STUDIES RELATED TO AMMONIA METABOLISM IN MAMMALIAN TISSUES

A thesis submitted to the University of Oxford
in part fulfilment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY

by

John T. Brosnan, M.Sc. (N.U.Ireland).

St. John Baptist College,
Oxford.

Michaelmas Term
1968

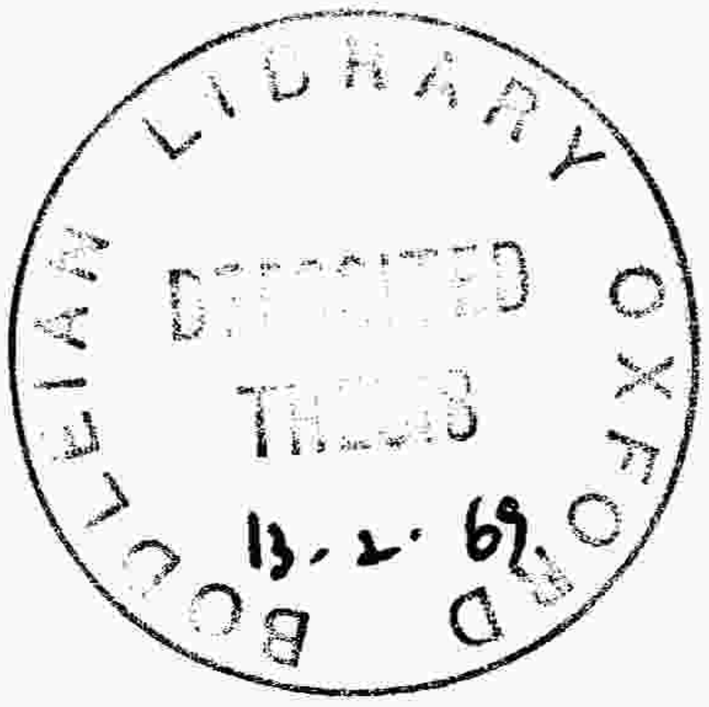


TABLE OF CONTENTS

		Page
ACKNOWLEDGEMENTS		
ABBREVIATIONS		
CHAPTER I	INTRODUCTION	1
CHAPTER II	MATERIALS AND METHODS	15
CHAPTER III	METABOLISM OF AMMONIA AND RELATED INTERMEDIATES IN THE ISCHAEMIC RAT LIVER	30
	Discussion	49
CHAPTER IV	RESPONSE OF RAT LIVER METABOLITES TO AN AMMONIA LOAD	57
	Discussion	88
CHAPTER V	AMMONIA METABOLISM AND THE KIDNEY	101
	Discussion	113
CHAPTER VI	THE CONCENTRATIONS OF AMMONIA IN BLOOD AND TISSUES	124
	Discussion	133
CHAPTER VII	GENERAL DISCUSSION	141
APPENDIX		150
REFERENCES		157
ABSTRACT		(i)

ACKNOWLEDGEMENTS

I am indebted to my supervisor, Dr. D.H. Williamson, for his constant patience and tolerant supervision which have sustained the work of this Thesis.

I am deeply grateful to Professor Sir Hans Krebs, F.R.S., for providing me with the facilities for carrying out my research and for his kindly interest and encouragement.

I have benefitted particularly from the moral support of my colleagues: Douglas Hems, Reg Hems, Patricia Lund, Luisa Raljman, Marion Spry, Dulce Veloso, Richard Veech and Barbara Walker.

I also wish to thank the Agricultural Institute of Ireland for an Agricultural Research Scholarship.

This Thesis was typed by Angela Roberts, to whose patience I owe a special debt.

CHAPTER I

INTRODUCTION

Historical introduction

According to Dr. Johnson (1755), ammonia is described by Pliny & Discorides as a "native salt, generated in those large inns or caravanseras, where the crouds of pilgrims, coming from the temple of Jupiter Ammon, used to lodge; who, in those parts, travelling upon camels, and those creatures when in Cyrene, a province of Egypt, where the celebrated temple stood, urining in the stables, or say some, in the parched sands, out of this urine, which is remarkably strong, arose a kind of salt, denominated from the temple, Ammoniac.". It is, perhaps, appropriate that the etymology of the word should derive from the recognition of ammonia as a urinary constituent since a great deal of biochemical and physiological research has been directed to a study of its excretion in one form or another. Indeed as recently as 1935 ammonia was described in Harrow & Sherwin's 'Textbook of Biochemistry'

