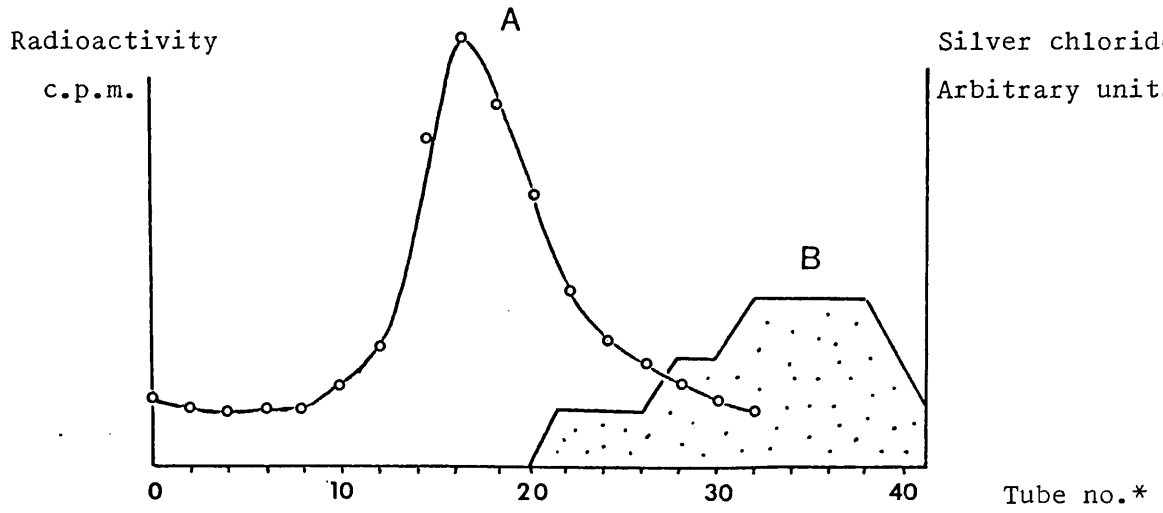
A comparison of $^{14}CO_2$ recovery values for both FL* and *FL provides further evidence that the fructosyl moiety of FL is considerably more available as an energy source for E.coli than is the lysyl moiety. Possibly the very low recoveries of $^{14}CO_2$ from FL* which were observed were artefacts of the techniques used and the lysyl moiety was not, therefore, utilised at all.

Part IV - Examination of breakdown products of FL produced by E. coli.

Mixtures containing breakdown products obtained by incubating induced E.coli cells with radioactive FL were examined either by paper chromatography or by high-voltage paper electrophoresis. After resolution the papers were analysed by scanning the chromatograms for radioactivity and/or by spraying with ninhydrin or $AgNO_3$/NaOH reagents.

Aerobic breakdown products of FL.

The preparation of samples prior to analysis was varied according to whether the radioactive substrate was strongly or weakly labelled. Those culture samples previously taken from the Warburg flasks at the end of the respiration experiments (Part III) which possessed a low radioactivity (e.g. see Exp. 16 in both Tables 2.3 and 2.4) were sonicated and then filtered with an 'Amicon' ultrafiltration chamber to remove all compounds with mol.wt. > 1000. The recovery of radioactivity in the filtrates (representing contents of cells and media) was ca. 80% of that in the original samples. The filtrates contained sulphate (from the sulphuric acid used for acidification) and salts which came from the original minimal medium. These salts were present in sufficient amounts to cause serious disruption of resolution of the mixtures and hence they were removed with an ion retardation resin by the following procedure. Samples were concentrated (by rotary evaporation) and applied to a column of the resin which was then eluted with water. The fraction containing organic compounds (including FL and its breakdown products) was located by counting the radioactivity in aliquots taken from each fraction of the eluate and the salt fraction was located by detecting chloride ions with silver nitrate solution. A typical result is shown in Fig. 2.21. Resolution was incomplete but usually resulted in the removal of sufficient inorganic material to allow further analysis. Selected fractions containing the organic compounds were pooled and concentrated to dryness by rotary evaporation and then redissolved in water. The resulting syrups were examined by paper chromatography. Separation and scanning of the mixtures produced in all cases a number of radioactive peaks including one with an Rf value corresponding to that of FL (e.g. Fig. 2.22 and Fig. 2.23). The traces obtained with 24 hr. digests using *FL were qualitatively similar to that shown in Fig. 2.22 for a 5 hr. digest. However, quantitative



Peak (A) = Radioactivity in organic fraction

Peak (B) = Chloride ions, representing salt fraction

* 3ml fractions were collected

Fig. 2.21 Desalting of radioactive samples with ion-retardation resin.

differences were apparent: peaks No. (2) and (4) in the case of the 24 hr. digests were reduced by approximately half, relative to the suspected FL peak and peak No. (5) was absent. The 24 hr. pattern for the FL* breakdown products was also similar to that for the corresponding 5 hr. pattern (Fig. 2.23) but peaks (a) and (c), relative to the suspected FL peak, had increased to approximately three times the previous size in the former case. It was observed that peaks (c) and (d) exhibited Rf values corresponding to those of lysine and FL, respectively. It was also noted at this stage that the minor peaks (a) and (b) possessed Rf values equivalent to those of FL sulphate and lysine sulphate, respectively (see p.26). These compounds would presumably not be absorbed by the ion-retardation column or, alternatively, free FL and lysine in the eluates from the column reacted with excess sulphate which had not been retained. The FL sulphate area in the trace for the *FL digests (see Fig. 2.22) may have been the small shoulder occurring between peaks (1) and (2); lysine or lysine sulphate would not, of course, be detectable in this latter case.

In the case of digests with high radioactive counts (i.e. > 100,000 cpm; e.g. Exps. 17, 18, 19, in both Tables 2.3 and 2.4) where only small

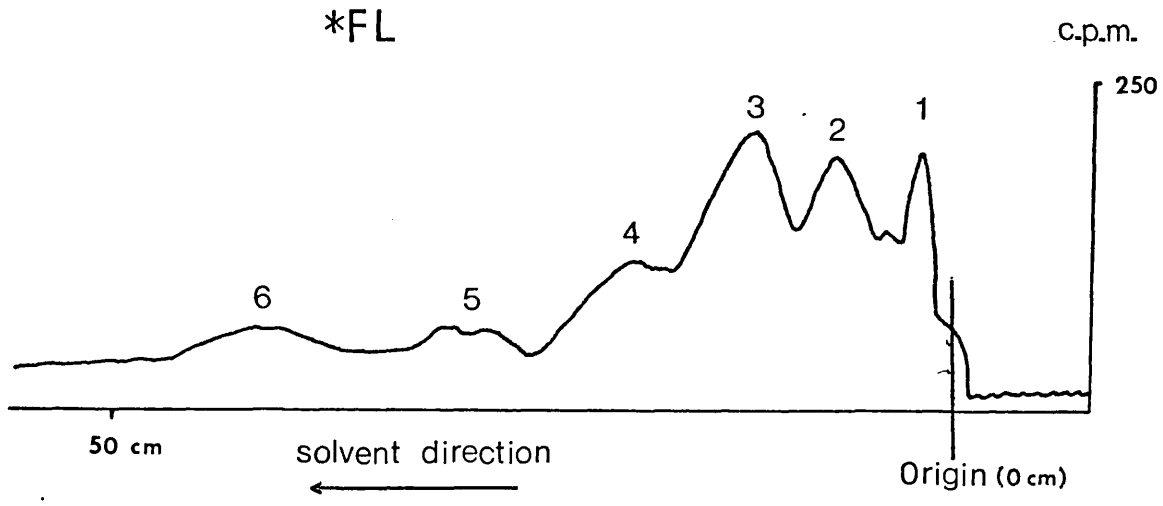


Fig. 2.22 Products from *FL (medium and cell contents) obtained by incubation (5hr.) with E.coli under aerobic conditions

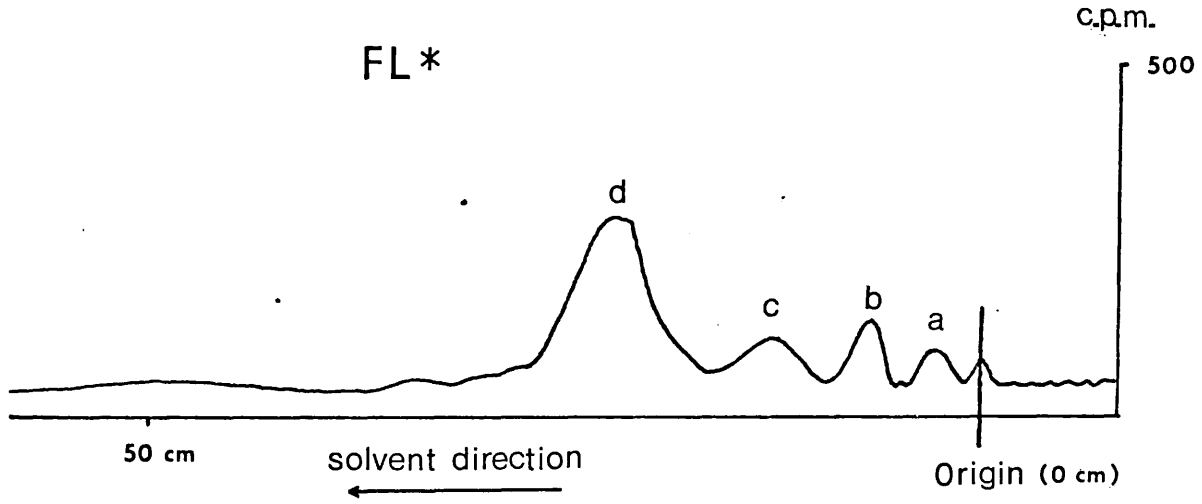


Fig. 2.23 Products from FL* (medium and cell contents) obtained by incubation (5hr.) with E.coli under aerobic conditions.

volumes of solutions were required for paper chromatography and scanning and where sulphuric acid was not added, the ion retardation procedure was unnecessary. Samples were fractionated as before but culture media and cell contents were, in this case, examined separately. The patterns of radioactive peaks for 5 (or 6), 12 and 24 hr. incubation periods with *FL confirmed the numbers and Rf values of peaks found with experiments using the resin-treated samples where 5 and 24 hr. incubations were carried out (see above). The chromatograms showed in addition that the compound with an Rf value corresponding to that of FL (No. (3) Fig. 2.22) was present only in the culture medium and that compound(s) with similar chromatographic behaviour to monosaccharides (No. (6) Fig. 2.22) were also found mostly in the medium; the four other compounds, corresponding to peaks 1, 2, 4 and 5 (Fig. 2.22), were found only in the cell contents. These findings support the theory that a derivative with properties similar to a monosaccharide may be released by extracellular FL cleavage and, after penetration of the cell, this is further metabolised. It was also noted that peaks No. (5) and (6) were absent at 12 hr. The reappearance of activity in the free sugar area (peak 6, Fig. 2.22) seen in the 24 hr. trace remains unexplained.

The radioactive traces for FL* digests (those which did not require resin treatment) confirmed the presence of peaks corresponding to lysine and FL and the chromatograms also showed that these compounds were present only in the medium and not in the cell contents. Peaks corresponding to lysine sulphate and FL sulphate, as expected, did not occur. The approximate ratios of the supposed FL and lysine peaks were 4:1, 1.5:1 and 1:1 (FL:lysine) after 5, 12 and 24 hr., respectively. These findings support previous evidence that the extracellular cleavage of FL results in the release of lysine which then remains in the culture medium unchanged.

Samples of media from cultures grown with FL* (previously used for the chromatographic analysis) were also resolved by electrophoresis in both formate (pH 3.8) and borate (pH 9.6) buffers and in all cases the radioactive scan profiles confirmed the presence of two compounds with electrophoretic mobilities of FL and lysine, respectively. The cells from stock cultures entering the stationary phase of growth on unlabelled FL (2.5mM) were isolated and recultured in an equivalent volume of minimal medium containing FL* (2mM) under aerobic conditions. Examination of the media by paper

chromatography and electrophoresis after growing the cultures for 4 hr. also suggested the presence of FL and lysine which were found in the approximate ratio of 1:1.

Anaerobic breakdown products of FL.

Samples of media from induced cell cultures grown under essentially anaerobic conditions were analysed, as above, by the 'high cpm procedure', by both paper chromatography and electrophoresis. The cultures examined were either grown under the usual conditions conducive to complete growth of colonies in approximately 12 hr. (see section (1); Table 2.6†) or were innoculated with the induced cells isolated from fully grown cultures (see e.g. Exp. 14) which, as previously shown, were capable of complete FL (2mM) breakdown in approximately 2 hr. (Table 2.6††). The various growth conditions and sampling times used for the cultures examined are summarised in Table 2.6. The radioactive scan profiles of the separated degradation products of *FL (Fig. 2.24) were similar to those for the corresponding 12 hr. aerobic digests, cf. Fig. 2.22 and text p. 72 , and showed three compounds in the cell contents and only FL in the medium. Analysis of the cultures incubated with FL* (Fig. 2.25) revealed, in all cases, the presence in the medium of two compounds with electrophoretic and paper chromatographic mobilities identical with those of lysine and FL. The FL and lysine areas from FL* incubations were also eluted from paper chromatograms and their electrophoretic mobilities confirmed separately.

Further characterisation of the radioactive compound in the media, from FL*, which was thought to be lysine was attempted using lysine decarboxylase. The compound was eluted separately from individual paper chromatograms of both aerobic and anaerobic digests which had been incubated for times up to 40 hr. The eluates were freeze dried and then incubated with the decarboxylase in Warburg flasks containing KOH papers to collect any $^{14}CO_2$ evolved. Significant amounts of radioactivity were recovered from all the samples tested and, since lysine decarboxylase is highly specific for lysine (or hydroxylysine), this provided strong evidence that lysine was present in all of the digests examined.

An experiment where FL-induced E.coli cells were incubated under anaerobic conditions with a commercial sample of [U-^{14}C] lysine as sole carbon source showed that all the amino acid remained in the medium for up

TABLE 2.6 Analysis of products from FL* and *FL obtained by incubation with E.coli under anaerobic conditions (cf. Figs. 2.24 and 2.25).

Exp. No.	Label (all > 100,000 c.p.m., i.e. 'high counts')	Sampling Time (hr.)	Approximate ratio (c.p.m.) of FL: Lysine found on chromatograms	Reaction with Ninhydrin ≠	Borsook reaction of sample
20 ≠	FL*	6	4:1	+	+
21 ≠	FL*	12	2:1	weak	weak
22 ≠	FL*	24	1:1	-	-
23 ≠≠	FL*	2½	15:1	-	-
24 ≠≠	FL*	4	1:1	-	-
25 ≠≠	FL*	20	1:1	-	-
26 ≠≠	FL*	40	1:1	-	-
27 ≠≠	FL*	45	No lysine	-	-
28 ≠≠	*FL	6		-	-

≠≠ Incubations carried out with high cell counts, i.e. high FL-ase activity (see p.73)

≠≠ Incubations carried out with low cell counts, i.e. relatively low FL-ase activity (see p.73)

≠ The sign given denotes either a positive or negative reaction when the radioactive area, on paper chromatograms, corresponding to the Rf of FL was sprayed with ninhydrin.

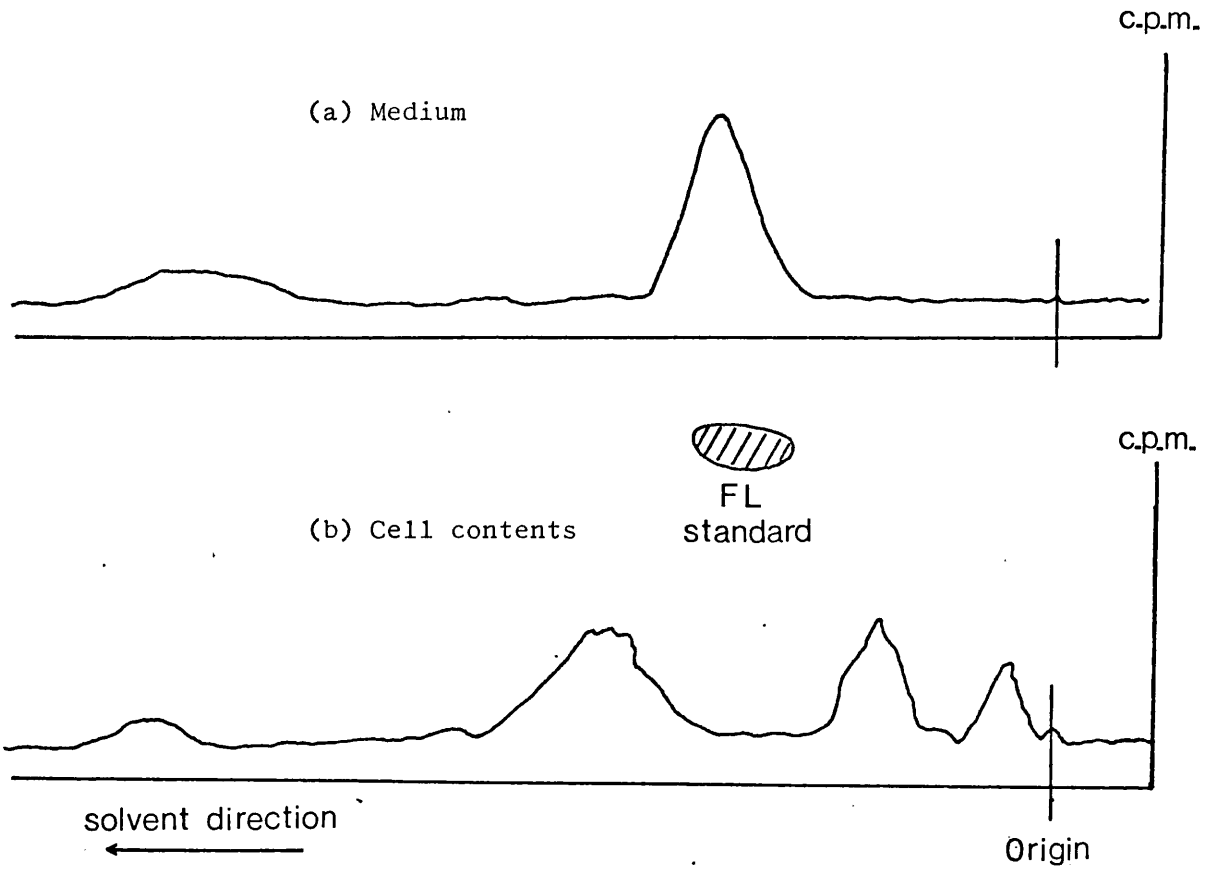


Fig. 2.24 Products from *FL obtained by incubation with E.coli under aerobic conditions (see Exp. 17)

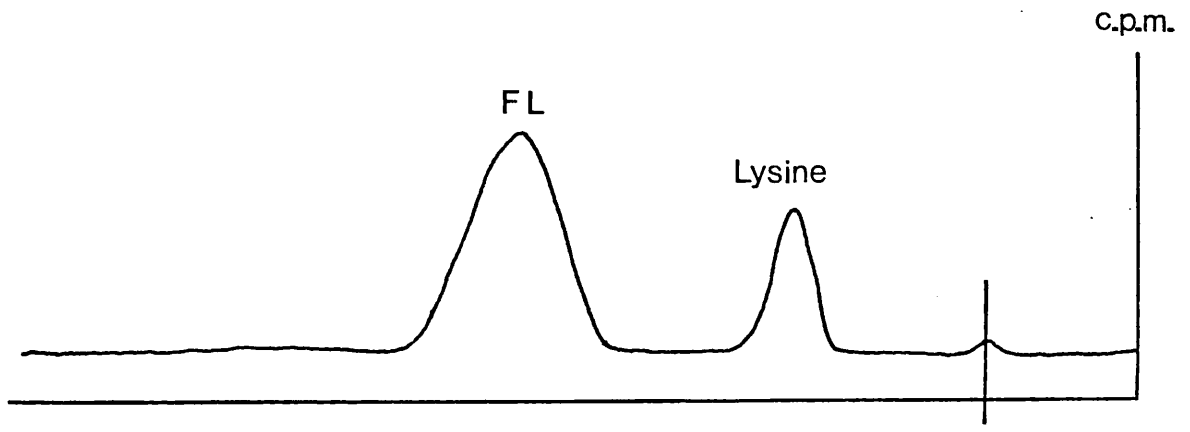


Fig. 2.25 Products present in the medium from FL* obtained by incubation with E.coli under anaerobic conditions

to 40 hr. However, in Exp. 27 (see Table 2.6) where FL* had been incubated anaerobically with induced cells some of the total radioactivity in the medium after 40 hr. had disappeared and paper chromatography after a further 5 hr. indicated that this was due to the complete loss of lysine. In this particular instance it is presumed that in the presence of a better carbon source a lysine permease was produced at approximately 40 hr. causing accumulation of ^{14}C-labelled lysine by the cells. The cell contents of this culture were analysed on chromatograms at 48 hr. and Fig. 2.26 is a trace of the radioactive scan obtained: the two main peaks (i and ii) on this trace corresponded to ninhydrin-positive areas on the paper. It is likely that all the peaks in Fig. 2.26 are lysine metabolites.