ABBREVATIONS

NAD	Nicotinamide adenine dinucleotide.
NADH	Nicotinamide adenine dinucleotide, reduced form.
NADP	Nicotinamide adenine dinucleotide phosphate.
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form.
ATP	Adenosine-5'-triphosphate.
ADP	Adenosine-5'-diphosphate.
AMP	Adenosine-5'-monophosphate.
Pi	Inorganic phosphate.
GPT	Alanine aminotransferase.
GOT	Aspartate aminotransferase.
LDH	Lactic dehydrogenase.
MDH	Malic dehydrogenase.
ICDH	Isocitrate dehydrogenase.
ME	Malic enzyme.
mito.	mitochondrion.
cyto.	cytoplasm.

as "essentially an excretory product, occurring, as nitrogen, on the average of 0.75 gm. per twenty-four hours". Nevertheless, much recent work has shown that ammonia is more than just a toxic end-product of nitrogen metabolism to be excreted as rapidly as possible. In particular, the classic N^{15} studies of Schoenheimer (1949) have amply demonstrated the rapid intracellular metabolism of ammonia. However, some more recent investigators (Brown et al., 1957) have suggested that the concentration of ammonia within liver cells is effectively zero. It is a main thesis of the present work that liver and other animal tissues commonly contain appreciable quantities of ammonia and that these concentrations are important for its normal metabolism.

The study of ammonia metabolism has always been difficult because of the lack of good analytical methods for its determination. In the early methods it was necessary to separate the ammonia from the other constituents in the tissue or blood extract. This was accomplished by steam distillation (Parnas & Heller, 1924) or by microdiffusion, either as described by Conway (1962) or as adapted by Seligson & Hirahara (1951). After this preliminary separation, ammonia was measured either by titration or by colorimetry (principally with Nessler's

reagent). Titrimetric methods can be used because ammonia is a basic substance and can be titrated with acid. Of course titration measures not only ammonia but any volatile base and, since Richter (1937) has shown that a number of volatile bases known to occur in blood (e.g. -butylamine, isoamylamine) can diffuse in a Conway apparatus at about the same rate as ammonia, the use of titrimetric methods may give artifactually high values. While Nessler's reagent does not determine these aliphatic amines as ammonia, the absolute specificity of colorimetric reactions must always be in doubt. Alkali-labile groups in certain nitrogenous substances may cause artifactually high ammonia values in any micro-diffusion method. The recent introduction of an enzymatic procedure (Kirsten et al., 1963), however, dispenses with the necessity to separate ammonia from the other constituents and, in addition, takes advantage of the high specificity of the enzyme (glutamate dehydrogenase) for ammonia.

Another difficulty in the study of ammonia metabolism lies in the lack of a suitable radioactive isotope. There is no radioactive isotope of nitrogen although the stable N^{15} may be employed. This, however, requires a mass spectrometer for its measurement and relatively large

quantities are necessary for an accurate determination. Thus experiments with trace quantities of ammonia are extremely difficult. Tritiated ammonia cannot be used as the tritium will rapidly exchange with the hydrogen atoms of water.

Toxicity of Ammonia

Folin & Denis (1912) first implicated the gut as the major source of blood ammonia when they showed that portal venous blood contained far greater amounts of ammonia than did blood in the systemic circulation. Earlier, Hahn et al. (1893) had shown that force-feeding of meat to dogs in which the portal vein had been anastomosed to the inferior vena cava (Eck fistula) resulted in a characteristic complex of symptoms (dizziness, atoxic gait, anorexia and blindness) eventually leading to stupor and death. As intravenous administration of ammonium salts caused similar symptoms to appear, the hypothesis was formulated that the 'meat intoxication' in dogs with Eck fistulae was due to ammonia. Van Caulaert et al. (1932) showed that patients with cirrhosis of the liver had a decreased tolerance to ammonium salts and correlated the advent of symptoms such as stupor with an elevated blood ammonia level. This was

followed by Kirk's (1936) studies on the effects of oral administration of ammonium salts to cirrhotic patients and control subjects, which demonstrated that these patients could metabolise ammonia normally provided that there was no shunt between the portal vein and the systemic circulation. Thus the concept arose that it is the portacaval shunts, caused by portal hypertension in diseases such as cirrhosis and hepatitis, that are responsible for the elevated blood ammonia levels and central nervous symptoms seen in these diseases rather than biochemical lesions in the liver itself.