Discussion

When FL is incubated with E.coli the compound gradually disappears from the medium and this is accompanied by the appearance of lysine (see Table 2.6). These results suggest that FL is cleaved by E.coli to lysine, which tends to remain in the medium, and a hexose or hexose derivative which can be taken up by the cells and metabolised. The results of studies with *FL suggest that an intermediate (A) in the degradation by the bacterium may accumulate in the medium. Thus in Fig. 2.19 it can be clearly seen that there is a rapid loss of FL from the medium, as judged by the Borsook assay, but loss of radioactivity originally associated with the fructose moiety is relatively slow, with the rate of disappearance approaching zero after 5 hr.

This observation could be interpreted in a number of ways: (1), FL could be converted directly to lysine, which remains in the medium, and a simple labelled hexose, presumably glucose or fructose. However, such sugars would almost certainly be rapidly accumulated by the cells and hence the curve in Fig. 2.19 representing loss of label from the medium would in this case be expected to approximately parallel the decrease in total FL (decrease in Borsook colour); (2), the FL in the medium could be rapidly converted (in 2 hr., Fig. 2.19) without major degradation to a closely related Borsook negative derivative (A) which in turn is cleaved by a rate-limiting reaction, represented by the curve for radioactivity, to a simple hexose plus lysine.

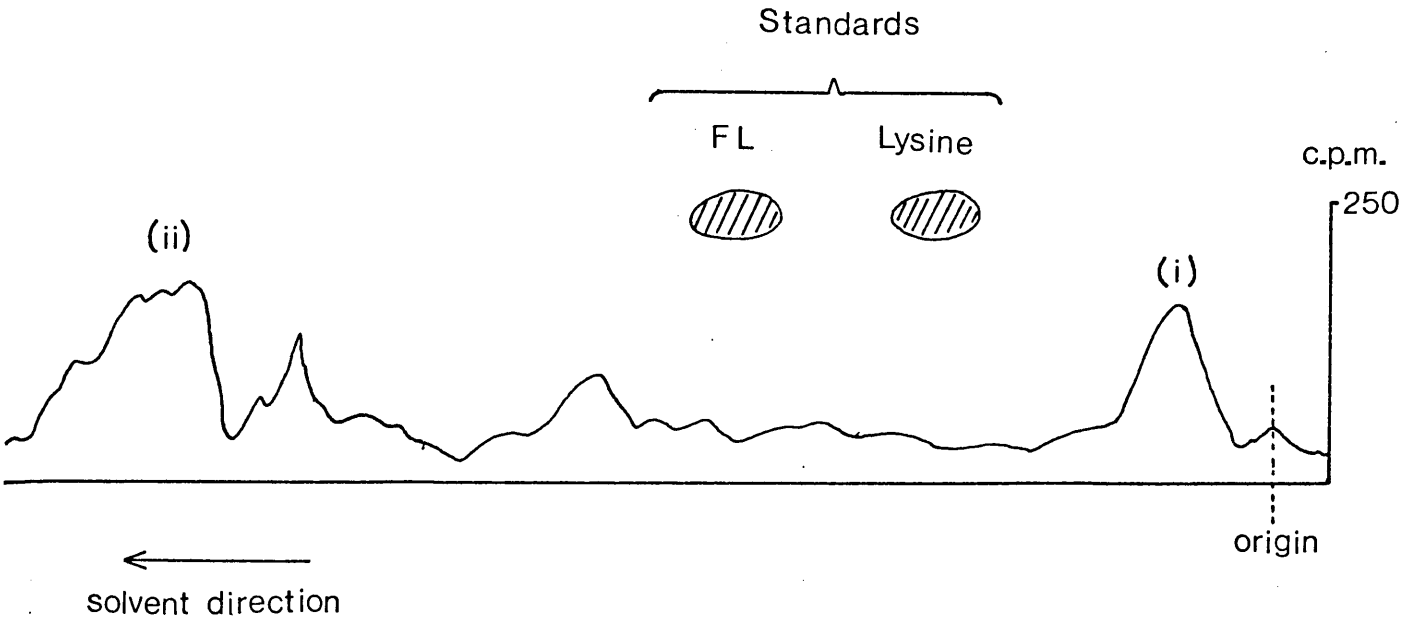


Fig. 2.26 Radioactive trace of the cell contents of E.coli cells grown anaerobically (48 hr.) on FL*

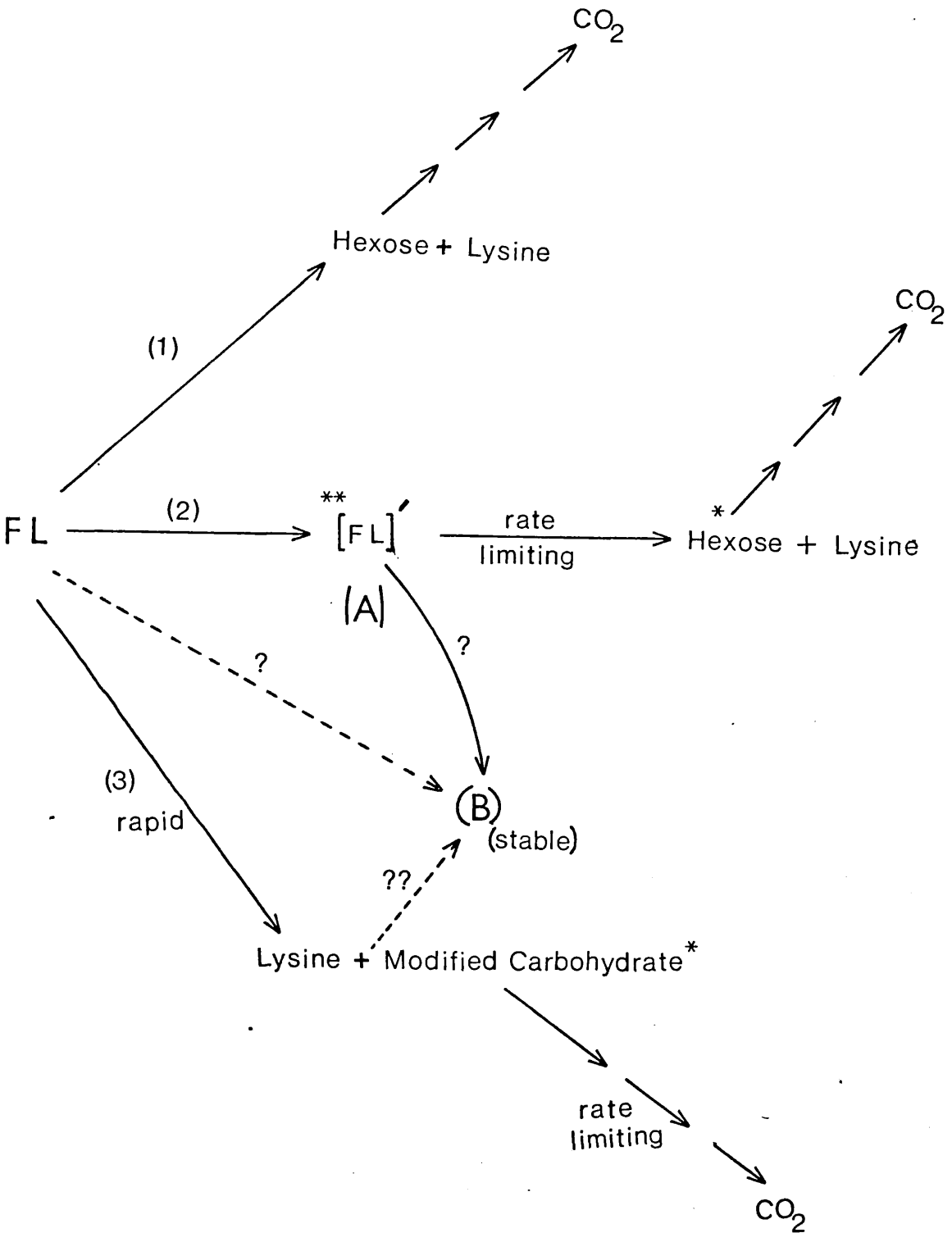


Fig. 2.27 Possible pathways for the metabolism of FL by E.coli

* Possibly carbohydrate(s) or mixture of various compounds (derived from the hexose moiety of FL)

** A postulated intermediate in FL breakdown

The postulated hexose could then be quickly accumulated by the organism; (3), FL could be rapidly degraded to lysine and a labelled hexose derivative other than a simple sugar and the latter compound then accumulated slowly. The three possible alternatives are illustrated in Fig. 2.27.

In addition, studies with FL*, which are represented in Table 2.6, also support the view that an intermediate (A) in the breakdown of FL together with a stable metabolite (B) is present in the medium. At the point in time when all the FL has disappeared from the medium (according to the Borsook assay) lysine can be detected by paper chromatography together with a labelled peak (A + B?) with similar chromatographic properties to FL but different chemical properties i.e. giving negative reactions with ninhydrin, phenyl tetrazolium chloride and $AgNO_3$/NaOH. The ratio at this time, in terms of c.p.m., of the unknown peak to lysine is 2:1 (see Table 2.6, Exp. 21). If the reaction is allowed to continue the ratio approaches 1:1 which presumably represents the disappearance of the intermediate (A). However, this ratio remains constant for periods up to 40 hr. which suggests that a non-metabolisable compound (B) is produced during incubation with the organism. The presence of (B) is also supported by studies with *FL where again the 'inert' compound can be shown to be present in the medium by paper chromatography after a 40 hr. incubation.

All of these observations (see Fig. 2.27) may be explained by the assumption that an intermediate (A) is produced in the medium from FL and this is slowly utilised. At the same time a second compound (B) is also being formed from FL or from (A) and this is reflected by the final radioactive count in the unknown peak after a long term incubation. As mentioned above the stable compound (B) showed many similarities with FL and from a study of molecular models it seems possible that the latter is able to form an internal ester between the α-carboxyl group of the lysyl moiety and one of the alcoholic groups of the fructose moiety. Reaction of carboxyl with the -OH group at C-6 would, from stereochemical considerations, be most favoured giving a 13-membered stable ring structure. The resulting 'cyclic fructose-lysine' is shown below in Fig. 2.28 and this, or a similar molecule, may represent compound (B).

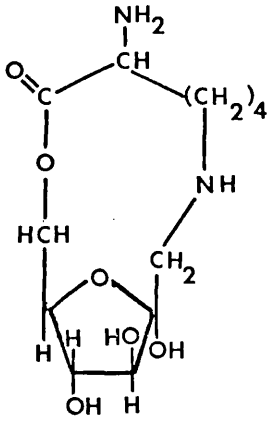


Fig. 2.28 The hypothetical cyclic compound (possibly (B)) formed by reaction of the C-6 hydroxyl of fructose (furanose) with the lysyl α-carboxyl group.

The above molecule would be expected to be ninhydrin -negative due to the absence of the free α-carboxyl group which probably participates in the positive ninhydrin reaction. The compound is of similar molecular size to FL and still posesses a free $-NH_2$ group and may well exhibit a similar chromatographic mobility to FL as was the case with compound (B); from the point of view of charge the lack of the α-carboxyl group would be unimportant at acid pH. The existence of a free amino group in compound (B) was possible since (B) was thought to form an amine sulphate (see 24 hr. trace p. 70). The changes resulting from the cyclisation of FL could result in a compound which gives a negative Borsook reaction; the alkali treatment employed in this assay (approximately 10^{-2}M-NaOH) would presumably be ineffective in breaking the ester link.

Although the cyclic ester is, in theory, a stable structure it is unlikely to be formed from FL in good yields unless the reaction is catalysed, presumably by an E.coli enzyme. There is no direct evidence to support this latter hypothesis, however.

The occurrence of (B) could also be explained if the radioactive preparation of FL*, as used, was contaminated with 50% of the α-isomer, i.e. 1-(α-N-[U-^{14}C] lysyl)-1-deoxy-D-fructose (N.B. both isomers have the same Rf value (12) in the chromatographic solvent used for examining digests), which was (unexpectedly) not susceptible to degradation by E.coli; after 40 hr. all of the ε-derivative would have been degraded and the α-FL* molecules remaining in very low concentration may not have been detectable by chemical means. However, the FL* sample used in these studies did appear to be homogeneous when examined on paper chromatograms and electrophoretograms (see section I, pp28-30). It should also be noted that contamination of ε-FL with α-FL could explain the fact that only 25% of the total radioactivity is lost from the medium when *FL is incubated with E.coli (see Fig. 2.19); again the α-FL would have to be non-metabolisable. The unlikely possibility that the labelled ε-*FL was contaminated with the α-isomer has already been discussed on page 30 . To account for the 25% loss of radioactivity on the basis of this contaminant it would be necessary for the *FL to be composed of 75% α-isomer and only 25% ε-isomer (Fig. 2.29(i)). A number of other explanations are possible, two of which are shown in Fig. 2.29. Fig. 2.29(ii) shows the case where a relatively small contamination of the ε-*FL by the α-isomer exists and this remains in the medium together with other labelled compounds arising from the breakdown of *FL which the cell fails to utilise. Fig. 2.29(iii) portrays a modification of this latter idea involving the formation of the stable end-product (B) together with contamination by the α-isomer.

If, as is believed, contamination of ε-*FL by α-*FL did not in fact occur then another explanation is needed to account for the incomplete uptake (25%) of radioactivity from the medium by E.coli. The studies with ε-FL* showed that after long term incubations (12 hr.) compound (B) was present as 50% of the total activity of the medium (together with an equal amount of lysine). Hence, it would be expected that after a similar period of time *FL would be degraded to unlabelled lysine, (B) (representing 50% of the total radioactivity added as *FL) and a further 50% activity, representing the hexose moiety, which could be taken in and metabolised by the cell. However, the observed value was a 25% disappearance of radioactivity (see Fig. 2.19) and this could be a result of the breakdown of the hexose moiety of ε-*FL, by the E.coli, to various products, not appearing as distinct

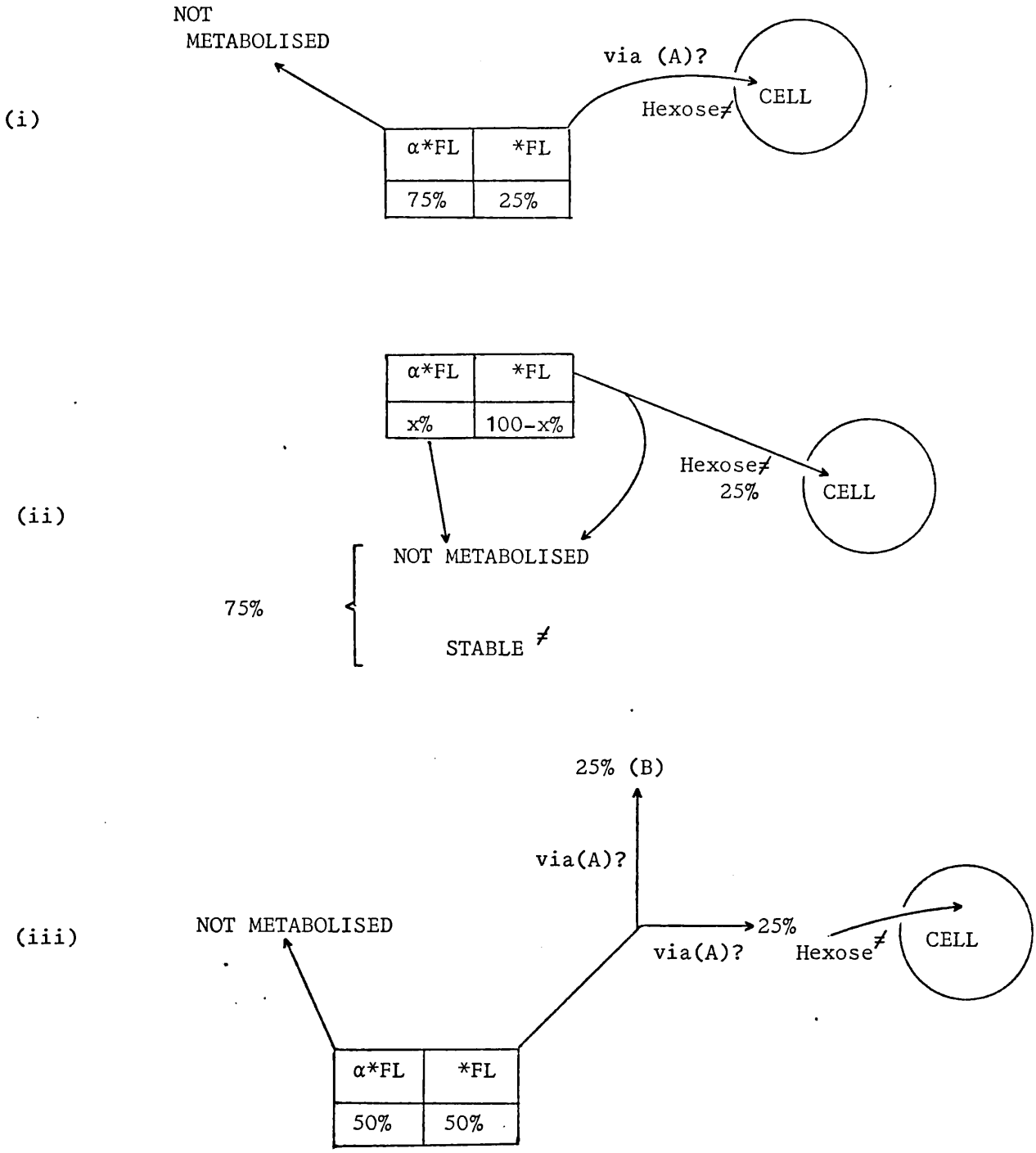


Fig. 2.29 Theoretical effect of contamination of labelled -FL by the α-isomer in relation to the incomplete uptake of radioactivity from the medium by E.coli

≠ Carbohydrate or mixture of various compounds (derived from hexose moiety of FL)

compounds on chromatograms, of which only half (i.e. 25% of the total radioactivity) are incorporated into the cell contents.

S E C T I O N 3

Studies with Rat Liver Preparations

As FL appears in the urine after a diet of powdered milk it is presumably absorbed in small amounts from the intestinal lumen into the circulatory system probably by means of passive diffusion. There is evidence that FL reaches the liver (via the hepatic portal vein) and here it was believed that detoxication could occur.

In this organ FL could, hence, be partially or totally degraded by typical detoxication reactions such as oxidation, reduction, hydrolysis, acetylation, methylation, etc. or it may be conjugated with other compounds. Both the sugar and the amino acid moieties could be attacked and might be expected, in particular, to be susceptible to oxidation as are most aliphatic foreign compounds. Possibly, also, the C-N bond linking the amino acid to the sugar could be hydrolysed.

Experiments were, therefore, carried out to investigate the possible metabolism of FL by liver slices and homogenates in vitro. In an initial experiment, FL was incubated at 37°C with a rat liver homogenate prepared in Krebs Ringer-bicarbonate solution. The level of FL in the digest was observed to slowly decrease over the 12 hr. period of incubation (Fig. 3.1). A number of other experiments were carried out using either *FL or FL* as substrate (see Exps. 3.1 - 3.4 in Tables 3.1 and 3.2). Liver slices or homogenates were incubated with the labelled FL samples in Warburg flasks and the $^{14}CO_2$ evolved was trapped with KOH papers in the centre well and assayed as described in Materials and Methods.

The results of these experiments are shown in Table 3.1 (*FL) and Table 3.2 (FL*). Measurable radioactivity was recovered (as $^{14}CO_2$) from all the digests and the values obtained normally ranged from 0.1 (after 4hr. incubation) to 2.5% (after 30 hr. incubation) of the initial radioactivity added. Laborit et al. (51) have stated that CO_2 is an essential metabolic substrate for liver slices respiring in vitro and this may explain why generally (see Tables 3.1 and 3.2), the proportion of labelled CO_2 liberated from solution by acid treatment, compared with gaseous $^{14}CO_2$, decreased with the incubation time.