There is no satisfactory explanation for the toxic action of ammonia. Beesman & Beesman (1955) have proposed that the formation of glutamine in the brain during hyperammonemia depletes the citric acid cycle of oxoglutarate. It is easy to visualise how such an occurrence could cause coma. Supporting evidence for this theory comes from the experiment of Warren & Schenker (1964) who showed that if mice were treated with methionine sulfoximine (a competitive inhibitor of glutamine synthetase) and subsequently injected with ammonium chloride, the brain ammonia level was almost double that found in normal rats injected with the same dose of ammonium chloride. Nevertheless, the toxicity of

ammonia was much less in the methionine sulfoximine treated group (as judged by mortality). Thus ammonia intoxication may not depend on the mere presence of high cerebral ammonia levels but on the mechanism used to remove it. Schenker et al. (1967) have recently reported that administration to rats of sufficient ammonium acetate to cause drowsiness and coma resulted in large decreases in the ATP and creatine phosphate content of the base of the brain but no changes in cortex. According to Schenker et al. (1967) the neurological basis of many of the symptoms of hepatic coma may be malfunctioning of specific structures in the base of the brain.

Intracellular metabolism of ammonia

Ammonia is the major nitrogenous end product of the catabolism of amino acids in cells. Bessman (1956) has listed the many enzymes that can give rise to ammonia in animal cells. In contrast, there are only three enzymes sufficiently active to be of importance in the removal of ammonia: carbamyl phosphate synthase, glutamine synthetase and glutamate dehydrogenase. The role of carbamyl phosphate synthase in the hepatic synthesis of urea has been well established and glutamine formation is considered to be the principal extrahepatic mechanism for

ammonia removal. The reductive amination of oxoglutarate as a means of ammonia removal has not been adequately studied but experiments in this thesis suggest that it can be of quantitative importance.

Besides the synthesis and excretion of urea, a small fraction (derived mainly from circulating glutamine) of the total excreted nitrogen is disposed of as ammonia by the kidney. This renal ammonia excretion is extremely important for the conservation of cations such as Na^+ and K^+. Smaller quantities of ammonia are excreted in sweat (Brusilow & Gordes, 1968) and minute amounts in exhaled air (Bromberg et al., 1960).

Ammonia may be used anabolically either directly or through the mediation of glutamine. Glutamine serves as a nitrogen donor in many reactions (e.g. formation of the imidazole ring of histidine, biosynthesis of purines, synthesis of glucosamine-6-phosphate, amidation of desamido-NAD to NAD, etc.). Ammonia can directly participate in similar reactions such as the reductive amination of oxoglutarate, the synthesis of carbamyl phosphate (which may be subsequently utilised for pyrimidine synthesis) and the conversion of UTP to CTP.

Equilibria in animal tissues

The recent finding that the reactants of the glutamate dehydrogenase system are close to equilibrium in rat liver (Williamson et al., 1967b) stimulated the investigation, in this thesis, of the relationships between ammonia metabolism and related 'equilibrium enzymes'.

All reactions tend towards an equilibrium position. While it is very doubtful that any real equilibrium exists *in vivo*, many reactions appear to be relatively close to the equilibrium position. To deal with this situation a working definition of 'equilibrium' must be formulated:

consider the reaction

$$A + B \rightleftharpoons C + D$$

The 'mass action ratio' (Γ) measured at any stage during the reaction is $\frac{[C]\,[D]}{[A]\,[B]}$ and is a convenient index of how close to equilibrium the reaction is. At equilibrium $\Gamma = K_{eq.}$. Arbitrary limits may be set within which the reaction is considered to be 'at equilibrium'. In this thesis values of $\frac{\Gamma}{K_{eq.}}$ between 0.33 and 3.0 are taken to represent an intracellular reaction 'at equilibrium'. It is realised that this involves a serious misuse of the term 'equilibrium' but it should be borne in mind that

this is regarded merely as a convenient working definition. It is in this loose connotation that enzyme systems are claimed to be at equilibrium in this thesis.