A comparison of the percentage of labelled CO_2 produced from *FL (Table 3.1) with that from FL* (Table 3.2) suggests that more $^{14}CO_2$ is derived from the fructose moiety than from the lysyl moiety. The $^{14}CO_2$ recovery values from FL* range from 25-95% of the corresponding values

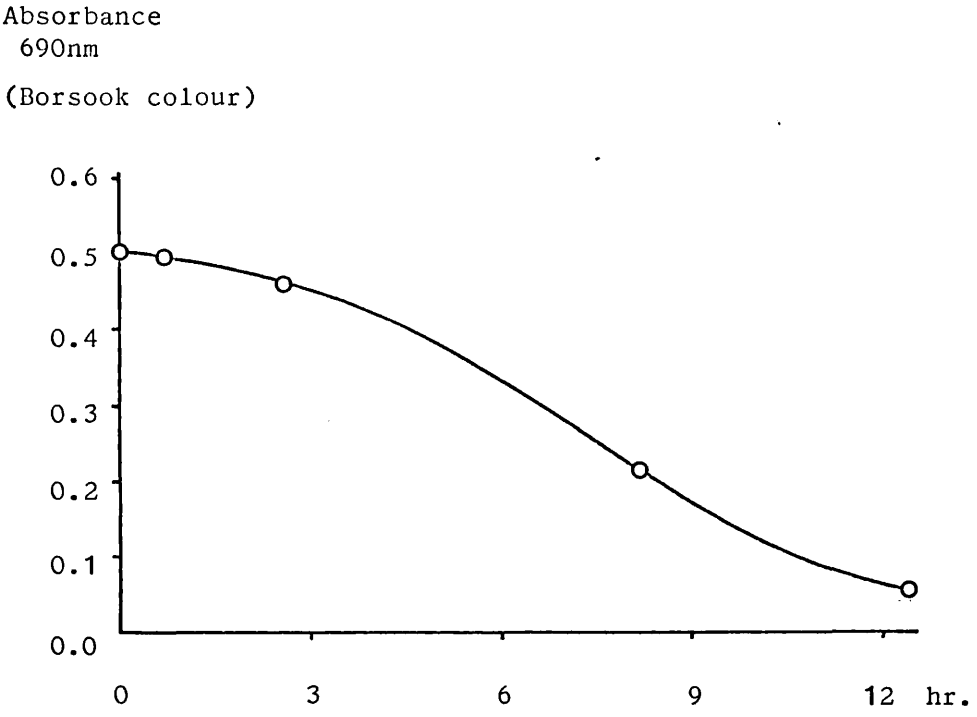


Fig. 3.1 The disappearance of FL added to a rat liver homogenate at 37^{o} under aerobic conditions

TABLE 3.1 $^{14}CO_2$ production from *FL by liver preparations

Experiment No.	Liver Preparation: Homogenate (H) or slices (S)	Flask No.	c.p.m. (*FL) added	pH check on completion of incubation	Time (hr.) at which KOH paper was removed##	$^{14}CO_2$ (% c.p.m.#) recovered on KOH paper - 'A'	$^{14}CO_2$ (% c.p.m.#) liberated on acidification of the medium on completion of incubation - 'B'	Total% c.p.m.# ('A' + 'B')
3.1	H	(1)	10^5	-	4	0.5	0.3	0.8
	H	(2)	10^5	-	4	0.6		
				-	30	1.5	0.45	1.95
	S	(3)	10^5	-	4	0.55	0.2	0.75
3.2	H	(1)	4×10^6	5.2	16	0.2	1.4	1.6
3.3	H	(1)	2.5×10^6	8.0	4	0.15	1.95	2.1
	H	(2)	2.5×10^6	7.8	4	0.15		
				-	16	0.5	1.6	2.1
	H	(3)	2.5×10^6	8.0	4	0.1		
				-	16	0.5		
				4.2	30	8.3	8.9	17.2
3.4	H	(1)	4×10^6	7.2	4	0.05	0.05	0.1
	H	(2)	4×10^6	7.4	4	0.05		
				4.4	16	0.2	0.1	0.3
	H	(3)	4×10^6	7.4	4	0.05		
				4.8	16	0.2		
				4.4	30	1.9	0.3	2.2

Results expressed as % of c.p.m. originally added as labelled FL

The values given for each flask are the total recorded c.p.m. after start of incubation (e.g. Flask (3) of Experiment 3.3; the 30 hr. value is the sum of the KOH papers collected at 4, 16 and 30 hr.)

TABLE 3.2 $^{14}CO_2$ production from FL* by liver preparations

Experiment No.	Liver Preparation: Homogenate (H) or slices (S)	Flask No.	c.p.m. (FL*) added	pH check on completion of incubation	Time (hr.) at which KOH paper was removed ##	$^{14}CO_2$ (%c.p.m.†) recovered on KOH paper - 'A'	$^{14}CO_2$ (%c.p.m.†) liberated on acidification of the medium on completion of incubation - 'B'	Total % c.p.m.† ('A' + 'B')
3.1	H	(4)	3×10^5	-	4	0.2	0.1	0.3
	H	(5)	3×10^5	-	4	0.2		
				-	30	0.4	0.1	0.5
	S	(6)	3×10^5	-	4	0.2	0.1	0.3
	S	(7)	4×10^6	-	4	0.3		
				-	30	0.5	0.1	0.6
3.2	H	(2)	2.5×10^6	5.1	16	0.15	1.3	1.45
3.3	H	(4)	2.5×10^6	9.0	4	0.1	1.45	1.55
	H	(5)	2.5×10^6	7.9	4	0.1		
				-	16	0.3	0.9	1.2
	H	(6)	2.5×10^6	9.0	4	0.1		
				-	16	0.3		
				4.2	30	0.5	0.3	0.8
3.4	H	(4)	2.5×10^6	7.2	4	0.1	0.05	0.15
	H	(5)	2.5×10^6	7.2	4	0.1		
				4.6	16	0.2	0.1	0.3
	H	(6)	2.5×10^6	7.4	4	0.1		
				4.4	16	0.3		
				4.4	30	2.2	0.3	2.5

†, ## see explanation for Table 3.1

from *FL. Exp.3.4, however, shows the reverse situation where FL* yielded more CO_2 than *FL. The values representing $^{14}CO_2$ evolution for each liver in general are fairly consistent. However, the expected variations in data obtained with preparations from different livers were observed. Similar variations were observed when the rates of oxygen uptake of the liver preparations were measured (Table 3.3).

TABLE 3.3 O_2 uptakes of liver slices and homogenates incubated with FL

Experiment No. (see Tables 3.1 and 3.2)	O_2 uptake after 1 hr. incubation (cu.mm)	O_2 uptake after 16 hr. incubation (cu.mm)
3.1 (slices)	// 205 (±5)	3,200 (±200)
3.1 (homogenate)	/ 190 (±5)	3,000 (±200)
3.2 (homogenate)	// 140 (±10)	2,400 (±400)
3.3 (homogenate)	/ 20 (±5)	800 (±300)
3.4 (homogenate)	/ 90 (±7)	2,800 (±100)

/ average value for 6 flasks
// average value for 2 flasks
see 'Materials and Methods' p.118

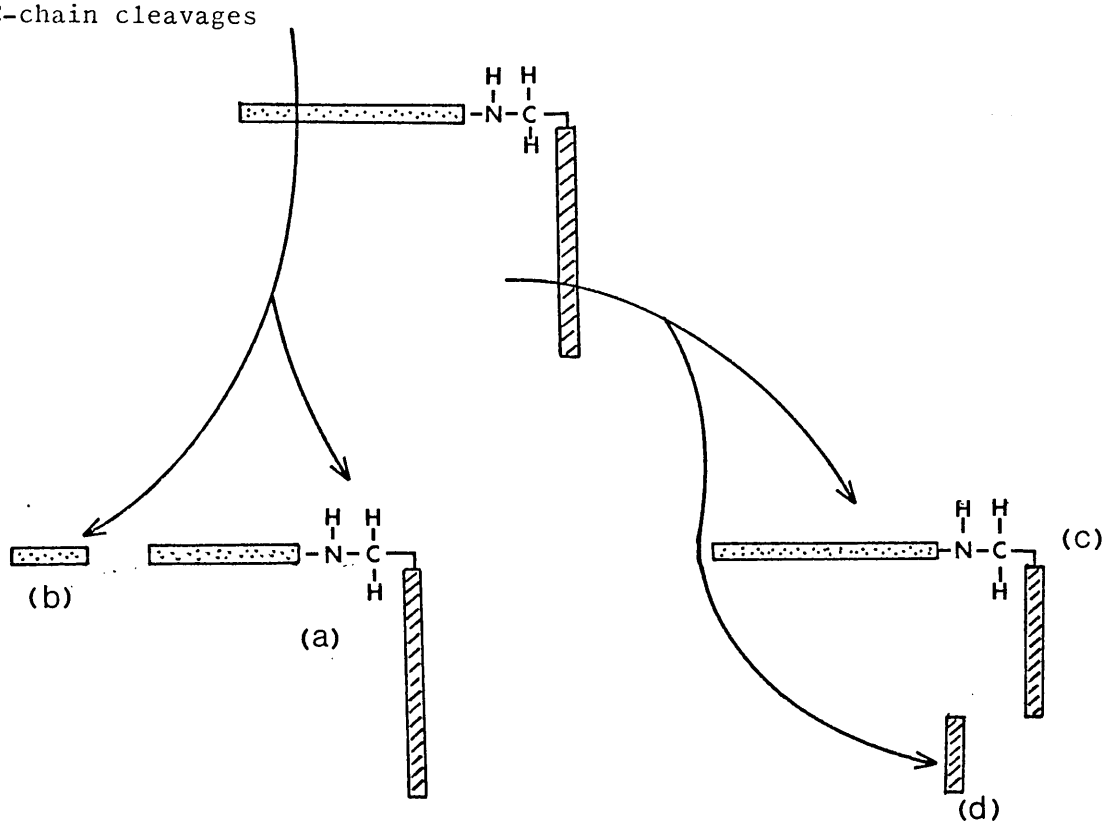
The homogenates and slices of liver which had been incubated with radioactive FL were examined for the presence of products of FL metabolism after 4, 16 or 30 hr. periods. The digests were first centrifuged at 3000g and then the supernatant solutions filtered through an 'Amicon' membrane to remove additional particulate matter and polymeric substances. All solutes of molecular weight <1000 were collected in the filtrates which were then reduced in volume by evaporation and small aliquots applied to Whatman No.1

papers which were developed with pyridine/acetic acid/water (9:1:2 v/v). The resulting chromatograms were scanned for radioactivity.

The traces so obtained confirmed the idea gained from the CO_2 evolution study that the different liver preparations had different biochemical activities: although the patterns of radioactive peaks within each experiment were basically similar, individual experiments where different livers had been used could not be compared. The tracer also suggested that, for each of the four livers, many of the compounds obtained from the breakdown of *FL also appeared as products of FL*. This would indicate that cleavage (or alteration) of the FL molecule was occurring at some point other than the C-N bond linking the amino-acid and sugar moieties; this hypothesis is illustrated in Fig. 3.2. If cleavage of the carbon chain of either lysine or fructose were to occur, then the fragment containing the C-N bond, i.e. compounds such as (a) or (c), respectively, in Fig. 3.2 would be labelled whether the radioactive FL sample was *FL or FL*. Peaks having the same R_{FL} values would therefore appear in radioactive traces of both *FL and FL* digests. Quantitative differences would of course occur since, depending on the site of the C-chain cleavage, one or other of the radioactive compounds would lose some of its radioactivity in the form of small molecules (eg. (b) or (d); Fig. 3.2). The latter compounds should appear as extra radioactive peaks in the trace. Any alteration other than C-chain cleavage would also produce the same radioactive compound from both *FL and FL*. If, alternatively, (as was the case with E.coli; see Chapter 2) FL was cleaved at the C-N bond, yielding hexose and lysine, then the radioactive traces from *FL digests would show only the ^{14}C-hexose or its breakdown products and the FL* digests would result in a different group of compounds representing ^{14}C-lysine metabolism.

However, the radioactive traces obtained showed that the composition of the *FL and FL* digests were essentially similar for all the liver samples i.e. in keeping with the former theory that the sugar-amino acid C-N bonds remain intact during the early stages of incubation. Fig. 3.3 provides a comparison between the FL* and *FL digest traces both obtained after a 4 hr. incubation period (Exp. 3.4; see Tables 3.1 and 3.2). In the diagrams peaks (i) and (ii), metabolites of FL, are seen in both traces; peak (i) may be a product resulting from the loss of part of the lysine chain since it is

(i) C-chain cleavages



(ii) C-N bond cleavage (e.g. hydrolysis)

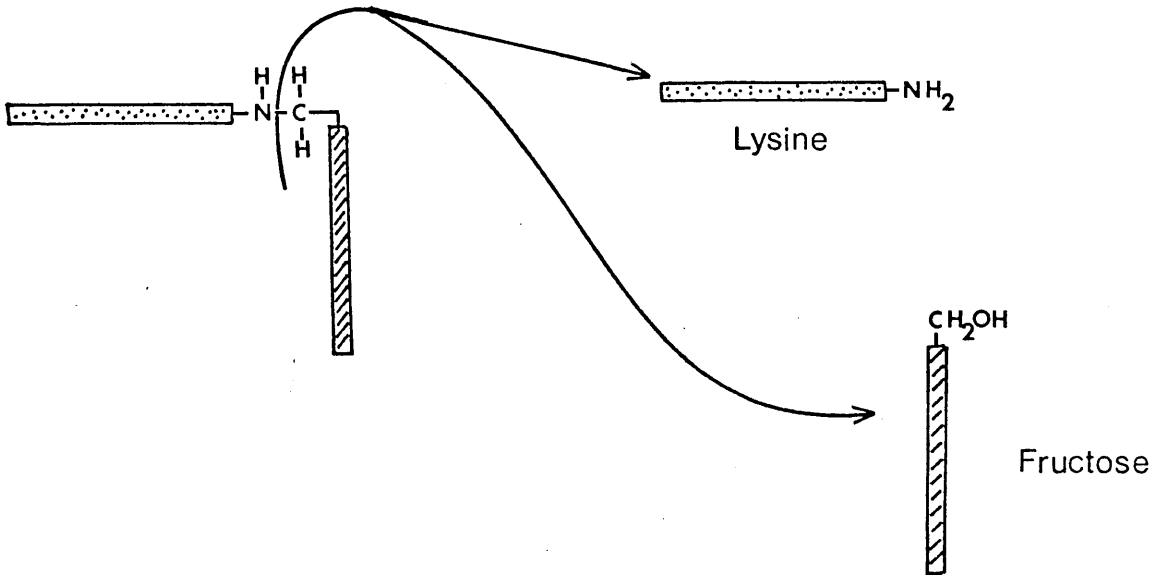


Fig. 3.2 Schematic representation of possible types of FL degradation reactions in liver cells

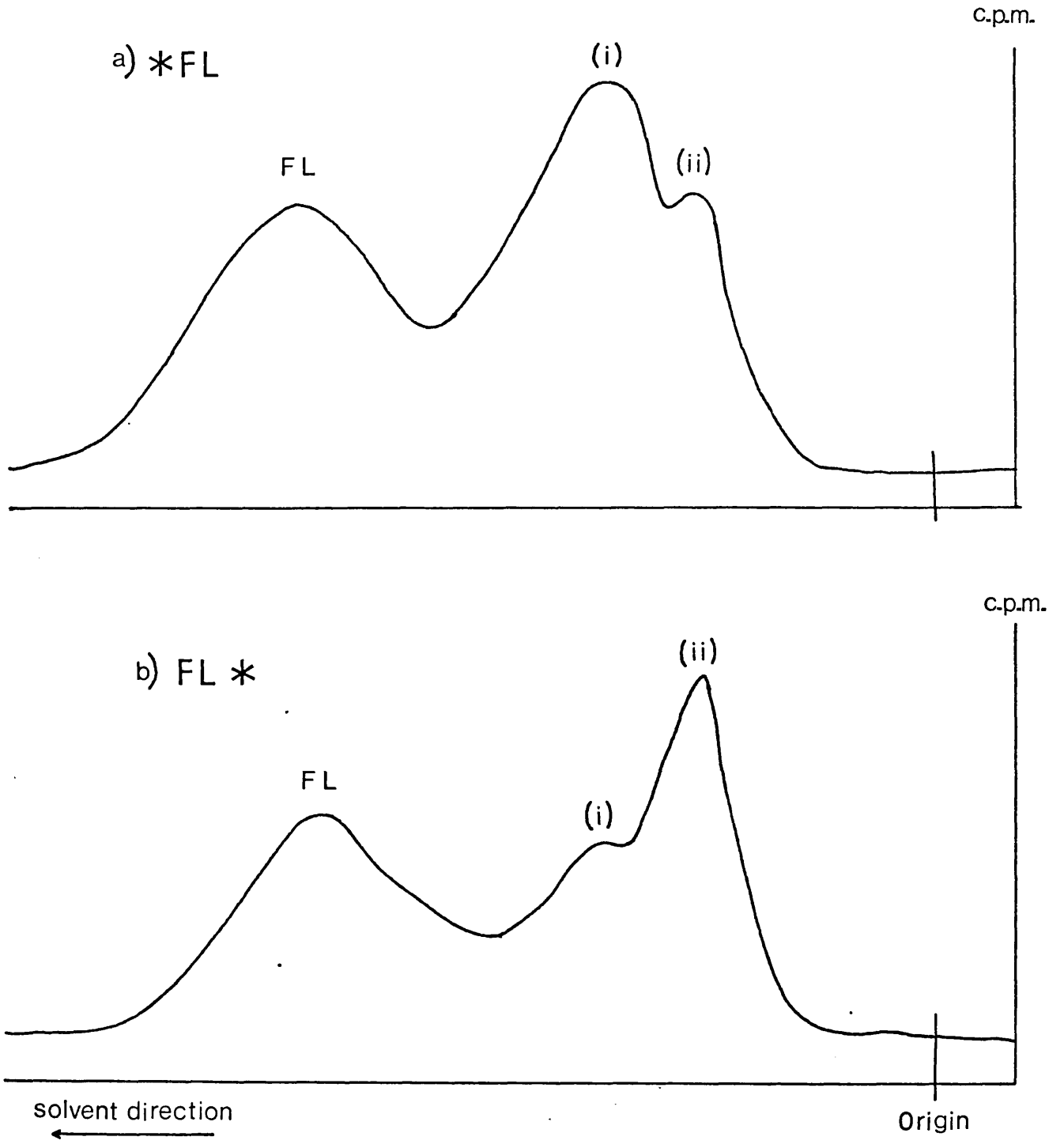


Fig. 3.3 Radioactive traces of digests (4hr.) of rat liver homogenate with either *FL or FL*

attenuated in the FL* digest (relative to the FL* peak) and (ii) may be a result of loss of part of the carbon chain of the sugar since it is attenuated in the *FL digest (relative to the *FL peak). It was noted that these two liver homogenates utilised almost exactly the same volume of O_2 during the 4 hr. incubation period hence suggesting that they had equivalent 'physiological activities'. When the incubations described above were continued for 16 hr. and 20 hr. it was observed in the case of both *FL and FL* digests, that peak (i) and FL decreased and almost disappeared and that peak (ii) increased. Hence it is possible that compound (ii) was produced from FL via compound (i) and, in view of the possible nature of (i) and (ii) discussed above, this in turn would imply that compound (ii) was a molecule similar to FL but with first the amino acid and then the sugar carbon chains shortened by successive cleavages. The *FL digests for 4 and 16 hr. showed trace peaks at high R_{FL} values (in the free sugar area, i.e. R_{FL} 3.6 - 4.2) which may have represented the labelled fragment removed from the labelled fructosyl moiety.

The radioactive traces obtained from Exp. 3.1 were ill-defined and accurate quantitative comparisons were difficult since the c.p.m. used for this experiment were relatively low. However, three peaks with lower mobilities than FL were obtained with most of the digests and one of these peaks was thought to be FL sulphate since these samples (Exp. 3.1 only) had been treated with sulphuric acid, (see p.118). For Exp. 3.1 the three peaks were present in both *FL and FL* digests and also a broad, flattened peak (unidentified) with R_{FL} of approximately 2.0 was apparent in all 30 hr. traces. The results obtained with liver slices were basically the same as those with homogenates.

The radioactive traces obtained with the liver preparations in Exps. 3.2 and 3.3 differed considerably from those described above. In both experiments the pattern was the same for all incubation times and for both *FL and FL*, and only one peak at the R_f of FL was observed. Electrophoretograms (borate buffer pH 9.1) of the digest in Exp. 3.2 showed FL together with small amounts of two other compounds both with higher negative charges than FL. These compounds were present in both the *FL and FL* digests. Electrophoretic separation of the same digest in formate buffer (pH 3.8) revealed a small 'shoulder' on the peak corresponding to FL and slower moving in the case of both the *FL and FL* digests.

Although it appears in Exps. 3.2 and 3.3 that FL remained almost unchanged, it should be noted that the radioactive $^{14}CO_2$ obtained in these two experiments tended to be higher than in the case of the two experiments previously described (3.1 and 3.4, see Tables 3.1 and 3.2) but this remains unexplained.

The results in general indicate that the liver samples possess several alternative biochemical pathways for FL degradation in vitro. FL degradation was slow in vitro (usually incomplete after 30 hr.) and hence, in vivo FL may be only partially detoxified in the liver, the remainder being excreted by the kidney with the appearance (as previously mentioned) of FL in the urine.