Establishing an equilibrium in vivo

The approach necessary for establishing that an equilibrium exists *in vivo* depends on the enzyme in question. For instance, in the case of a transaminase, it is only necessary to measure the four substrates in rapidly frozen tissue and compare the mass action ratio to the equilibrium constant. For dehydrogenases an added complication is the fact that there are at least two distinct pools of pyridine nucleotides in cells so that measurement of NAD and NADH will not be meaningful in this context. This difficulty can be overcome if there are two dehydrogenases at equilibrium in the same compartment. For instance, Williamson et al. (1967b) have shown that both glutamate dehydrogenase and hydroxybutyrate dehydrogenase are in equilibrium with the same pool of nucleotides. This was accomplished by freeze-clamping liver and estimating its content of the relevant metabolites (3-hydroxybutyrate, acetoacetate, glutamate, 2-oxoglutarate and ammonia). By this means

one can arrive at an estimate of the ratio $\frac{[NAD]}{[NADH]}^*$ in rat liver mitochondria as follows:

If the following equation represents a typical dehydrogenase:

$$AH_2 + NAD^+ \rightleftharpoons A + NADH + H^+$$

then $\frac{[NAD]}{[NADH]} = \frac{[A][H^+]}{[AH_2]} \times \frac{1}{K_1}$ where K_1 is the apparent equilibrium constant.

This simplifies further (if we assume a pH of 7.0) to $\frac{[NAD]}{[NADH]} = \frac{[A]}{[AH_2]} \times \frac{1}{K_2}$ where $K_2 = \frac{K_1}{10^{-7}}$

Calculations on this basis give a ratio of $\frac{[NAD]}{[NADH]}$ of 7.3 when calculated from the reactants of glutamate dehydrogenase and 7.8 when calculated from the reactants of hydroxybutyrate dehydrogenase in the livers of fed rats. This excellent agreement between the two estimates of the $\frac{[NAD]}{[NADH]}$ ratio is good evidence that both enzymes are in equilibrium with the same pool of NAD and NADH.

The measurement of $\frac{[NAD]}{[NADH]}$ ratios by this indirect method implies certain assumptions:

(a) that the amounts of metabolites present in a perchloric acid extract of frozen-clamped liver removed from an unanaesthetised rat within 10 sec. of cervical dislocation reflect the <u>in vivo</u> content.

(b) that μmoles/g. fresh wt. of metabolite measured in

* [NAD] and [NADH] here refer to the concentrations of NAD and NADH <u>in solution</u> in the compartment where the enzyme is situated. Since the thermodynamic activity of bound nucleotides is zero, these ratios must, perforce, refer to nucleotides in solution. The term '<u>free</u>' nucleotides is commonly used to denote these nucleotides in solution.

tissue is equal to 10^{-3} M/L.

(c) that the total substrates measured in the liver represent the proportions at the enzyme site. Equal distribution of the relevant metebolites throughout the cell best meets this criterion.

(d) certain intracellular properties not amenable to direct measurement must be assumed. These assumptions are based on the best indirect evidence available -

(i) intracellular pH is assumed to be 7.0 in both cytoplasm and mitochondrion,

(ii) intracellular ionic strength is assumed to be 0.25 in both cytoplasm and mitochondrion.

Early workers in this field recognised the possibility that the overall amount of a metabolite measured in a tissue may be quite different from the concentration of that metabolite in various compartments within the cell. Hohorst et al. (1959) used the word 'gehalt' to describe the amount measured in the tissue. This convention will be observed in this thesis - 'gehalt' is translated either as tissue 'content' or tissue 'level'. Whenever intracellular events are interpreted from these measurements, however, one invokes the above assumptions to equate 'content' with 'concentration'.

Enzyme reactions other than glutamate dehydrogenase and

Table I.1 Equilibrium constants of some enzymes at pH 7.0, 38° and I = 0.25

The concentration of water is taken to be unity. In the cases of malic enzyme, isocitrate dehydrogenase and the transaminases K is independent of pH.

Enzyme System	Definition of constant	Value of constant	Reference
Malic enzyme	$\frac{[\text{Pyruvate}][CO_2][\text{NADPH}]}{[\text{Malate}][\text{NADP}^+]}$	3.44×10^{-2} M	Veech (1968)
Isocitrate dehydrogenase (NADP)	$\frac{[\alpha\text{-Oxoglutarate}][CO_2][\text{NADPH}]}{[\text{isocitrate}][\text{NADP}^+]}$	0.91 M	Veech (1968)
Lactate dehydrogenase	$\frac{[\text{Pyruvate}][\text{NADH}]}{[\text{Lactate}][\text{NAD}^+]}$	1.11×10^{-4}	Williamson et al. (1967b)
β-Hydroxybutyrate dehydrogenase	$\frac{[\text{Acetoacetate}][\text{NADH}]}{[\beta\text{-Hydroxybutyrate}][\text{NAD}^+]}$	4.33×10^{-2}	Williamson et al. (1967b)
Glutamate dehydrogenase (NAD)	$\frac{[2\text{-Oxoglutarate}][NH_4^+][\text{NADH}]}{[\text{Glutamate}][\text{NAD}^+]}$	3.87×10^{-6} M	Engel & Dalziel (1967)
Glutamate dehydrogenase (NADP)	$\frac{[\alpha\text{-Oxoglutarate}][NH_4^+][\text{NADPH}]}{[\text{Glutamate}][\text{NADP}^+]}$	2.49×10^{-6} M	Engel & Dalziel (1967)
Malate dehydrogenase	$\frac{[\text{Oxaloacetate}][\text{NADH}]}{[\text{Malate}][\text{NAD}]}$	2.78×10^{-5}	Williamson et al. (1967b)
Aspartate aminotransferase	$\frac{[\alpha\text{-Oxoglutarate}][\text{Aspartate}]}{[\text{Glutamate}][\text{Oxaloacetate}]}$	6.61	Stubbs (unpublished)
Alanine aminotransferase	$\frac{[\alpha\text{-Oxoglutarate}][\text{Alanine}]}{[\text{Glutamate}][\text{Pyruvate}]}$	1.46	Grosman (unpublished)

hydroxybutyrate dehydrogenase have also been shown to be close to equilibrium in rat liver. These include alanine aminotransferase and aspartate aminotransferase (Williamson et al., 1967a), lactate dehydrogenase and cytoplasmic malate dehydrogenase (Hohorst et al., 1959). Veech (1968) has recently shown that the cytoplasmic NADP-specific enzymes, isocitrate dehydrogenase and malic enzyme are also close to equilibrium with a common NADP pool. As CO_2 is a reactant for both enzymes and its intracellular concentration is not amenable to direct measurement, calculations of $\frac{[NADP]}{[NADPH]}$ ratios from these enzymes require an assumed CO_2 concentration. Veech (1968), by measuring the CO_2 pressure in the hepatic vein and assuming that the intracellular pressure was the same, estimated the CO_2 concentration in tissue water to be 1.16mM.

Table I.1 gives the apparent equilibrium constants of some of the enzymes that are known to be close to equilibrium <u>in vivo</u>. They were all measured with low enzyme concentration, at pH 7.0 and at ionic strength, I = 0.25.

Present approach

The concept that glutamate dehydrogenase catalyses

an equilibrium within liver cells served as a starting point for this work. As ammonia is a reactant of this enzyme, the existence of this equilibrium establishes ammonia as an important cell metabolite, the concentration of which could conceivably alter the redox state of the mitochondrial pyridine nucleotide system. The experiments in this thesis were designed to evaluate the role of the pool of hepatic ammonia. Its metabolism was studied in the liver both <u>in vivo</u> and during ischaemia, using rapid freezing techniques. Ammonia loads were administered to rats to define the role of mechanisms other than the urea cycle in the disposal of ammonia and to study the alterations in the redox state of the mitochondrial pyridine nucleotides. The behaviour of equilibrium reactions were also studied in these acute situations since most of the previous work has compared physiological states (e.g. fed versus starved rats) in which the transition period has been days rather than minutes. It was considered that this study of hepatic metabolism during periods of acute perturbation might yield more meaningful information on the response of certain 'equilibrium'enzymes to rapid changes in flux.

Ammonia metabolism in the kidney, especially during acidosis was also investigated. The objects of the study

were to gain some information on:

(a) equilibrium enzymes in kidney, and

(b) the mechanism by which renal gluconeogenesis is stimulated during acidosis.

Finally, experiments were conducted to establish whether the liver maintains an ammonia gradient against plasma and to investigate the mechanism by which this may occur.

CHAPTER II

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, chemicals were of analytical grade purchased from British Drug Houses Ltd., Poole, Dorset.

Coenzymes and nucleotides. ATP.Na_2.$3H_2O$, ADP.Na_3, NAD, NADH.Na_2, NADP.Na were purchased from Boehringer Corporation (London).