Preliminary Experiments with Isolated Liver Mitochondria.

The site of FL metabolism within the cell may be the microsomal 'smooth' endoplasmic reticulum where detoxication reactions typically occur. However, FL may undergo metabolic changes in other parts of the cell; the mitochondria are centres of high metabolic activity devoted mainly to producing energy from cell 'nutrients' and, hence, preparations of this organelle were investigated for their ability to metabolise FL. The mitochondria were isolated from rat livers and maintained in sucrose - EDTA suspension containing materials necessary to support oxidative phosphorylation (see 'Materials and Methods' p.138). The mitochondrial suspensions were shaken in Warburg flasks (at 37°) with FL and the oxygen uptake and CO_2 evolution monitored.

An initial experiment showed that FL slowly disappeared from a medium containing mitochondria, as judged by the Borsook assay (see Fig. 3.4). The experiment was carried out with *FL added as a radioactive tracer and, over the 4 hr. period of the experiment, the recovery of label as $^{14}CO_2$ was 0.24%

Samples of media (separated from the organelle fraction by centrifugation) were examined by paper chromatography and the chromatograms were scanned for radioactivity. The traces obtained showed a single peak with an R_f value corresponding to that of FL. The fact that $^{14}CO_2$ was evolved suggests that FL was metabolised, and that the product(s) must either have the same R_f as FL in the solvent used or be present in too low a concentration for detection. Alternatively, some of the FL may be absorbed by the mitochondria and then be metabolised inside the organelle.

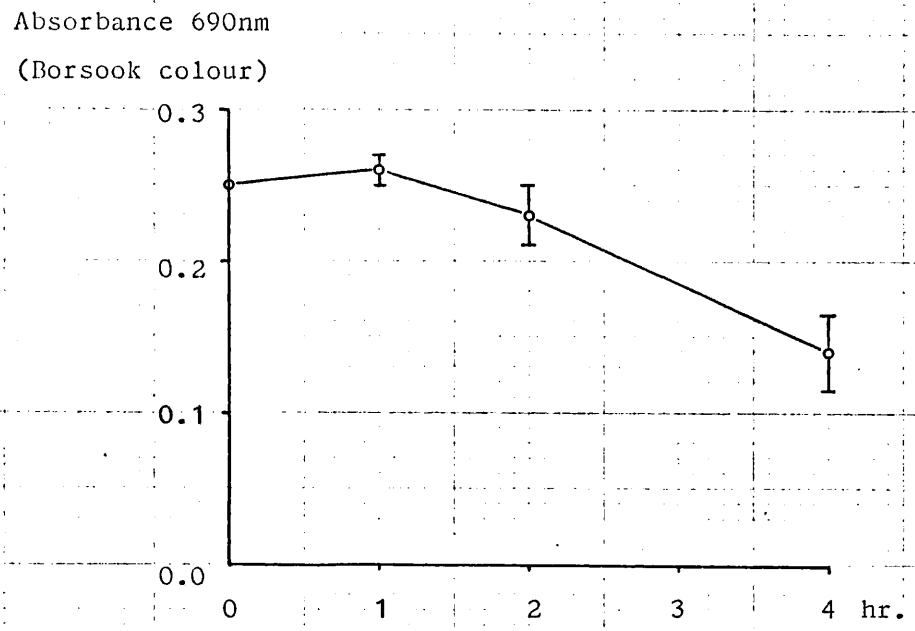


Fig. 3.4 The utilisation of FL by isolated liver mitochondria (each point is the average of two digests and is corrected for changes occurring in control (without FL) digests)

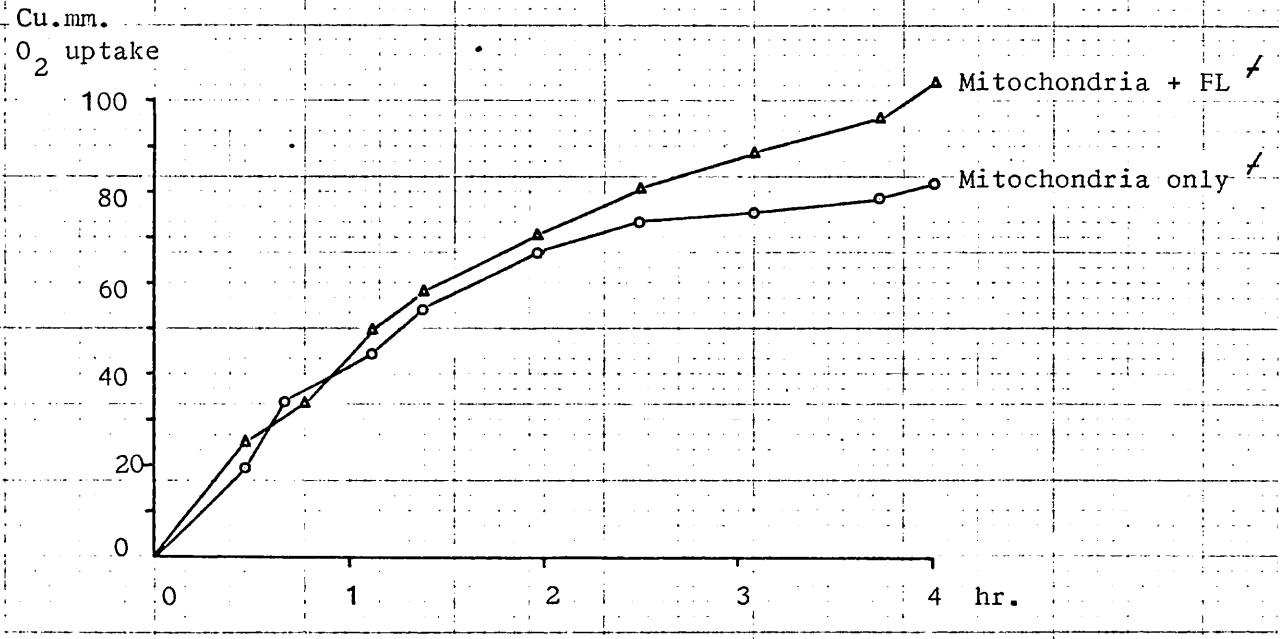


Fig. 3.5 The effect of FL on the uptake of O_2 by liver mitochondria

≠ Each point is the average of two flasks ($\pm$2 cu.mm)

Oxygen uptake for the first 2½ hr. of incubation was similar in control mitochondrial preparations to those containing FL. However, from 2½-4 hr., the rate began to increase in those flasks containing FL (see Fig. 3.5). Thus the results for Borsook-assay measurements, oxygen uptake and $^{14}CO_2$ evolution suggest that FL can be utilised and respired by rat liver mitochondria.

Some further experiments were carried out with isolated mitochondria to determine whether FL would interfere with mitochondrial oxidation of natural substrates, e.g. succinate and glutamate. Model systems, using mitochondrial suspensions, were set up in Warburg flasks as described in Materials and Methods p.138. The effect of adding FL to the respiring mitochondria whilst utilising either glutamate or succinate was investigated by following the oxygen uptake manometrically. The CO_2 evolved was removed from the atmosphere with KOH papers in the centre well.

The resulting oxygen uptake curves are shown in Fig. 3.6. They show that the addition of FL (equimolar to the natural substrate) to digests containing glutamate as substrate caused a slight depression of the rate of oxygen uptake (approximately 15%). Glutamate is normally oxidised in mitochondria by a highly active glutamic dehydrogenase (EC 1.4.1.3). A later experiment (see p.106) showed that FL did not affect a model, in vitro system using commercially purified glutamic dehydrogenase and, since the enzyme activity in vivo is located within the mitochondrial matrix (52), it is possible that FL may hinder the passage of glutamate through the impermeable inner membrane. Hence, Triton X-100 was added to a mitochondrial preparation containing FL and glutamate in order to disrupt the mitochondrial architecture and allow direct access of the glutamate to the dehydrogenase enzyme (53). Under these conditions the FL did not appear to inhibit oxygen uptake in the presence of glutamate.

FL had no effect on mitochondrial oxygen uptake when glutamate was replaced by succinate.

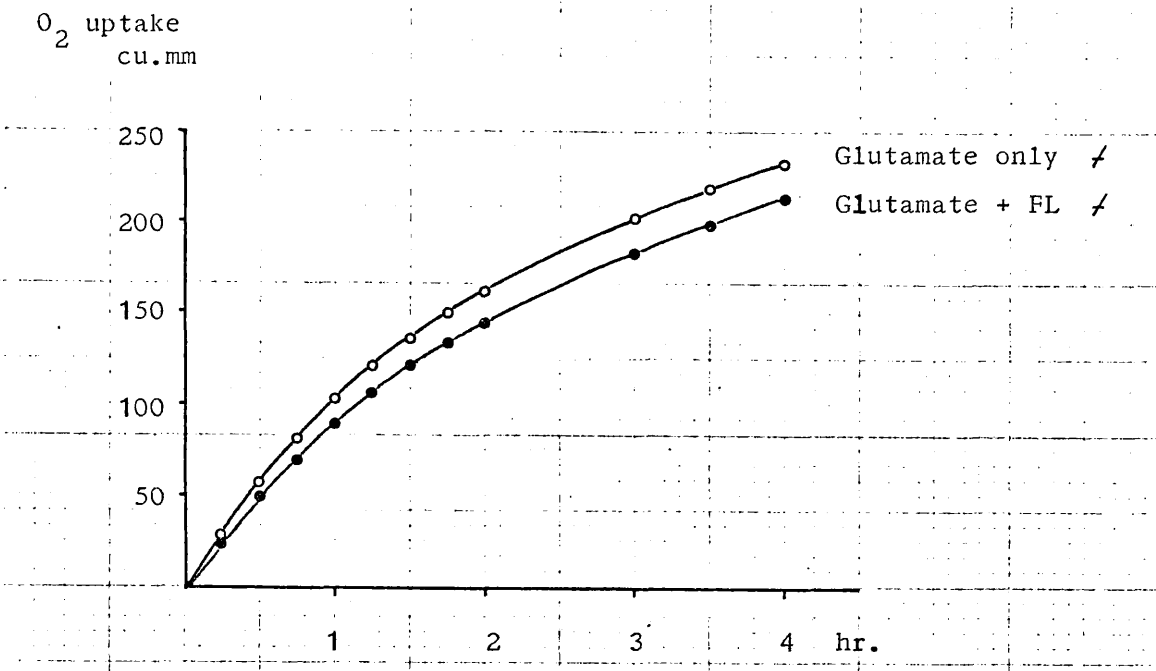


Fig. 3.6 The effect of FL on the mitochondrial uptake of O_2 with glutamate as substrate

≠ Each point was the average reading of two flasks (maximum mean deviation: 5 cu.mm)

S E C T I O N 4

Studies with Enzyme Systems in vitro.

As FL and LL possess both amino acid and carbohydrate moieties, they could well interfere with carbohydrate- protein- or amino acid-metabolising enzymes in both the gut and body organs. It was considered that FL or LL might readily serve as competetive inhibitors of enzymes involved in the reactions of normal cell metabolites. Inhibition of allosteric enzymes of the 'non-' or 'uncompetitive' type was also considered to be possible.

In the small intestine, FL and LL would normally be present in low concentrations compared with natural substrates such as disaccharides, and hence competitive inhibition may be of little significance. However, in the case of intestinal disease where, for example, disaccharidase activity is deficient any inhibition, no matter how small, may be of importance. For example in coeliac disease the intestinal maltase,isomaltase, sucrase and lactase activity are depressed (33) and in such a case carbohydrate-amino acid derivatives could enhance disaccharide intolerance. In view of these possibilities the inhibitory effects of FL and LL on several enzymes were investigated. In some instances where enzymes from animal sources were not readily available other sources, mainly microbial, were used (see Table 4.3)

1. Carbohydrate-Metabolising Enzymes

α-Glucosidase (E.C. 3.2.1.20) was assayed in the presence and absence of FL using p-nitrophenyl α-D-glucoside as substrate. The presence of FL, at a concentration 5 times greater than that of the substrate, was found to produce a 24% depression of the enzyme activity. The value for the inhibition was increased marginally to 26% when the enzyme was preincubated with FL for 10 min. Two-dimensional chromatographic examination of the reaction mixtures indicated that FL was not hydrolysed by the enzyme.

α-D-Glucose, a known competitive inhibitor of α-glucosidase, was observed to have a similar inhibitory effect on the enzymes as FL. In the case of the latter compound, inhibition was also assumed to be competitive due to the fructosyl moiety.

β-Fructofuranosidase (E.C. 3.2.1.26) is highly specific for the hydrolysis of substrates possessing non reducing β-D-fructofuranosyl groups; it was, therefore, expected that FL, a substrate analogue, might act as a competitive inhibitor of the enzyme. The enzyme was incubated under standard

conditions in hermetically sealed capillary tubes in the presence or absence of FL using sucrose as substrate and reaction rates were assayed using the glucose oxidase method (54). The glucose oxidase test itself was shown to be mildly affected by FL (see below) and appropriate corrections were therefore made. The presence of FL in the incubation mixtures (at the same concentration as the substrate, 10^{-1}M) was found to produce a 68% inhibition of the β-fructofuranosidase. When the concentration of FL was reduced by a half 23% inhibition resulted (also Table 4.1). A partially-purified sample of LL also inhibited the reaction (by 27%) when present in a concentration 4 times greater than that of the substrate. Chromatographic examination of the FL- and LL- containing incubation mixtures showed that neither compound was hydrolysed by the enzyme.

Although strong inhibition of the yeast enzyme was demonstrated *in vitro* the same need not, of course, apply to the human intestinal sucrase which exists as a complex (also having maltase and isomaltase activities) in the tissues.

As the glucose oxidase method was used to assay β-fructofuranosidase in the above study, the effect of varying concentrations of FL on this enzyme was also investigated. Minor inhibition was observed (see Table 4.2) which could have been due to FL affecting glucose oxidase itself or the peroxidase component of the test combination.

β-Galactosidase (E.C. 3.2.1.23) FL and LL were examined as possible substrates and/or inhibitors of β-galactosidase (*E.coli*). The activity of the enzyme was assayed by determination of the galactose-released using galactose dehydrogenase (*ex.* P.fluorescens); the accompanying reduction of NAD was followed spectrophotometrically. The effect of FL and LL on galactose dehydrogenase itself was shown to be unimportant (see below). A solution of purified LL (10^{-1}M) was incubated with the enzyme at pH 7.5 for 1 hr. Subsequent assay with galactose dehydrogenase revealed the presence of large amounts of β-galactose in the digest thus indicating that hydrolysis of the glycosidic linkage of LL had occurred. A control experiment with galactose dehydrogenase showed that the LL sample was not contaminated with galactose. Assay of the 1hr. digest for glucose using the glucose oxidase method (54) confirmed that this sugar was absent and thus indicated that the original LL sample was not contaminated with lactose since this, on incubation with β-galactosidase, would have yielded glucose and galactose. A two-dimensional

TABLE 4.1 The effect of varying substrate concentration on the inhibition by FL of sucrose hydrolysis with β-fructofuranosidase

Final Molar Concentration* of substrate	Final Molar Concentration* of Inhibitor	Percentage Inhibition
0.5×10^{-1}	10^{-1}	74
2.5×10^{-1}	10^{-1}	37
5×10^{-1}	10^{-1}	10
1.0	10^{-1}	2
2.0	10^{-1}	0

*Substrate alone at these concentrations did not produce enzyme inhibition.

TABLE 4.2 Effect of FL on the assay of glucose (5×10^{-4}M) by the glucose oxidase method (54)

Final FL concentration	Inhibition (%)
10^{-2}M	10
10^{-3}M	5
10^{-4}M	2

chromatogram showed the presence of ε-FL in the reaction mixture after incubation with β-galactosidase. The ε-FL did not disappear from the digests on long term incubation thus suggesting that the sugar-amino acid C-N bond was not hydrolysed by β-galactosidase.

An initial study of the hydrolysis of LL showed that under the conditions used the rate was zero order up to approximately 30 min. at 37°. A Lineweaver-Burke plot obtained by varying the substrate concentrations is shown in Fig. 4.1. The Km for the enzyme with LL as substrate, calculated from the graph, was 5.6×10^{-2}M.

Preliminary studies of the hydrolysis of lactose with β-galactosidase using LL and FL as inhibitors showed that FL did not cause inhibition, even when present at 10 times the concentration of the substrate, but that LL produced a 37% inhibition when present in equimolar proportions (1.7×10^{-2}M) with the substrate. The kinetics of this inhibition are summarised in Fig. 4.2. Linear kinetics, under the conditions used, were observed for times up to 12 min. A comparison of the curves obtained in the presence of (I x1) and absence of LL shows that at this inhibitor concentration a competitive situation exists. There is some departure from linearity at the higher inhibitor/substrate ratios and this effect is presumably due to the inhibitor itself acting as substrate.

From the Lineweaver-Burke plots in Fig. 4.1 and Fig. 4.2 for LL and lactose, respectively, it is apparent that the V_{max} for the β-galactosidase-catalysed hydrolysis of lactose is 9.00 times greater than that for the hydrolysis of LL. It can be estimated from the results that β-galactosidase hydrolyses lactose at 56 times the rate for LL at concentrations of 10^{-2}M.

It was also observed that β-galactosidase had a high affinity for the synthetic substrate, p-nitrophenyl β-D-galactoside (Km 4.45×10^{-4}; Tris buffer, pH 7.6; 20°C) and that LL (3.3×10^{-3}M final concentration) inhibited the initial rate of hydrolysis of this substrate (3.3×10^{-2}mM), by 26%

The β-galactosidase preparation used for these experiments was a commercial sample obtained from E.coli. However, human intestinal lactase is presumably also capable of hydrolysing LL to FL and galactose. The reaction in vivo in this case is probably incomplete since LL has been detected in faecal specimens from infants (27-31). This could be a function of the relatively low hydrolytic rate and lactase deficiency due to some intestinal disorder.