Other chemicals. 2-Oxoglutaric acid, oxaloacetic acid, sodium pyruvate and sodium phosphoenolpyruvate.H_2O were obtained from Boehringer Corporation (London). Florisil (100-200 US mesh), dihydroxyacetone, Nessler's Reagent and L-arginine.HCl were obtained from British Drug Houses Ltd.; Heparin (1000 units/ml.) was from Boots Ltd., Nottingham; 'Nembutal', veterinary pentobarbitone sodium B.P. (60 mg./ml.) was supplied by Abbott Laboratories Ltd., Queensborough, Kent. L-Cycloserine (4-amino-3-isoxozolidine) was purchased from Calbiochem, Los Angeles; 5-bromofuroic

acid was supplied by Aldrich Chemical Co. Inc., Milwaukee. Oleic acid (puriss.) was a product of Fluka AG, Buchs, Switzerland. Crystalline bovine albumin (Fraction V) was from Armour Laboratories Ltd., London and was stored dry at 4° until use. Norite (activated charcoal) was purchased from Harrington Brothers, Weir Road, Balham, London.

Enzymes

The following enzymes were obtained from Boehringer Corporation (London): citrate synthase, glutamate dehydrogenase, aspartate aminotransferase, α-glycerophosphate dehydrogenase, malate dehydrogenase, lactate dehydrogenase, hexokinase, adenylate kinase, pyruvate kinase, aldolase, glucose-6-phosphate dehydrogenase, α-glycerophosphate dehydrogenase/triosephosphate isomerase, isocitrate dehydrogenase, enolase, phosphoglyceromutase, phosphoglucose isomerase, phosphoglucomutase.

Glutaminase (type III) and urease (type III) were purchased from Sigma Chemical Co., St. Louis.

3-Hydroxybutyrate dehydrogenase was isolated from extracts of Rhodopseudomonas spheroides (N.C.I.B. 8253) according to the method of Williamson et al. (1962) and further purified by chromatography on DEAE-Sephadex A-50

in a manner similar to that described for the crystalline enzyme (Bergmeyer et al., 1967). The final preparation had a specific activity of 3-7 I.U./mg. protein and contained about 1-2% malate dehydrogenase relative to the hydroxybutyrate dehydrogenase activity. It was virtually free of lactate dehydrogenase and NADH-oxidase activity.

Alanine dehydrogenase was isolated from extracts of Bacillus subtilis essentially according to Yoshida and Freese (1964) except that chromatography on calcium phosphate gel was omitted and the protein solution from the initial ammonium sulphate fractionation was chromatographed directly on DEAE-Sephadex. After stepwise elution with 0.05M phosphate buffer, pH 7.4 and 0.05M phosphate buffer, pH 7.4, containing 0.2M NaCl, the enzyme was eluted with the same buffer containing 0.3M NaCl. The active fractions were combined and the protein precipitated by the addition of solid ammonium sulphate to 90% saturation. Since at this stage the preparation contained no appreciable lactate or glutamate dehydrogenase activities, further purification was not attempted.

Solutions and Glassware

Solutions were made up with glass-distilled water and

neutralised where necessary, using a glass-electrode for large volumes and narrow-range pH indicator paper for small volumes. Dirty glassware was soaked overnight in a solution of 1% 'Pyroneg' (Diversey (U.K.) Ltd., Cockfosters, Hefts.), rinsed thoroughly in tap water, then in deionised water and dried at 105°. Pipettes were rinsed out with tap water in a pipette washer for at least twelve hours, allowed to soak in concentrated H_2SO_4 (technical grade) for a similar period, rinsed out with tap water overnight in a pipette washer and finally rinsed thoroughly with deionised water and dried as above.

Animals

Male rats of the Wistar strain were used. On completion of part of this work rats had to be obtained from another breeding colony. This may explain why the levels of certain metabolites in livers from control rats vary in different experiments. Commercial rat cubes were used as a normal diet. These had the following composition: approximately 15% of protein, 3% of fat and the remainder consisting mainly of carbohydrate plus minerals and vitamin supplements. Except where otherwise indicated injections were via the intraperitoneal route and the

solution to be injected was neutralised to pH 7.

Anaesthesia. Where necessary, this was accomplished either by injection (i.p.) of Nembutal, (60 mg./ml.; 0.1 ml. per 100 g. body wt.) or by passing an ether-air mixture through a plastic box which contained the rat.