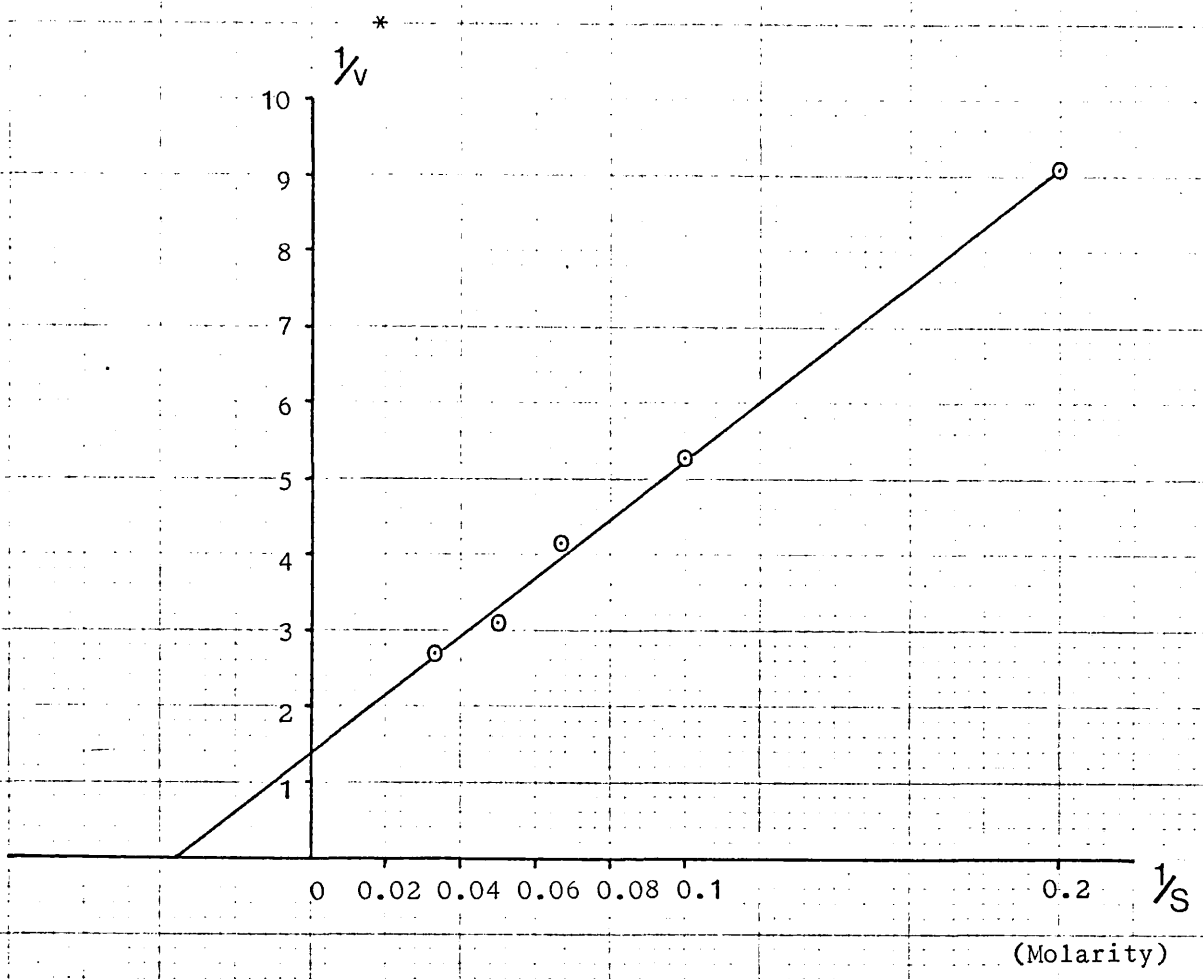


Fig. 4.1 Lineweaver-Burke plot for the hydrolysis of lactulose-lysine by β-galactosidase at 37° (o.2 m-phosphate buffer, pH 7.5, 15 min. incubations)

* Arbitrary units based on the absorbance at 340 nm recorded in the galactose dehydrogenase assay for liberated galactose (see text for full details)

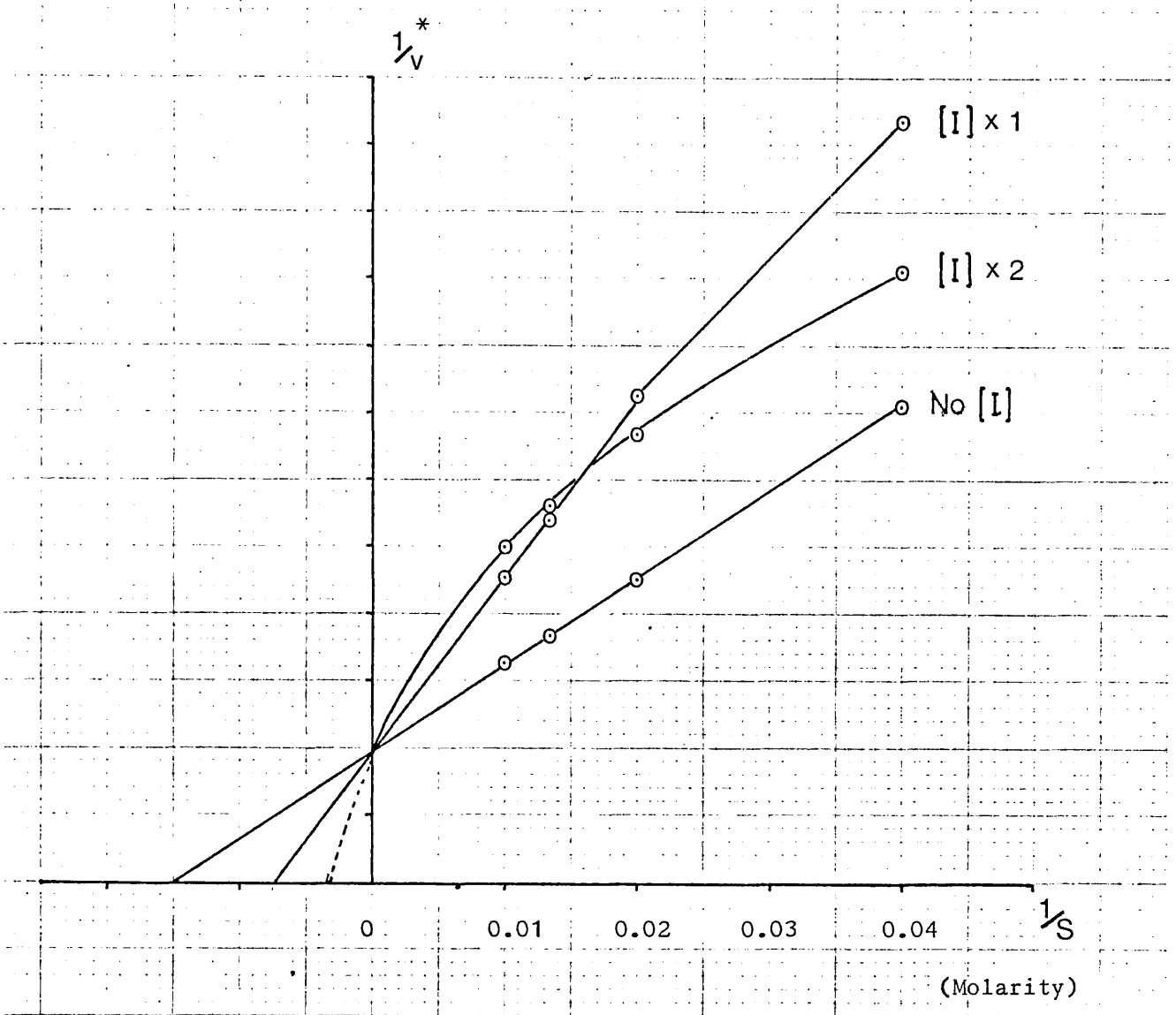


Fig. 4.2 Inhibition by lactulose-lysine of lactose hydrolysis by β-galactosidase (6 min. incubations, 0.2 m-phosphate buffer, pH 7.5, 37°)

* Arbitrary units based on the absorbance at 340 nm recorded in the galactose dehydrogenase assay of liberated galactose (see text for full details)

The Galactose Dehydrogenase used in the β-galactosidase assay was also examined for possible inhibitory effects by FL or LL (equimolar with the substrate, galactose; final concn. 2.5×10^{-2}M). It was observed that FL caused a 21% decrease in the initial rate of reaction and with LL there was a 38% decrease. The final absorbance value was, however, unaffected and hence the presence of FL and LL would not affect the quantitative determination of galactose by this method.

Phosphorylase a (E.C. 2.4.1.1; from rabbit muscle) was assayed by measuring the liberation of inorganic phosphate from G-1-P in the presence of glycogen as a glucose acceptor. The addition of FL to the enzyme at concentrations equal to or 10 times greater than the concentration of G-1-P caused no detectable inhibition of glycogen synthesis. Chromatographic examination of the digests showed that the FL itself remained unchanged during this reaction.

Acid Phosphatase (E.C. 3.1.3.2). The hydrolysis of sodium β-L-glycerophosphate (1 mM) by hydrolysis with acid phosphatase (prepared from rat-liver lysosomes) was not affected by the presence of FL at final concentrations of 10^{-5} - 10^{-2}M. The same results were obtained using acid phosphatase from potato.

As expected, the FL remained unchanged in the presence of the phosphatase.

2. Amino Acid-Metabolising Enzymes

Lysine Decarboxylase (E.C. 4.1.1.18) is a common intracellular enzyme of bacteria (e.g. Lactobacillus spp.) and inhibition of this enzyme could be important since its function is vital for the normal growth of some intestinal microorganisms. The polyamine produced by the decarboxylates of lysine, i.e. cadaverine, can act as a growth factor by stabilising bacterial membranes. Hence, the possible inhibition of the enzymes by FL, which might compete for active sites by acting as a substrate analogue, could indirectly affect the growth of some colonic bacteria.

Mixtures containing lysine decarboxylase (ex B.cadaveris) and lysine were therefore incubated in the presence and absence of FL and the CO_2 production compared by a manometric method.

Decarboxylation of FL by the enzyme could not be detected by Warburg manometry or by the use of ^{14}C-labelled FL. In addition, no changes in FL after incubation with the decarboxylase could be detected by chromatography.

Fig. 4.3 depicts the observed evolution of CO_2 from lysine and shows that FL, equimolar with the substrate (10mM) caused slight inhibition of the CO_2 production. From the total CO_2 produced after 50 min. incubation, obtained by acidification of the digests, it was calculated that an overall 15% inhibition of the decarboxylase had occurred.

Glutamate Dehydrogenase (E.C. 1.4.1.2), prepared commercially from beef liver was incubated with ammonium acetate and α-oxoglutarate in the presence of NADH:

$$\alpha\text{-oxoglutarate} + NH_3 + NADH^+ \rightleftharpoons \text{L-glutamate} + H_2O + NAD^+$$

The above reaction was followed spectrophotometrically. FL was added to the reaction at concentrations of $^1/_5$ or equal to the concentration of the oxoglutarate (3mM) but no detectable change in the rate of reaction occurred.

L-amino acid oxidase (E.C. 1.4.3.2), a flavoprotein enzyme, is found in mammalian liver and catalyses the oxidative deamination of several amino acids:

$$\text{L-amino acids} + H_2O + O_2 \longrightarrow \text{2-oxo acids} + NH_3 + H_2O_2$$

The enzyme (rat kidney or snake venom) does not react with L-lysine but, since L-amino acid oxidase is a relatively unspecific enzyme (regarding the side chain of the L-amino acid), activity with FL is not precluded. A commerical preparation of l-amino acid oxidase derived from snake venom (Crotalus adamanteus) was therefore examined for its ability to oxidise FL. The enzyme activity was assayed by following the disappearance of oxygen from the reaction mixture using an oxygen electrode. Reactions were carried out in oxygen-saturated buffer (0.1M Tris-HCl, pH 7.5 at 30°C) and the initial velocities for the reactions expressed as the change in % oxygen saturation per minute. The enzyme (0.5 mg/ml) was inactive in the presence of lysine but FL appeared to act as a substrate causing a slow disappearance of oxygen in the reaction chamber (Fig. 4.4). A control experiment confirmed that this change did not occur in the absence of enzyme. The initial rate of oxygen uptake for 10^{-3}M-FL was 1.050% saturation per min. ($\pm$ 0.05% in repeat experiments) as compared with 10.00% saturation per min. for 2 x 10^{-5}M-tryptophan (a natural substrate). The oxidation was not inhibited by substrate (a characteristic of this enzyme) at concentrations of FL up to 5 x 10^{-3}M. The addition of lysine, equimolar with FL, to the reaction mixture during the oxidation of FL caused a slight inhibition of the oxygen uptake rate (it was reduced by 0.16% saturation per min.) and, since this effect was reversed by

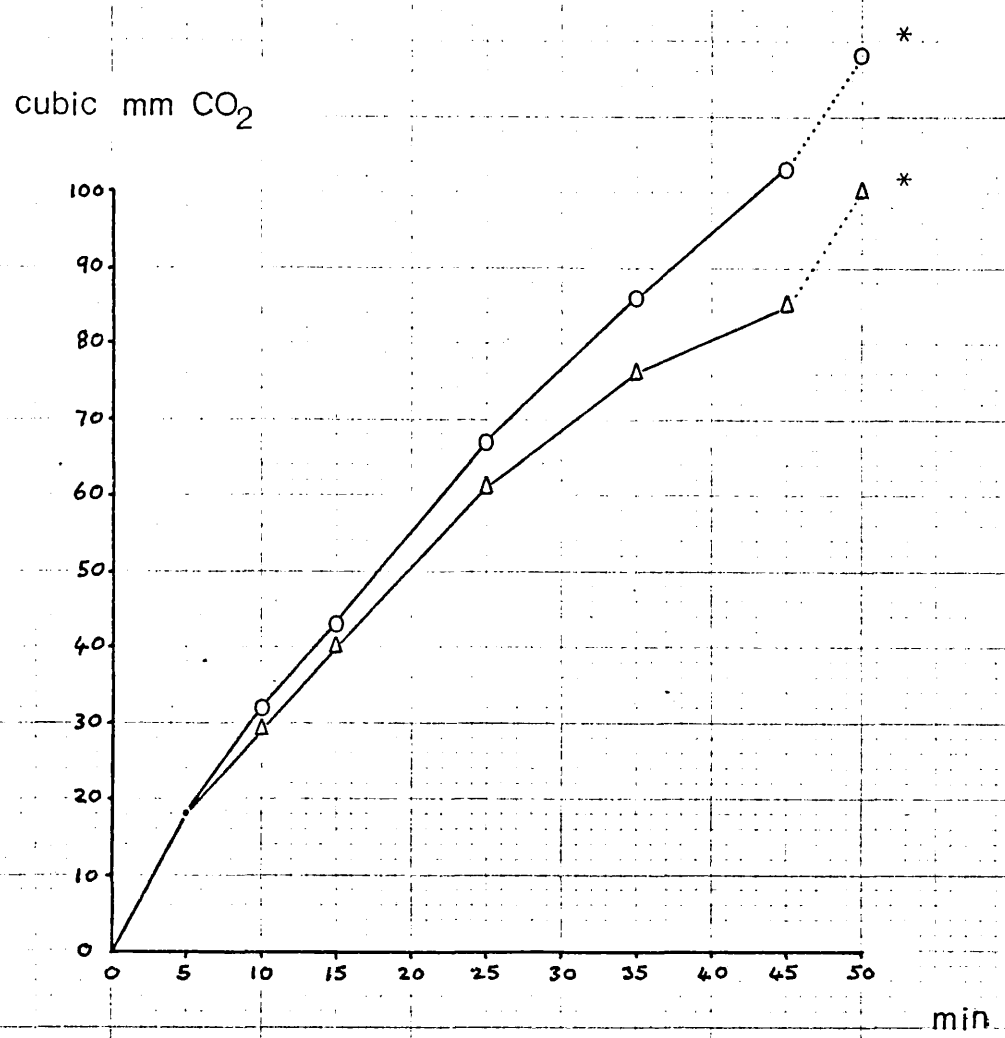


Fig. 4.3 Inhibition of lysine decarboxylase activity by FL

* These values were obtained after acidification of the flask contents

o—o Lysine (10 mM)

Δ—Δ Lysine + FL (both 10mM)

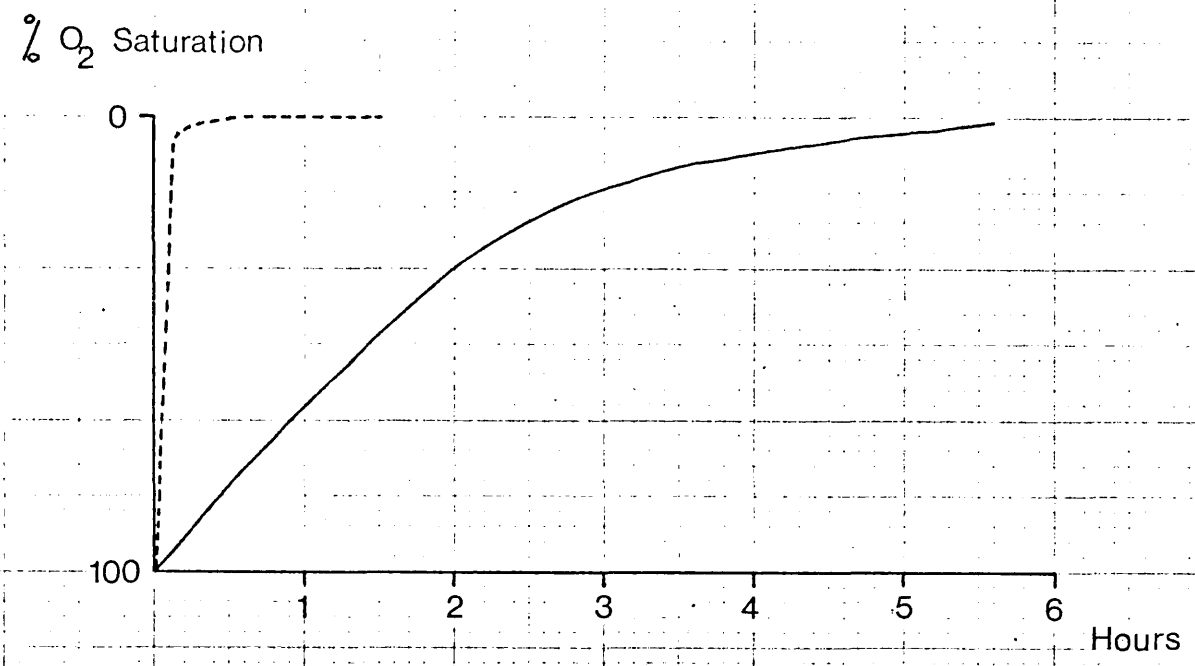
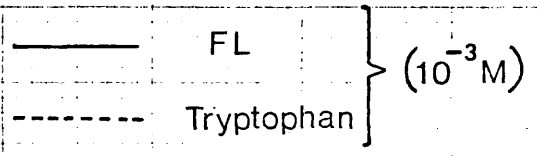


Fig. 4.4 The uptake of oxygen in solution by FL and tryptophan in the presence of L-amino acid oxidase.



the addition of further FL, the inhibition may have been of the competitive type. When a lysine/FL mixture was added to the enzyme, the normal rate of FL oxidation was reduced by 0.58% saturation per min. and, when lysine was preincubated with the enzyme, subsequent FL oxidation was reduced by 0.74% saturation per min. In the latter cases lysine is presumably able to compete more successfully than FL for the active sites of the enzyme. FL (10^{-3}M) caused reversible inhibition of tryptophan (2×10^{-5}M) deamination (it was reduced from 10.00 to 5.60% saturation per min.), when both were added simultaneously to the enzyme; lysine exerted a similar effect.

The oxidation of FL was repeated using FL* and, after obtaining the characteristic reaction curve over 4 hr. with the oxygen electrode, the digest was concentrated and examined on a paper chromatogram using a pyridine/acetic acid/water solvent (9:1:2, v/v). Scanning the paper for radioactivity revealed, in addition to a small amount of residual FL, a large peak of radioactivity remaining at the origin. A radioactive scan of the components of the digest separated by electrophoresis in ammonium formate buffer (pH 3.8) showed the presence of a large peak, with a positive charge, close to the origin (M_{FL} ca. 0.5). An electrophoretogram of the digest using borate buffer (pH 9.1) showed a large peak with the same degree of electrophoretic mobility as FL but moving in the opposite direction i.e. with a positive charge. This compound, although theoretically present in detectable amounts, was not ninhydrin positive, hence suggesting the absence of a free amino group. The compound which in theory should result from the oxidative deamination of FL is shown below. The structure given satisfies the acidic properties shown by chromatography and electrophoresis in formate buffer. It is difficult to comment on the behaviour of the compound in borate buffer in view of possible borate complex formation and even degradation under strongly alkaline conditions.

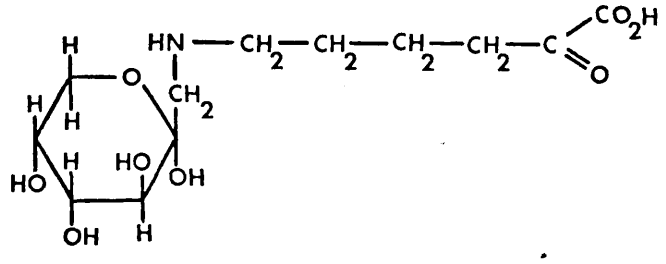


Fig. 4.5 Possible product of oxidative deamination of FL

No compound with the chromatographic and electrophoretic properties described above has been detected in FL digests with liver preparations (see Section 3) and the absence of such a compound may reflect the reputedly low order of L-amino acid oxidase activity in the liver.

Summary

FL has been examined with respect to its potential role as an enzyme inhibitor and as a possible substrate. No significant inhibition of a number of important metabolic enzymes has been observed and the compound shows only mild inhibition of some glycosidases related to those normally occurring in the intestine. As expected, for those enzymes where FL is a close structural analogue of the natural substrate, the greatest inhibition was observed and it appeared, in every case, to be of the competitive type. The C-N bond linking the sugar and amino acid moieties was not hydrolysed by any of the enzymes investigated and FL was used as substrate only by L-amino acid oxidase. Table 4.3 summarises all of the work on the interaction of FL and LL with enzymes.

TABLE 4.3 The reaction of FL and LL as substrates and inhibitors of various enzymes of metabolic importance.

ENZYME	ENZYME SOURCE	SUBSTRATE	Inhibitory Properties		Behaviour as substrate	
			FL	LL	FL	LL
α-Glucosidase (ex yeast)	Yeast	p-nitrophenyl α- D-glucoside	-		-	
β-Fructofuranosidase	Yeast	sucrose	+	+	-	-
β-Galactosidase	E.coli	lactose	-	+	-	+
L-lysine decarboxylase	Bacillus cadaveris	L-lysine (free base)	+		-	
Phosphorylase a	Rabbit muscle	glycogen	-		-	
Acid phosphatase I	potato	sodium β-glycerophosphate	-		-	
Acid phosphatase II	rat liver lysosomes	sodium β-L glycerophosphate	-		-	
Glucose oxidase/ Peroxidase (Boehringer test kit)	Aspergillus niger	glucose	+		-	
Galactose dehydrogenase	Pseudomonas fluorescens	galactose	-	-	-	-
L-Amino acid oxidase	snake venom	tryptophan	+		+	
Glutamic dehydrogenase	bovine liver	glutamic acid	-		-	
Lysosyme	egg white				-	
Pronase	Streptomyces griseus				-	
Acylase	Porcine kidney				-	
Acylase	Porcine kidney				*-	
Chymotrypsin	Bovine pancreas				*-	
Peptidase	Porcine intestine				*-	

* Studies with these enzymes were conducted using α-formyl-FL as substrate.