Treatment of Tissues for the Determination of Metabolites

Liver. The rats were killed by cervical fracture. A portion of liver was rapidly excised and clamped between aluminium tongs previously cooled in liquid nitrogen (Wollenberger et al., 1960). The time elapsing between dislocation of the neck and deep-freezing the liver was 8-10 sec., although the time required for the transfer of the piece of liver was considerably shorter (1-2 sec.). The frozen liver was pulverized to a fine powder in a mortar, with frequent additions of liquid nitrogen. The powder was transferred to a cooled, tared, plastic centrifuge tube which was then rapidly reweighed. Ice-cold perchloric acid (6% (w/v), 4 ml. per gram powder) was added and the tube contents intimately mixed. The resulting slurry was immediately homogenised in the centrifuge tube with a glass pestle driven by a low speed motor. Denatured protein was removed by centrifugation

in the cold at 30,000 g for 10 min. The supernatant fluid was adjusted to pH 5-6 with 20% (w/v) KOH and, after standing in the cold for 30 min., the precipitate of potassium perchlorate was centrifuged off. The yellow supernatant was then shaken for 30 sec. with Florisil (0.1 g./ml.). This treatment reduced the slow non-enzymic oxidation of NADH observed with untreated samples, possibly because it removed flavins from the solution. The Florisil was removed by centrifugation and the supernatant fluid was used for the determination of metabolites. Florisil was not employed for the determination of some metabolites because of poor recovery of these metabolites. The metabolites concerned for which Florosil was not used were ammonia, oxaloacetate, ATP, ADP, AMP, inorganic phosphate, NAD, NADH and the glycolytic intermediates (except pyruvate).

When isocitrate was to be determined, liquid nitrogen (through which carbon monoxide had been bubbled see Veech (1968)) was added to the frozen powder continuously for about 10 min. and allowed to evaporate. This treatment improved the recovery of isocitrate (Veech, 1968).

For the determination of NADH a separate sample of frozen liver powder was extracted with hot ethanolic

-KOH according to the method of Klingenberg (1963b.).

Other tissues (except brain) were treated exactly as the liver except that occasionally it was necessary to use anaesthesia. Where this was so it is specifically mentioned in the text.

Brain. The rats were decapitated with a large pair of scissors so that the head dropped directly into liquid nitrogen. When it had frozen solid it was transferred to a large mortar containing liquid nitrogen and divided in two by a hammer blow on a deep-cooled chisel. The whole brain was then chipped out with a smaller chisel (also cooled in liquid nitrogen). The frozen brain tissue was ground up and treated in the same way as liver except that 1 mM E.D.T.A. was incorporated into the perchloric acid so as to remove any calcium that may have been accidentally included from chipped cranium.

Blood. Samples of blood were taken, under anaesthesia, from the appropriate blood vessel into a heparinised syringe and immediately added to an equal quantity of 12% perchloric acid in a stout-walled test tube. This mixture was then homogenised using a low-speed overhead drive homogeniser and centrifuged in a bench centrifuge for 10 min. The supernatant was neutralised with 20% (w/v) KOH and, after removal of the potassium perchlorate, was used for the required estimations. It was never necessary to

use Florisil on blood samples as they caused negligible non-enzymic oxidation of NADH.

It was especially difficult to obtain blood from the hepatic vein because of its short length and its proximity to the diaphragm. Two different methods were used and both yielded very similar blood ammonia levels.

(a) The right-hand jugular vein was exposed and freed from surrounding tissue. Two ligatures were placed around it and the anterior one tied. The vein was nicked (but not cut through) just below the tied ligature and a catheter (plastic tubing. PPO 20) stiffened by a thin wire (5 amp fuse wire. 35 SWG) inserted. The catheter was manoeuvered into the superior vena cava, through the heart (right auricle) and into the inferior vena cava. At this stage the wire was removed from the catheter which was worked further down until it entered the hepatic vein and was impeded by the liver. The catheter was then tied in place with the second ligature around the jugular vein and blood was withdrawn into a syringe. Each animal was afterwards killed and the position of the catheter verified.

(b) Hepatic venous blood was obtained directly from the hepatic vein by the method of Rosado et al. (1962) using a hypodermic syringe with a bent needle.

Perfusion medium. 2 ml. of perfusion medium was added to 0.1 ml. 60% perchloric acid. This was then treated in the same way as blood.