FINAL CONCLUSIONS

A broadly based study with FL[/], and to a lesser extent LL, has given some insight into the possible fate and pathology of these compounds in man. No evidence of serious biochemical toxicity was found but some observations suggest that the sugar-amino acid derivatives may be indirectly harmful to infants.

LL is probably released from heat-damaged milk protein or derived peptides either by proteolytic enzymes in the small intestine or possibly as a result of bacterial action in the large bowel. Some LL may be further hydrolysed to yield FL by the action of bacterial and/or intestinal lactase. It is fairly certain that small amounts of FL and LL enter the circulatory system from the intestine, probably by passive diffusion (22) and, hence, are presumably able to reach all organs of the body. However, no evidence was discovered in the present study to suggest that these compounds significantly interfere with normal intracellular enzyme function, and the findings suggest that the compounds can probably be detoxified by breakdown in the liver and by excretion in the kidneys (30). Lysine is the most vital of the 'essential' amino acids (with a dietary requirement for the intact molecule in man) and rat bioassay experiments by other workers have suggested that, although lysine complexed with carbohydrate can be utilised as a nitrogen source, symptons of lysine deficiency are still present Our results also indicate that FL is metabolised in the liver but without the appearance of free lysine.

The observed mild inhibition of some intestinal enzymes by FL and LL may be significant in the case of infants suffering from intestinal disorders, if the concentrations of these compounds reach a relatively high level in the intestinal lumen.

It is apparent from the present work that E.coli B is able to metabolise FL after induction of a suitable enzyme(s) (FL-ase). The characterisation of this extracellular FL-ase could, in future, be profitably extended. In view of the fact that E.coli B cultures are able to cleave FL with the release of

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Footnote

/ The FL sample used throughout this study has kindly been analysed by Dr. J. Seakins and Dr. R. Ersser, and shown to be chromatographically and electrophoretically identical to 'substance 2' (thought to be FL) which they detected in faecal extracts of babies fed HCND milk (27,31).

free lysine it is logical to suppose that this occurs to some extent in the colons of infants and is consistent with literature reports of unusually large amounts of lysine accompanying FL (and LL) in faecal extracts (30). The observed hydrolysis of LL by lactase (E.coli) *in vitro* further suggests that E.coli may metabolise this compound (releasing FL) possibly after assimilation by a non-specific β-galactoside permease. The CO_2 evolved and possible induced osmotic water loss resulting from FL and LL breakdown by bacteria could directly harm infants by causing diarrhoea. Also, if E.coli thrive on these compounds they can buffer the conditions (pH) to optimise their own growth and hence that of Coliforms in general thus encouraging likely pathogens. It is obvious that future work should include a study of the effect of LL and FL on a variety of gut organisms from infants, in particular Lactobocillus bifidus usually the most prevalent microorganism during the first 10 days of life.

Partial chemical decomposition of FL and LL involving melanoidin production would presumably render subsequent digestion by bacteria more difficult and indeed a FL sample partially degraded by heating was observed to exert a bacteriostatic effect on E.coli B.

In conclusion, our results suggest that dietary FL and LL exhibit some properties which could be consistent with the idea that they are involved in the worsening of diarrhoea in infants. This link should, of course, be confirmed by appropriate feeding experiments designed to correlate a dosage of LL or FL with effects on the diarrhoea syndrome.

MATERIALS AND METHODS

Materials: Analar grade chemicals were used whenever possible; other chemicals were of best available purity.
Glass distilled, deionised water was used for the preparation of solutions.

Animals: Wistar-strain rats from 4-17 months of age were used to provide liver specimens. The animals were fed on a commercial stock diet ad lib, and killed as needed by cervical fracture.

General Methods

1) Chromatography: Whatman No. 1 and No. 3 papers were used throughout for qualitative and preparative chromatography, respectively, and chromatograms were developed by the descending elution technique. All mixtures containing FL, LL or their derivatives were dried on the origin with a stream of cold air. Samples containing radioactivity were applied to the origin as a 4cm-wide band. Papers containing separated radioactive samples were scanned by cutting out 4cm-wide strips and assaying these with a Tracerlab 4 radioactive scanner.

'Schleicher and Schüll TLC-Ready Plastic Sheets' (F 1440, cellulose) were used for thin-layer chromatography and were developed by the ascending elution technique. Two-dimensional separations were carried out with solvent (i)(below) for the first direction and solvent (ii)(below) for the second direction (12).

Solvents

(i) Methyl ethyl ketone/acetic acid/water (5:3:2; v/v)
(ii) Pyridine/acetic acid/water (9:1:2; v/v)
(iii) Ethyl acetate/acetic acid/formic acid/water (18:3:1:4; v/v)

Locating Reagents

a) Carbohydrates on paper were located with a silver nitrate solution in acetone followed by ethanolic sodium hydroxide (0.5-2.0%, (58)). Carbohydrates on thin-layer plates were located by spraying with silver nitrate/ammonia solution followed by heating at 100°C.

b) Amino compounds were detected with ninhydrin in an ethanol, acetic acid, 2-4-6 collidine, cupric nitrate mixture (59) used in a 'dip tray' (for papers) or as a spray (for TLC) and followed by heating. The positive areas were then

fixed by spraying a 7% solution of nickel sulphate.

c) Amadori products and sugars were visualised with triphenyl tetrazolium chloride (58).

d) O-Dinitrobenzene in ethanol/4M-NaOH (0.2:10:1; w/v/v) was used as a spray reagent for Amadori products.

e) FL and LL were detected with NaOH and 0.1% potassium ferricyanide followed by ferric sulphate reagent as described by Borsook (5).

Other reagents were prepared and applied as described in a standard text (60).

All thin-layer chromatograms, after the separated samples had been treated with a locating reagent were sprayed with vinyl solution to fix the cellulose and to stabilise the colours. For chromatographic separations of radioactive samples from E.coli experiments or liver preparations it was noted that, due to the large number of components in the mixtures, all Rf values were reduced (to 70% for liver samples and to 90% for E.coli samples) compared with the standards. Hence quantities of FL* or *FL were mixed with appropriate samples of the E.coli or liver digests and were applied to all radioactive chromatograms, and later scanned, to enable precise assessment of the degree of retardation occurring.

2) Electrophoresis: Whatman No. 1 paper was used for high-voltage electrophoresis (2000-5000 volts; 0-90mAmps) and thin layer plastic sheets for low-voltage electrophoresis (300-900 volts). Radioactive samples were applied as a 4cm band and, after electrophoretic separation, were assayed with a 'Tracerlab' 4π radioactive scanner. During the separation, picric acid was used as a visual marker to estimate the progress of sample movement.

Solvents

(i) Ammonium formate buffer (pH 3.8, 0.1M)

(ii) Acetic acid (1M)

(iii) Borate buffer (pH 9.1)

The same locating reagents were used as previously described in the section on paper chromatography.

3) Radioactivity: All radioactivity was measured on paper and counted to a 3% error with a Beckmann CPM 100 liquid scintillation counter, using a toluene scintillant (5g PPO : 0.3g POPOP : 1L toluene). The estimated recovery of radioactivity in c.p.m. was 65-75% of the d.p.m. in all cases.

4) Assay of N-substituted 1-amino-1-deoxy-2-ketoses: The method was based on that described by Borsook et al (5). Samples (0.2ml) were mixed with 2ml of potassium ferricyanide solution (0.1% w/v) and then 1M-NaOH (0.25ml) added. After reaction for 10 min, ferric sulphate reagent (2ml) was added (32ml phosphoric acid, 2.5g anhydrous ferric sulphate made up to 500ml with water). After a further 5 min period, the mixture was diluted with 10ml H_2O and the absorbance$_{690nm}$ measured within 30 min. The reactions were carried out in a constant temperature water bath.* The prussian blue colour obtained with FL followed the Beer-Lambert proportionality law relating absorbance and substrate concentration for absorbance readings between zero and 1.00. Where enzyme digests containing FL were assayed, the samples were first mixed with the NaOH to destroy the enzyme activity and the potassium ferricyanide solution was added immediately to start the reaction.

Column fractions were commonly assayed by using the Borsook reagents in a 'Bioanalyst' automatic analyser. *(25-35°c.)

5) Respirometry: The respirometer used was a constant volume, double capillary manomater, 'Warburg' apparatus. The general procedures for manometric technique were followed using a standard text (e.g. Umbreit et al., (71)). Precalibrated Warburg flasks with one or two side arms and with a centre-well were used. The flasks were all approximately 17ml and were normally used with test volumes of 4ml. Reaction mixtures were shaken in a constant temperature water bath at 37°C. and the first reading taken after ten minutes' equilibration.

Gas evolution or uptake was measured by adjusting the appropriate limb of the manometer to give a constant flask volume and by then reading the pressure change in the other limb. A thermobarometer was set up with every experiment. Pressure changes were corrected to give true volume changes (V) using the following formula:

V = K. (pressure change in experimental apparatus - pressure change in thermobarometer)

where K is the flask constant. Carbon dioxide was removed from the atmosphere in the flask, where necessary, with 30% w/v KOH (0.1ml) absorbed on to fluted filter paper wicks (1.5cm x 2.0cm) in the centre well. $^{14}CO_2$ collected in this way was estimated by drying the wicks in an oven and then counting the radioactivity in PPO (5% w/v)/toluene scintillation fluid.

For respiration experiments with E.coli cultures and liver slices

(or homogenates), the pH values of the reaction flask contents were checked whenever a sample aliquot or KOH paper was removed. The amount of $^{14}CO_2$ in solution when the medium was above pH 5.0 was estimated by treating a suitable aliquot (usually 1/10 of the test volume) with sulphuric acid (final pH value 2.0 - 3.0) added from the side-arm. The $^{14}CO_2$ liberated during a further incubation period (10 min) was collected with a fresh KOH paper and assayed in the usual way. For E.coli incubation mixtures containing 300,000 c.p.m. or less ('low counts' samples) of labelled FL, the dissolved $^{14}CO_2$ was liberated by acidification (final pH 2.0 - 3.0) of the whole mixture which was then incubated for a further 10 min. This treatment with acid did not cause dissolution of the cells and chromatographic analysis of controls showed that FL was not cleaved. Cultures were then neutralised with 0.1M NaOH and stored in deep-freeze. Rat liver preparations of slices or homogenates containing 300,000 c.p.m. or less of radioactive FL were also treated in this way.

The efficiency of $^{14}CO_2$ collection, by the use of KOH and filter-paper 'wicks', was estimated by treating a known amount of $NaH^{14}CO_3$ with sulphuric acid in the Warburg apparatus: after incubation (10 min), the recovery of the original counts on the KOH paper was found to be 100%.

Counts obtained for KOH paper from various experiments were subject to the following errors and were corrected accordingly:

(i) The background c.p.m. for the scintillation fluid were subtracted

(ii) The presence of KOH on the papers was found to cause 'alkaline chemo-luminescence' (see Kalbhen 1971 (63)) of the scintillation fluid. The counts recorded as a result of this decayed to a minimum after approximately 30 min.

All samples, therefore, were allowed to stand for 1 hr. before counting. Counts due to residual alkaline chemo-luminescence were then subtracted.

(iii) The presence of KOH was found to quench radioactivity on paper and, hence, to reduce the c.p.m. for filter paper wicks by 38%. All c.p.m. values were multiplied by 100/62 to compensate for this.

(iv) Control Warburg flasks containing radioactive FL and minimal medium only, showed that there was a continuous transfer of radioactivity, linear with time, from the flask contents to the wick in the centre-well, presumably due to 'splashing'. This amounted to 0.15% of the initial FL activity per 10 hr. and was subtracted accordingly.

6) Desalting by Ion Retardation: 'Bio-Rad' AG 11A8 ion retardation resin (61,62) was slurried into a column giving a bed volume of 1.9cm x 18cm (approx. 50 ml). The resin was washed with deionised water until the eluate was neutral. The sample (1ml. in aqueous solution) was fed into the bed at a flow rate of 0.1ml/cm^2/min and elution with deionised water was then carried out, at the same flow rate, at room temperature. The eluate was collected in 3ml. fractions.

Elution with water was expected to yield all organic substances in a fraction preceding the inorganic salts and (since the exchangeable groups of the resin are, ideally, self-absorbed) with a yield approaching 100%. However, the resin (50ml. bed-volume) was found to absorb samples of FL (1.5mg; i.e. an amount equivalent to that present in the Warburg flasks of respiration experiments) completely and irreversibly whether radioactively labelled or unlabelled and whether or not salt was also present. This total disappearance of FL was assumed to be due to absorption by unpaired exchangeable groups on the resin. Hence, these groups were neutralised by pre-conditioning the resin with a saturated solution of tryptophan which was passed through the column until it appeared in appreciable amounts in the eluate. Excess tryptophan was then removed by washing with water. Samples of FL (as above) then applied to the column could be successfully eluted from the resin with water and after pooling the fractions collected, were recovered in approximately 70% yield. Tryptophan was used in preference to other compounds because its appearance was readily monitored by passing the column eluate through a flow-through cell in a continuously recording spectrophotometer and measuring its Absorbance (260_{nm}).

During usage of the column, the application of a sample containing salt always resulted in some tryptophan being released from the column which could be recorded as a concentration peak by measurement of absorbance (260_{nm}). The position of this peak was a useful marker for estimating the positions of the organic and salt fractions prior to assay. The consequent presence of some tryptophan in the organic fraction of the separated sample did not appear to affect subsequent chromatographic or electrophoretic analyses.

After each separation of the radioactive samples the column was regenerated by washing with water until all salts had been removed and was then re-used as before. The application of further salt to the regenerated

column was found to cause the appearance of radioactivity in the eluate, and was presumably derived from the sample previously separated. Paper chromatograms of this material gave a separation pattern (but with grossly attenuated peaks) similar to that of the original sample. It was estimated that each sample (containing salt) separated on the column therefore became contaminated with approximately 5% of the preceding sample. This fact was taken into account during interpretation of the results of the subsequent chromatographic analyses.

The position of the salt fraction was estimated by locating chloride ions. One drop was taken from each fraction tube and mixed with one drop of silver nitrate solution (0.1M) on a glass plate. A visual estimation of the amount of white precipitate produced was made. The organic fraction was located by removing samples (0.2-0.5ml) from each fraction tube and absorbing them on to filter paper. The papers were dried and then assayed for radioactive counts with a scintillation counter.

Chemical Methods

Preparation of Fructose-Lysine

α-Lysine formate was prepared from L-lysine monohydrochloride by passage through a column of Amberlite IR-4B anion exchange resin (formate form). The eluate was passed through a second column to ensure complete conversion. The off-white solid obtained on evaporation of the eluate was chromatographically pure (in 18.3.1.4).

α-Formyl lysine was prepared by the method described by Hofmann (47). Crystallisation of the compound could not be easily achieved with ethanol as described by Hofmann but after occasional 'scratching' of the sides of the flask crystals were formed after a few days. Addition of small amounts of diethyl ether also facilitated the onset of crystallisation.

The preparation of FL was attempted by the method described by Finot and Mauron (12). Separation was effected using 'Bio-Rad' analytical grade cation exchange resin, AG 50W-X4, 100-200 mesh (effective pore size -'large') of the styrene sulphonic acid type. This was used in a column 80cm in length and 4cm in diameter (void volume, 250ml) and the eluate was monitored by passage through a Uvicord recording spectrophotometer containing a flow-through cell by which the $absorbance_{275nm}$ of the eluate was continuously

measured. Peaks of melanoidins were hence recorded and the position of fraction-tubes containing the required FL could be estimated from the positions of the melanoidin peaks. Crude preparations of α-formyl-FL characteristically showed four melanoidin peaks and crude deformylated products gave three. In both cases the FL derivative fraction directly followed the last melanoidin peak and was accurately located by Borsook assay of 0.2ml aliquots of the fraction tubes. Crude α-Formyl-FL was prepared and separated exactly as described by Finot and Mauron (12). Test deformylation reactions involving treatment with various concentrations of HCl and NaOH were carried out in sealed capillary tubes (50µl quantities) and were spotted on to thin layer plates for examination by two-dimensional chromatography. Enzymes used for attempted deformylation were 3mg/ml in phosphate buffer (pH 7.4) and were incubated in capillary tubes (1hr, 35°C) and examined by 2-D thin layer chromatography. Deformylation with ethanolic HCl was carried out under reflux and small amounts of water added to effect complete dissolution of the α-formyl-FL.

The modified procedure for preparing FL was as follows. Crude α-formyl-FL (12g) was dissolved in water (5.5ml) with gentle heating and then applied to the Dowex column (as used above) whilst elution (50ml.hr^{-1}) was in progress. Elution (pyridine formate buffer pH 3.25) was continued and the fraction tubes (10ml) assayed automatically with a 'Bioanalyst' using the 'Borsook' test. The tubes containing pure α-formyl-FL were pooled and evaporated to dryness by vacuum distillation at 35°C. The resulting yellowish solid (9.80g) was re-evaporated to dryness with water (50ml) to remove most of the pyridine and some of the formic acid. The resulting solid material was dissolved in 2M-HCl (50ml) and refluxed for 40 min in a round-bottomed flask (100ml) placed in a boiling water bath. The mixture was then reduced to dryness by rotary evaporation. The resulting white crystals and red-brown syrup smelling strongly of acid were redissolved in water (40ml) and again reduced to dryness. The mixture was again redissolved in water (20ml) and shaken with N-N'-dioctylmethylamine (10% v/v in chloroform, 40ml) in a separating funnel until the aqueous layer approached neutrality. The aqueous fraction was isolated and shaken twice with chloroform (50ml) to remove excess N-N'-dioctylmethylamine. The sample was evaporated to dryness and the resulting solid (4.48g) dissolved with gentle warming in 0.1M-pyridine acetate buffer, pH 3.25 (6ml). The solution was applied to the column, as

before, which was then eluted with 0.2M-pyridine acetate buffer (pH 3.25, 600ml) to remove mobile impurities. Elution was then continued with 0.4M-pyridine acetate buffer (pH 5.25) and the fraction containing FL located by automatic Borsook assay and isolated by rotary evaporation. The sample was redissolved in water (20ml) and treated with N-N'-dioctylmethylamine (20ml) as previously described for α-formyl-FL. The aqueous fraction was reduced to dryness and dissolved in methanol (20ml). FL was then precipitated with acetone (200ml) whilst stirring and then centrifuged (4000g, 1 min). The excess liquid was poured off, leaving the white solid damp with acetone. The tube was then carefully introduced into a large 'dry-bag' and the solid spread over a watch glass under conditions of zero relative humidity. After the remaining acetone had evaporated, a fine white powder highly charged with static electricity (as observed by Finot and Mauron (12)) remained. A small amount (approximately 15mg) was sealed in a tube and sent immediately to 'Alfred Bernhardt Microanclytisches Laboratorium' for elemental analysis. A small amount of powder (1-2mg) was sealed in a capillary tube for melting point determination. The remainder of the powder was removed from the dry-bag, weighed (1.48g) as quickly as possible (the powder became a syrup within approximately 30 sec exposure to the atmosphere due to its hygroscopicity). The FL was dissolved in water (to give a 1M solution) and stored at -20°C for use in further experiments. As FL was not recovered in large quantities, all further experiments using the compound were designed so that the minimum possible quantity was needed.

Solid-Phase Fusion Mixtures

(i) Lysine HCl and glucose were ground separately with a pestle and mortar into fine powders and then mixed in equimolar proportions. The mixture was melted by heating to 150°C in an oil bath and held at this temperature for 15 min. The fused solid was dissolved in water and samples examined by paper chromatography, both uni- and two-dimensional, or on thin-layer plates. Paper chromatograms were dipped in ninhydrin or $AgNO_3$/NaOH reagents and thin layer plates were sprayed with ninhydrin reagent.

(ii) Equimolar mixtures of powdered glucose, fructose or lactose and powdered lysine (free-base) were melted together (glucose/lysine, 140°C; lactose/lysine, 195°C; fructose/lysine, 100°C) and then heated at a variety of temperatures (90, 100, 110, 120, 140, 160°C) for 10 min and the fusion mixtures dissolved in water and analysed by chromatography as above.

(iii) The procedure described in (ii) was repeated but using 4 times the molar equivalents of the sugars.

Liquid-Phase Fusion Mixtures

(iv) A mixture of L-lysine (1g) and D-glucose (6g) was refluxed in methanol for four hours. The brown solution and the black solid residue which resulted were separated and then examined by thin layer chromatography.

(v) A mixture of L-lysine (1g) and D-glucose (6g) was dissolved in water and then reduced to a thick syrup by rotary evaporation. The mixture was allowed to stand at room temperature in an unstoppered flask for 15 days and samples were taken for chromatographic analysis on each day. The experiment was repeated using lactose and lysine (8:1 w/w).

(vi) L-lysine (1.5g) and D-glucose (10g) were dissolved in water (30ml), adjusted to pH 8 with HCl, and stirred in an evaporating dish at room temperature for 5 days either under ultra-violet light (a 'Hanovia' lamp placed 2" from the sample) or in the shade. Samples were removed at intervals and examined by thin layer chromatography. 5ml of water was added to the controls (shade) and 10ml to the UV-treated samples after each period of 20 hours to compensate for evaporation.

(vii) Lactose (16g) and L-lysine-HCl (2g) were dissolved in water (30ml). The mixture was divided into four portions: (a), was adjusted to pH 3.0 with HCl; (b), was adjusted to pH 9 with ammonia solution; (c), was treated with ammonium chloride (0.05M) and (d) was untreated. All mixtures were heated at 50°C and aliquots taken at 1, 2, 3½ and 5 hr. for examination by thin layer chromatography. The mixture (a 0.2ml sample) showing the optimum reaction conditions was used for attempted separation with a CM-52 cellulose column. The column (1" x 3") was used in the pyridine acetate form, obtained by equilibrating the ion exchange resin with 1M pyridine followed by 0.05M pyridine acetate, pH 4.0. The sample was applied to the column and eluted with a pH gradient from pH 4.0 to pH 6.4 with pyridine acetate buffer.

(viii) Mixtures of lactose (16g) and L-lysine (2g) were dissolved in water (20ml) and adjusted to pH 10.5 with ammonia solution and then heated at either 70°C or 100°C. Samples were taken at 0, 1, 2, 5 and 12 hr. for chromatographic examination. The experiment was repeated using glucose (8g) and L-lysine (2g) or L-lysine HCl (2g) dissolved in water (20ml). Separation of the mixture obtained at 2 hr., 100°C was attempted using CM 52 cellulose as above.

Samples of mixtures containing FL were separated on a column (50cm x 2cm) of Sephadex G50. The mixture (0.5ml) was applied to the column and eluted with water at a rate of 5ml/hr. Fractions were assayed for FL by the Borsook method. Separation of FL-containing mixtures was also attempted using an activated charcoal column. The carbon, after thorough washing with water, was either used alone or was mixed with up to twice its weight of 'Celite' (diatomaceous earth material). Samples were absorbed on to the columns (e.g. length 4cm, diamater 4cm) in a relatively large volume (200µl of reaction mixture syrup in 50ml of water) by allowing the solution to pass through due to its own head pressure. After recycling the eluate, several solvents were then used, by a batch elution technique, for desorbing the compounds e.g. water; 5, 10, 25 and 50% ethanol; 5, 10 and 50% ethanol pH 3 (i.e. 10mM formic acid). The eluates of each desorption were concentrated to a small volume by rotary evaporation and examined by thin-layer chromatography.

Preparation of Radioactively Labelled FL Samples

(i) 1-(ε-N-[U^{14}C]lysyl)-1-deoxy-D-fructose, (FL*):-

Radioactive L-lysine HCl-C14(U) (0.73mg) was dissolved in 50ml water using the ampoule in which the compound was supplied in order to reduce wastage to a minimum. D-glucose (40mg) was added and dissolved with gentle warming. The solution was brought to pH 10 with ammonia solution and taken up into a capillary tube which was then sealed at both ends. The mixture was heated in a water bath at 100°C for 100 min and then applied to Whatman No. 3 chromatography paper as a 30cm streak and developed with pyridine/acetic acid/water (9:1:2) for 36 hr. at room temperature. The position of the FL* was located by reference to unlabelled FL standards and by scanning the chromatogram for radioactivity. The band of FL* was eluted from the paper and the eluate evaporated to dryness. After redissolving in water (100µL) the FL* was applied to chromatography paper as a 15cm streak, separated with methyl ethyl ketone/acetic acid/water (5:3:2) for 24 hr. at room temperature and then located and eluted as before. The paper was allowed to dry thoroughly (24 hr.) before elution to ensure complete disappearance of the solvent. The eluate was freeze-dried, redissolved in water (20ml) and again freeze-dried, to ensure complete erradication of chromatographic solvent (which might have interfered with bacterial growth or enzyme reactions in subsequent experiments). The sample was dissolved in sterile water and stored at -20°C.

(ii) 1-(ε-N-lysyl)-1-deoxy-[U-^{14}C] D-fructose, (*FL):-

FL was prepared in a similar manner to FL (above). The radioactive [U-^{14}C] D-glucose (30mg) was dissolved with gentle warming in water (40 L) and unlabelled L-lysine HCl (15mg) added. The mixture was adjusted to pH 10 with ammonia solution and the reaction and separation of the *FL sample carried out as described for FL* (above).

The specific activities of the radioactive FL samples obtained were FL* = 10mCi/mmol. and *FL = 3mCi/mmol. The radioactive FL samples of lower activity, which were used as substrates in experiments with E.coli and liver preparations, were obtained by first adding the required number of c.p.m. of either the *FL or FL* samples to the reaction vessel and then diluting the compounds with a solution of unlabelled FL to give the required molarity of substrate.

Acid Hydrolysis

Acid Hydrolysis of FL and LL were carried out using small volumes (50µl) in sealed glass capillary tubes heated in a water bath. The hydrolysates were then shaken with 'Amberlite' 1R-4B anion exchange resin (1g) in water (1ml) to de-acidify, concentrated by evaporation and then analysed by thin layer chromatography or galactose dehydrogenase.

Titration Curves

10ml of FL solution (25 mM) was stirred constantly in a beaker (20ml) and was continuously monitored for pH changes using an 'EIL' direct reading pH meter. Titrations at alkaline pH were against 1M-NaOH added in 50, 100 or 200µl quantities. HCl (0.2M and 2M) was used in 10, 20, 50 or 100µl additions for titration at acid pH.

NMR Spectrophotometry

Proton-NMR studies were carried out with a 'Varian' spectrophotometer operating at 60MC and a C^{13}-NMR spectrum was obtained with a 'Bruker Spectrospin' operating at 22.63MHz. In both cases the FL sample (100mg) was examined in deuterium oxide (3ml).

Thanks are due to Dr. D. Gillies for producing the proton spectrum and to Dr. Ira Hughes for producing the C^{13} spectrum.

Microbiological Techniques

1) Materials:

a) The following growth medium (referred to as 'minimal medium') was used in all experiments:

NH_4Cl	5g	
NH_4NO_3	1g	
Na_2SO_4	2g	made up to 1 litre with deionised water. Final pH, 7.2
K_2HPO_4	3g	
$M_gSO_4.7H_2O$	0.4g	

Each salt was dissolved in the order listed. The solution was autoclaved at 15lb.in^2 for 10 min. When glucose was required, sterile glucose solution was added to give a final concentration of 0.2%.

b) Nutrient Agar: Potato dextrose agar (39g) was added to deionised water (1l) and allowed to soak for 15 min, followed by autoclaving at 15lb.in^2 (121°C) for 15 min. After thorough mixing, the agar was poured in to sterile plates.

c) Stock slope-culture: All E.coli samples grown in liquid medium were sub-cultured from a stock culture maintained on a peptone/agar slope stored at 4°C. using a sterilised wire loop

2) General Methods:

a) Sterile Technique: Sterile technique was observed, where necessary, in all experiments involving the use of E.coli cells and extracts. All growth media and substrate solutions were autoclaved at 15lb per square inch (121°) for 15 min. All glassware and centrifuge tubes were sterilised by placing in an oven at 140°C. for 1 hr. Glass cuvettes, Warburg flasks and plastic centrifuge tubes were sterilised by washing in ethanol and were then dried in a previously sterilised oven at 40°C. Sterile pipettes were kept in a sterile metal tube and plugged with sterile cotton wool. All operations involving inoculations, removal of samples or culture transfers were made in a 'Pathfinder' ultraviolet-sterilised cabinet. Conical flasks were plugged with non-absorbent cotton wool and covered with aluminium foil, prior to sterilisation.

FL, as it is labile, was not sterilised by heating. As an alternative, the FL syrup isolated from the purification procedure was placed in a sterile flask and diluted to 1M with sterile water; this was used as a stock solution and was stored at -20°C. It was checked at intervals for microbial contamination by diluting to 10^{-3}M and then spreading on nutrient agar plates which were incubated at 30°C. Solutions sterilised by millipore filtration were forced through the membrane (pore size : 0.46μ) with a sterile glass syringe; this method was suitable for volumes of 1-25ml.

b) Preparation of Overnight Cultures: Samples were taken from the stock slope using a wire loop and subcultured into 25ml. of minimal medium in a 250ml conical flask. The cells were incubated overnight at 35°C. on a mechanical shaker. Aliquots of cultures were used after 12-15 hr. incubation i.e. during the early part of the stationary phase of growth. These cultures are hereafter (see 'Results and Discussion' section) referred to as 'stock cultures'.

Standardisation of the stock culture growth conditions ensured that differences in growth curves in the subsequent experiments were not due to variations in the viability of the cultures.

Fresh stock cultures were prepared for each day of the experimental period with E.coli.

c) Use of Agar Plates: The integrity of the original stock slope-culture was checked at intervals by spreading on to plates of nutrient agar with a wire loop. The plates were then incubated at 30°C. Pure cultures showing colonies of shape and colour typical of E.coli were obtained throughout the experimental period.

Experiments

a) E.coli Growth Curves

A typical bacterial growth curve is shown in Fig. MM1. The increase in bacterial mass per unit volume is a direct function of cell number per unit volume. This in turn is a function of the turbidity of the culture. Growth of colonies was therefore monitored by following the increase of absorbance of cultures at 420nm. Under normal conditions the growth obtained by this method will be sigmoid. The 'log phase' is represented by the exponential phase of cell division and the parameters controlling the growth kinetics of

this region depend on the environment (energy sources etc.) and vary slightly according to the bacterial species studied.

Anaerobic Growth Curves. Standard cultures were set up under sterile conditions in glass spectrophotometer cuvettes, (path length 10mm). The cuvettes were half filled with minimal medium containing 0.2% (w/v) glucose followed by the addition of any other substrate(s) under investigation. Bacterial culture aliquots (0.1ml) were then introduced and the cuvettes filled to the top with glucose-containing minimal medium (final volume 4ml). This ensured thorough mixing of the contents. The cuvettes were then sealed with glass lids. This caused a slight overflow of the contents and hence excluded all air bubbles from the cuvette.

Most of the dissolved air is removed from the minimal medium during autoclaving and no further deaeration was carried out.

The progress of the growth curves at 37^{o} was followed by reading the optical densities of the cuvettes in a Unicam SP 800 continuous recording spectrophotometer. The spectrophotometer was programmed to record optical density values (at 420nm) for up to four cuvettes at regular intervals (usually 15 min). Thus it was possible to run control and experimental growth curves simultaneously and since both were innoculated with equal amounts of the same parent culture, the accuracy of the control was very high. Within a run of four cultures, reproducibility was well maintained so that smooth growth curves were obtained. A pilot experiment of four identical controls showed absorbance differences, between the curves, of not more than 2%. Natural cell sedimentation did not affect readings taken as far as the stationary phase as settling of cells did not occur until the beginning of the dying phase. This was shown when identical absorbance readings were obtained for the cells before and after stirring with a small glass rod. During the experiments the cells were probably adequately circulated by natural micro-convection currents. On occasions a Unicam SP 1800 spectrophotometer was used instead of the SP 800 model. The technique was essentially the same but a greater degree of versatility was offered in relation to scale expansion, chart speed, etc.

Most results in the Results and Discussion section are presented as simplified diagrams of the actual plots (Figs. 2.1-2.8).

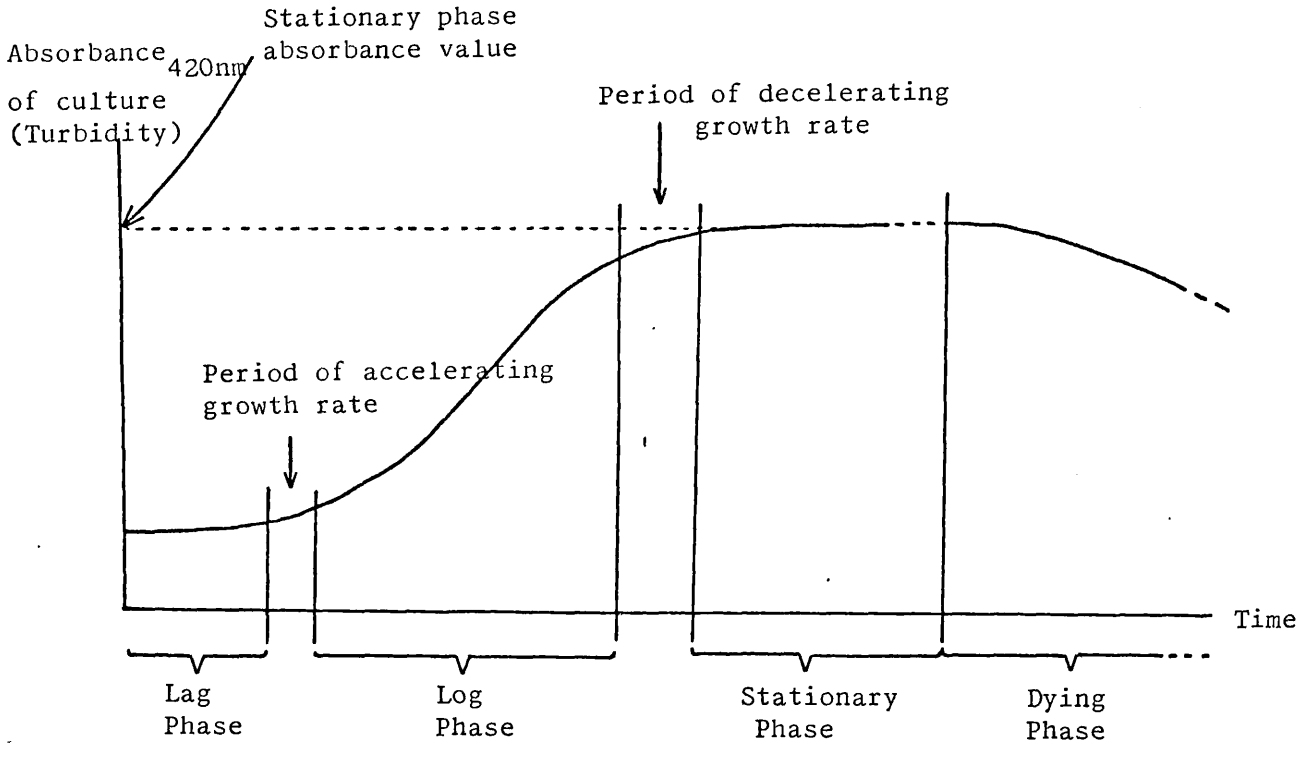

Fig. MM.1 Diagrammatic representation of a typical bacterial growth curve

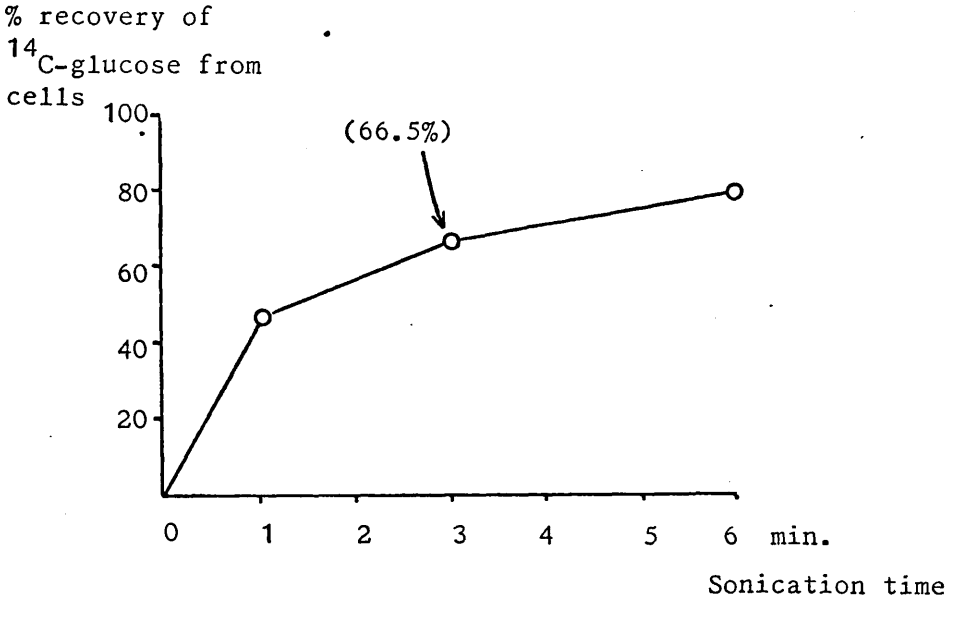

Fig. MM.2 Efficiency of E.coli B cell disintegration by sonication

Aerobic Growth Curves. Standard cultures were set up under sterile conditions in conical flasks. Aliquots (0.2ml.)of Overnight-cultures were added to 100ml. flasks containing minimal medium (10ml.) and 0.2% (w/v) glucose. The cultures were then incubated at 37° on a mechanical shaker. Growth was followed by rapidly transferring the culture (3ml.) under sterile conditions into a sterile cuvette. Absorbance readings at 420nm were made with a Unicam SP 500 spectrophotometer and the samples then quickly returned to the conical flask. Each reading took approximately 3 min but this caused no detectable interference with the growth curve. However, in order to minimise possible effects of the disturbance, readings were taken less frequently (usually at intervals of 1 hr.) than in the anaerobic experiments. A pilot experiment showed that identical controls differed in optical density readings by not more than 4%.

b) Induced Cultures

Cultures of induced cells capable of utilising FL were usually obtained by culturing an inoculum (0.1ml) of a normal stock culture (non-induced cells grown on glucose) in minimal medium containing FL (2.5mM) as the only substrate. The cultures (25ml) were grown in conical flasks (250ml) on a mechanical shaker at 37° and were harvested within 4hr. of reaching the stationary phase (20-25 hr.).

For experiments involving further growth of induced cells, e.g. for the experiments of Parts (II) and (IV) and the respiration experiments of Part (III), media were usually innoculated with 0.1ml aliquots of the above stock induced cultures and then incubated at 37° under aerobic or anaerobic conditions as required. Where the growth of cell numbers was monitored, measurements of cell turbidity at 420nm were made as previously described for non-induced cells. The level of FL in the medium during growth was estimated at suitable time intervals as follows: samples of the cultures (0.5ml) were removed and centrifuged in 5ml tubes at 4°C for 5 min at 5000g. and then 0.2ml of the supernatant solutions were taken and added to 1N NaOH (0.25ml), to stop enzyme reactions, and the Borsook assay then completed as usual. The levels of radioactive substrates in culture media were also estimated by taking samples (0.1ml) of the supernatant solutions (as above), which were then absorbed and dried on to filter paper and, finally, measured in a 'Beckman cpm-100' scintillation counter. The above samples taken for assay from the supernatant solution were removed carefully with a pipette from near the surface and were shown to be essentially free of cells, as a result of this procedure, when similar samples taken during a number of experiments and spread and incubated on nutrient agar plates showed little or no growth of colonies.

In an effort to conserve FL, a method using a reduced culture volume was attempted where the whole culture was centrifuged at 2,500g. and, after each removal of an aliquot of the supernatant for assay, the cells were resuspended and returned to their normal growth conditions. This repeated centrifugation was found to seriously depress the growth rate and, with non-induced cells, to inhibit the onset of induction. This method was not, therefore, used further.

Variations of the general methods used for studying growth and treatment of induced cells depended upon the special requirements of the experiment involved and are discussed below.

For investigations of the permanent nature of the FL-utilising system (see Part (I) p.44) the subcultures taken from Exps. 6 and 7 were made 8hr. and 10hr. respectively after the stationary phase of growth had been reached i.e. 8hr. and 10hr. after the utilisation of FL had been completed. During the last 15 min of this 8hr. and 10hr. period, the cells were centrifuged at 2,500g ($20^{\circ}C$; 5 min) and resuspended in their original volume of fresh minimal medium. Thus, any 'FL-utilising factors' (e.g. an extracellular enzyme) or antimetabolites, present in the external medium as a result of cell growth, were removed.

For investigations of glucose uptake in Exp. 9 (see p.51) a culture (25ml) was grown for 8hr. under identical conditions to those for the other cultures in this experiment. 25µl of glucose solution (2.5M) was then added to give a final concentration of 2.5mM and samples of the supernatant solution were obtained after centrifugation at 0, 5, 10, 20, 30 and 40 min. After suitable dilution with water, the samples were assayed for glucose using the glucose oxidase reagent (see p.100). Supernatant samples were shown to be essentially free of cells by incubation on nutrient agar plates. In a preliminary study of ^{14}C-labelled fructose uptake by cells under similar conditions where radioactivity in samples of the supernatant solution was counted, a reading taken after 1 hr. of incubation suggested a 95% accumulation of this sugar by the cells.

The culture used for the growth curve in Exp. 9 represented by Fig. 2.13 was obtained by resuspending cells grown on glucose (non-induced, early stationary phase, isolated by centrifugation, 5 min. 2,500g) in the original volume of minimal medium containing FL (2mM).

Cultures used for the studies of the accumulation of radioactive FL

(Exps. 14 and 15) and for Exps. 20 to 28 were obtained by isolating the cells from stock induced cultures in late log phase by centrifugation (20°C; 2,500g. for 5 min) and then resuspending the pellet in the original volume of fresh minimal medium. Cultures were then enriched with radioactive FL (usually 2.5mM) and the supernatant solutions assayed for FL and radioactivity at intervals as previously described.

Anaerobic conditions were used for some of the cultures (as denoted in the 'Results and Discussion' section) of the experiments in Part (IV) and also the cell extracts and cell cultures of the experiments in Part (II) (except those of Exp.?). This was effected by incubating the cells or cell extracts, without shaking, either in sealed test-tubes or, if the cells had been isolated by centrifugation, in the centrifuge tubes in which they were resuspended. Conditions were not strictly anaerobic as there was an air-space above the reaction mixtures and the tubes were opened periodically for sampling. The anaerobic cultures of Part (IV) all contained radioactive FL of >100,000 c.p.m. ('high-counts') and hence did not require ion-retardation resin treatment since only 1/10 of the volume was required for detection with 4π radioactive scans of chromatograms. Control samples (i.e. without E.coli cells) for all E.coli experiments both aerobic and anaerobic gave single peaks of FL after either paper chromatographic or electrophoretic separation. Occasionally, when incubation times were greater than 24 hr. a small amount of activity was also observed on the origin.

c) Cell-free Extracts

In the experiments of Part (II) cell-free extracts were prepared by a method shown in Fig. MM.3 where the filtrate obtained at the end of the procedure was used for further investigations. The procedure was designed to be as simple as possible in order to minimise the time taken for the preparation of the extract hence conserving possible enzyme activity. Also, operations were carried out in the cold where possible. It was shown that samples of the filtrates from the millipore filter were free of cells by incubating them on nutrient agar plates at 35°C when no growth of colonies was observed. The fractions referred to as 'Cell debris and unbroken cells' in Fig. MM.3 was used as the crude cell-wall preparation in Exp. 12.

In Exp. 8 four aliquots of 2ml. were taken from the filtrate and placed in sealed tubes. FL solution was added to two of these (final concentration, 2.5mM) and an equivalent amount of water (25µl) was added to the other tubes (controls).

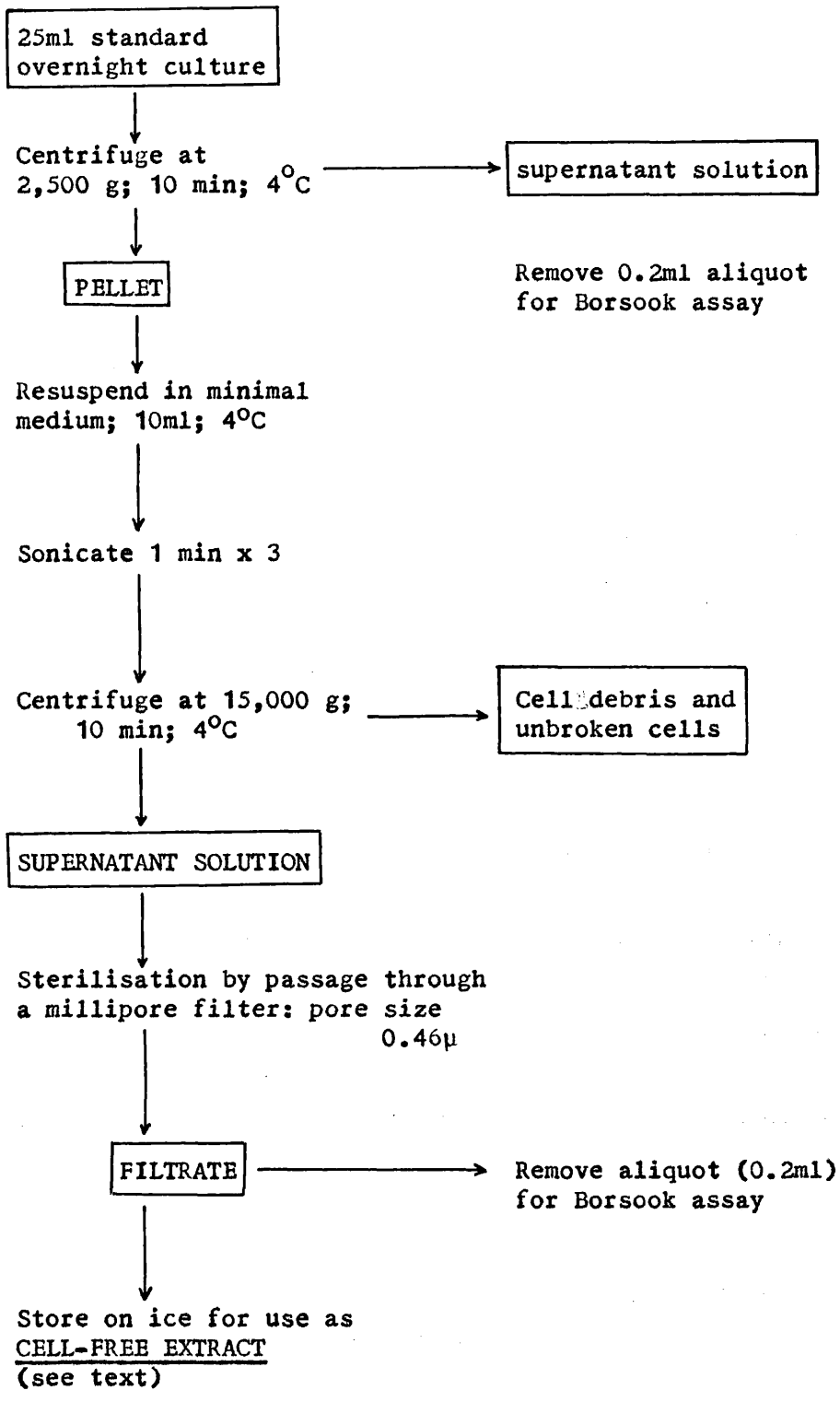

Fig. MM.3 Flow diagram for the preparation of cell-free extracts of E.coli

All tubes were then incubated at 37°C. in a water bath and samples (0.2ml) were taken at 0, 15, 30, 45, 60, 120 and 200 min and estimated by Borsook assay. The experiment was also repeated three times, as above, but where the cell pellet was resuspended in (i) water, (ii) phosphate-citrate buffer (pH 6.0, 0.05M) and, (iii) Tris-HCl buffer (pH 8.0, 0.05M).

Paper chromatographic examination of incubation mixtures was carried out using pyridine:acetic acid:water (9:1:2) solvent. After 200 min incubation periods, mixtures were freeze-dried and after redissolving the powders in water (100µl) an aliquot (20µl) was applied to the paper; this reduced the interference of Rf values by the presence of salt from the media to a tolerable level.

In a modification of the above procedure a more concentrated cell-free extract was prepared by resuspending the cell pellet in 2ml of minimal medium. The results obtained on using this extract for experiments with FL were complicated by the fact that sufficient solid material (probably protein) was precipitated by the NaOH in the Borsook assay to cause rapid flocculation and settling of the colloidal Borsook colour. Approximate readings were obtained by resuspending the colour with an 'automixer'.

The cell-free extract used in Exp. 10 was prepared by the method shown in Fig.MM.3 using a stock induced culture. It should be noted that if the sonication step released enzymes which may have been bound to the surface of the cell, activity could then have passed through the millipore filter either in association with cell wall fragments $<0.46\mu$ in size or as free molecules thereby resulting in contamination of the cell-contents preparation.

When an examination of the radioactive cell contents of cultures used in Parts (II), (III) and (IV) was required, the extracts were also obtained by following the procedure shown in Fig. MM.3. The millipore filtration step was, however, unnecessary since extracts were used immediately and analysed either quantitatively by applying known amounts to filter paper followed by scintillation counting or qualitatively, after freeze drying or rotary evaporation, by paper chromatographic or electrophoretic separation of suitable aliquots. Also, the cell pellet was resuspended in water rather than minimal medium in order to minimise interference of chromatographic separation by the presence of salts.

d) Disintegration of E.coli cells

E.coli cells were broken open by sonication of cultures with a 'PG 100

MSE Ultrasonic Disintegrator' (Model 150 W). The cultures were placed in glass vessels which were cooled in a bath of iced water during operation. Titanium probes operating at 20 KH_z were used and the transducer amplitude was adjusted to 12 microns (peak to peak) for each culture. A 'microprobe' was available for small volumes. The efficiency of the disintegration procedure was estimated as follows. E.coli cells in minimal medium were allowed to accumulate a known amount of radioactive glucose. The culture was then sonicated for three periods of one minute with an interval of one minute between each period to avoid overheating. The culture was then similarly treated for two periods of 1½ minutes' sonication. Samples of the culture were taken after 1, 3 and 6 minutes' sonication and centrifuged immediately (10,000g at 4°C for 5 min). Aliquots were removed from the surface of the supernatant solutions and assayed for radioactivity. The percentages of c.p.m. recovered are shown in Fig. MM.2 (p. 130).

The percentage of cells ruptured may have been higher than that estimated (66.5% for 3 min) if the unbroken cells had re-accumulated radioactivity before they were removed by centrifugation. 3 min was chosen as the most suitable time and was used for all cultures.

A further method of cell disintegration was attempted where cultures were homogenised in a Potter-Elvehjem glass homogeniser (0.005 in. clearance) held in an ice bucket. Treatment consisting of 50 complete strokes of the plunger took 10-15 min and gave an estimated maximum of approximately 50% ruptured cells. The use of this method was therefore discontinued.

Methods for bacteriolysis using lysosyme and/or chemical means were not attempted because the drastic procedures described in the literature (e.g. 64) could well have damaged FL or its products of metabolism.

Rat-Liver Preparations

Liver slices were prepared by removing the liver from a freshly killed rat and cutting thin sections (approx. 1mm) with a razor blade by hand. The slices (1.2g) were quickly put into Warburg flasks containing Krebs Ringer-bicarbonate solution (4ml). The stock solution of this medium was prepared as follows:

	Volumes
0.154M-NaCl -	100
0.154M-KCl -	4
0.11M-$CaCl_2$ -	3
0.154M-KH_2PO_4-	1
0.154M-$MgSO_4$ -	1
0.154M-$NaHCO_3$-	21

The $NaHCO_3$ was treated with CO_2 until acid to phenolphthalein, before mixing, to prevent formation and precipitation of $CaCO_3$. The medium also contained radioactive FL, when required, and unlabelled FL was added to give a final concentration of 2.5mM. Incubation with shaking at 37° was begun as soon as possible. Oxygen uptake was measured manometrically with reference to a thermobarometer. $^{14}CO_2$ was trapped and assayed as previously described (p.118). After incubation, the slices were removed and homogenised in the cold.

Liver homogenates were prepared by crushing a fresh rat-liver in ice-cold Krebs solution with a Potter-Elvehjem homogeniser using 5 complete passes of the teflon pestle (clearance, 0.025 in.). The homogenates were used immediately for incubation studies as described above for slices. Both slices and homogenates were prepared for incubation under sterile conditions.

Finally, all homogenates were centrifuged (3000g, 10 min. 4°C) and the supernatant solutions filtered through an Amicon membrane (allowing the passage of compounds with molecular weight < 1000) and the filtrates retained. Samples (1/10 of the original volume) of the mixtures were then reduced to a small volume by evaporation and applied to Whatman No. 1 paper as a 4cm band and separated with pyridine/acetic acid/water (9:1:2). The papers were scanned for radioactivity as previously described. Neutralised samples containing 'low counts' (< 300,000 c.p.m.) were separated on an ion retardation resin column as described for E.coli (p.120) and the whole sample was then used for the chromatographic analysis.

The disappearance of unlabelled FL added to liver homogenates shaken in Warburg flasks (37°) was estimated after various time intervals: aliquots (0.2ml) of the supernatant obtained after centrifugation (3000g, 5 min. 4°C) of a sample (1ml) of the digests were assayed by the Borsook method and values for control homogenates (no FL) subtracted.

Isolation of Liver Mitochondria. A rat-liver homogenate was prepared using equal weights of liver tissue and ice-cold 0.25M-sucrose: 0.001M-EDTA solution. The homogenate was centrifuged (600g for 15 min, 4°C) to remove cellular debris and cell nuclei. The supernatant was then centrifuged at 9000g for 10 min and the resulting supernatant discarded. The precipitate was gently resuspended, using a rubber policeman, in a volume of ice-cold 0.25M sucrose in 0.001M EDTA solution equal to the discarded supernatant solution and again centrifuged at 9000g (10 min, 4°C). After discarding the washings the pellet was resuspended as before. Aliquots (2ml) of this preparation were added immediately to the following incubation medium:

1. Mixture of ADP (3mM)
   ATP (1mM)
   $MgCl_2$ (15mM)
   Glucose (150mM) - 1ml.
   NAD (0.3mM)
   EDTA (3mM)
2. 0.1M potassium phosphate (pH 7.4) - 0.5ml.
3. 0.25M sucrose in 0.001M EDTA - 0.35ml.
4. 1% Hexokinase solution in 0.1% glucose solution-0.05ml.

Samples of substrate solutions (0.1ml, 0.2M) i.e. sodium hydrogen glutamate, sodium succinate and/or FL, were added as required. Samples of media were obtained by centrifugation of aliquots of the incubation media (0.5ml, 9000g, 5 min, 4°C) and were assayed for FL by the Borsook assay as previously described.

In vitro Enzyme Experiments

α-Glucosidase. The following reaction mixture was incubated at 30°C for 20 min:

0.2ml-FL (final conc. 10mM) in 0.1M phosphate buffer pH 7.0

0.4ml-p-nitrophenyl-α-D-glucoside (final conc. 2mM)

0.4ml-α-glucosidase (0.5×10^{-2} mg/ml)

Control reaction mixtures contained no FL. The reaction was terminated, with the development of the colour due to liberated paranitrophenol, by adding 0.1M-sodium carbonate solution (5ml). The absorbance at 405nm was measured against a blank containing boiled enzyme solution.

The β-fructofuranosidase concentrate (BDH 39020) was diluted 1:10,000 with 0.1M sodium acetate buffer pH 5.0 so that a 10 min incubation period gave an absorbance of approximately 0.6 with the standard glucose oxidase test (54). In order to conserve FL, a micro method was used and the reaction mixture was as follows:

diluted enzyme -(20µl)

10^{-1}M-sucrose in acetate buffer -(20µl)

FL solution (10^{-1}M; 20µl)

Water was used in place of FL solution for controls. The reactions were initiated by mixing the reactants on 'parafilm' spread on a chilled petri-dish and the mixture was taken up into a capillary tube which was quickly sealed, and incubated at 37° for 10 min. The reaction was terminated by placing the tubes in a boiling water bath for 10 min, (samples examined by two-dimensional chromatography were not boiled). The digests were then added to 1ml of water and assayed for glucose with the glucose oxidase test (54) using a boiled enzyme blank. For kinetic studies the concentrations of the above reactants were altered as required.

Reaction mixtures containing β-galactosidase were incubated in sealed capillary tubes (37° water bath) after mixing the reactants on parafilm. The digests for the kinetic studies of LL hydrolysis were as follows: enzyme (0.1mg/ml 0.1M-phosphate buffer, pH 7.5, 20µL), 0.1 M-aqueous LL (5-30µL) and 0.1M-phosphate buffer, pH 7.5 to a total volume of 500L. The reaction mixtures for the kinetic studies with lactase as substrate were as follows: enzyme (0.1mg/ml 0.1M phosphate buffer, pH 7.5, 20µL), lactose (6mg/ml aqueous solution, 25-100µL) and 0.1M-phosphate buffer (pH 7.5) to a total volume of 200µL. LL (10^{-1}M), used as the inhibitor, was added in 10µL (I x1, see Fig. 4.5) or 20µl (I x2, see Fig. 4.5) quantities with the corresponding decrease in the buffer volumes given above. At the end of the reaction time, mixtures were quickly blown from the capillary tubes (and washed out by sucking the liquid up and down a few times) into cuvettes containing the following:

0.1M Tris buffer pH 8.6 - 3.0ml

0.013M-NAD - 0.1ml

Galactose dehydrogenase - 0.02ml.

The reagents were as supplied with the 'Boehringer' Galactose test kit.

The mixtures were incubated at 35°C in an SP 1800 continuous recording spectrophotometer and the absorbance at 340_{nm} of $NADH^+$ (measured against a suitable blank) followed. The absorbance value at which the reaction curve flattened out was recorded. The act of adding the α-galactosidase reaction mixture to the pH 8.6 Tris buffer had the effect of terminating this reaction since the enzyme activity at this pH is negligible. The test volume for the hydrolysis of p-nitrophenyl-α-d-galactoside (pnp gal.) contained the following ingredients: 0.1M-phosphate buffer (pH 7.5, 3ml), pnp gal. (0.1mg/ml aqueous solution, 5μL), α-galactosidase (0.1mg/ml aqueous solution, 50μL), LL (10^{-1}M aqueous solution; 10μL) or water (10μL). The absorbance change at 405_{nm} due to the liberation of p-nitrophenol (37°C) was followed using an SP 1800 continuous recording spectrophotometer.

The method of assay used for Phosphorylase a was based on that described by Whelan (55). The following reagents were present in the test volume: glycogen (5% w/v in water, 0.1ml), glucose-1-phosphate (37mg/ml, 0.5ml), phosphorylase a (ex rabbit muscle, 0.1mg/ml of buffer, 0.9 ml), 0.5M-citric acid-NaOH buffer (pH 6.0, 0.25ml) containing FL as required. The reaction was terminated by TCA addition and the phosphate was measured as described (55) with reference to suitable controls.

For the Acid Phosphatase assay, the method was based on that described by Illingworth-Brown et al (56) and the lysosomes were purified by zonal centrifugation as described by Clarke (57).

The reagents used in the assay of Glutamic Dehydrogenase were mixed in silica cuvettes contained in an SP 1800 continuous recording spectrophotometer and were as follows: 0.87M-imidazole HCl buffer (pH 7.3, 1.5ml), 0.3M ammonium acetate (0.5ml), 0.003M EDTA (0.3ml), 2.4mM $NADH_2$ (0.1ml), 0.05M oxoglutarate (0.2ml), Enzyme (30μg/ml, 0.1ml), buffer containing FL as required (0.1ml). The reaction was started by adding the enzyme and followed by measuring the absorbance change at 340_{nm} at 35°C.

L-Amino Acid Oxidase. The oxidative deamination of FL and tryptophan was carried out using a 'Rank' oxygen electrode apparatus. Changes in oxygen tension at the silver electrode were monitored continuously with a chart recorder and full-scale-deflection represented 0-100% oxygen saturation. Removal of oxygen from solution for calibration purposes was effected by adding a crystal of sodium dithionite. The reaction chamber contained 5ml of Tris/HCl buffer, pH 7.5, pre-saturated with oxygen by continuous shaking with air and containing the enzyme (0.5mg/ml). After stabilisation of the

electrode substrates were added, with capillary tubes, in 20µL quantities through a small hole in the top of the reaction chamber. The reactants were thoroughly mixed by continuous stirring and incubated at 30°C. The trace obtained on the chart recorder showed a background rate of oxygen disappearance (presumably due to reduction at the electrode) and this was subtracted from the values obtained from the oxidative deamination of added substrates.

Lysine Decarboxylase; Assay of Lysine. The following reaction mixtures were used to assay lysine decarboxylase and were incubated in Warburg flasks: enzyme (2mg/ml in 0.2M-phosphate-citrate buffer, pH 6.0, 1ml), lysine (4mg/ml in buffer, 1ml), buffer or FL solution (final concentration 10^{-2}M) in buffer (0.6ml). 5N-H_2SO_4 (0.4ml) was placed in the side arm. The digests were incubated with shaking at 37°C. and the gas volume changes recorded manometrically with reference to a thermobarometer. The acid was tipped in from the side-arm after 50 min and the incubation continued until a steady manometer reading was obtained. The general method was originally described by Gale (65).

The radioactive lysine samples which were eluted from paper chromatograms as described in Section 2, part IV were dissolved in 1ml of water and added to the Warburg flasks containing enzyme and buffer, as shown above. KOH papers were placed in the centre wells to absorb the $^{14}CO_2$ evolved. After a 100 min incubation period followed by acidification and a further 10 min incubation, the KOH papers were recovered, dried and assayed for radioactivity as previously described (see p.118). Control digests (boiled enzyme) using commercial radioactive lysine were set up to determine the percentage of radioactivity reaching the KOH papers due to splashing of the flask contents.

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