Liver Perfusion

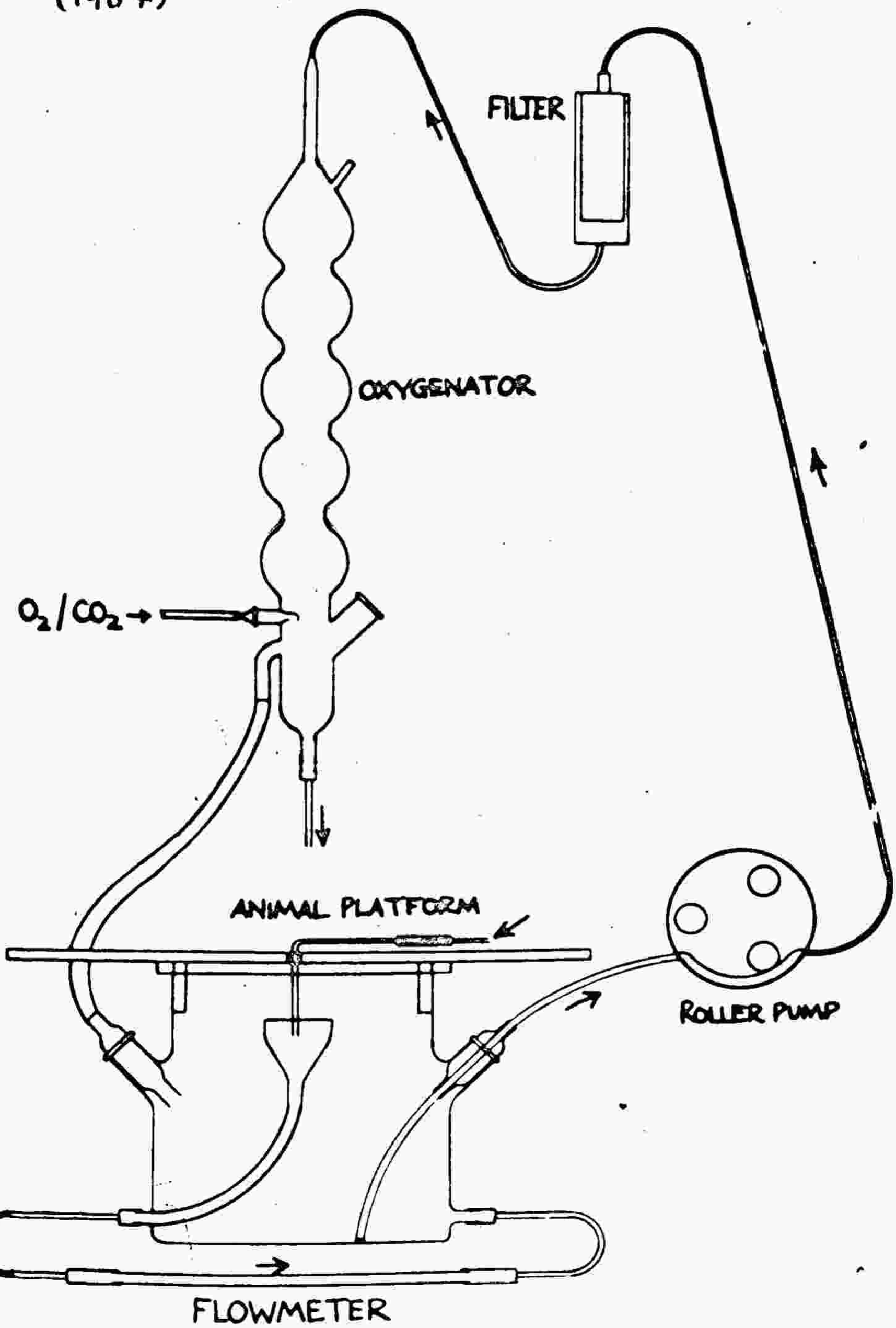
The object of perfusion is to provide a constant flow of oxygenated medium to the liver by the vascular system in order to maintain the physiological function of the liver in isolation from the rest of the animal. The technique of Hems et al. (1966) was used except that the bile duct was not cannulated. The design of the glassware and platform for supporting the animal and the arrangement of the apparatus are shown in Figure 2.1 while Figure 2.2 illustrates the operative procedure. The perfusion medium contained physiological saline (Krebs & Henseleit, 1932), bovine serum albumin powder fraction V and aged (4-5 weeks) washed human blood cells and was prepared as described by Hems et al. (1966). The total volume of the perfusion medium was 150 ml.

Determination of Metabolites

The enzymic methods used for the determination of the metabolites listed below are mainly standard procedures, involving spectrophotometric measurement (at 340 mμ) of

FIGURE II.1

after Ross (1967)



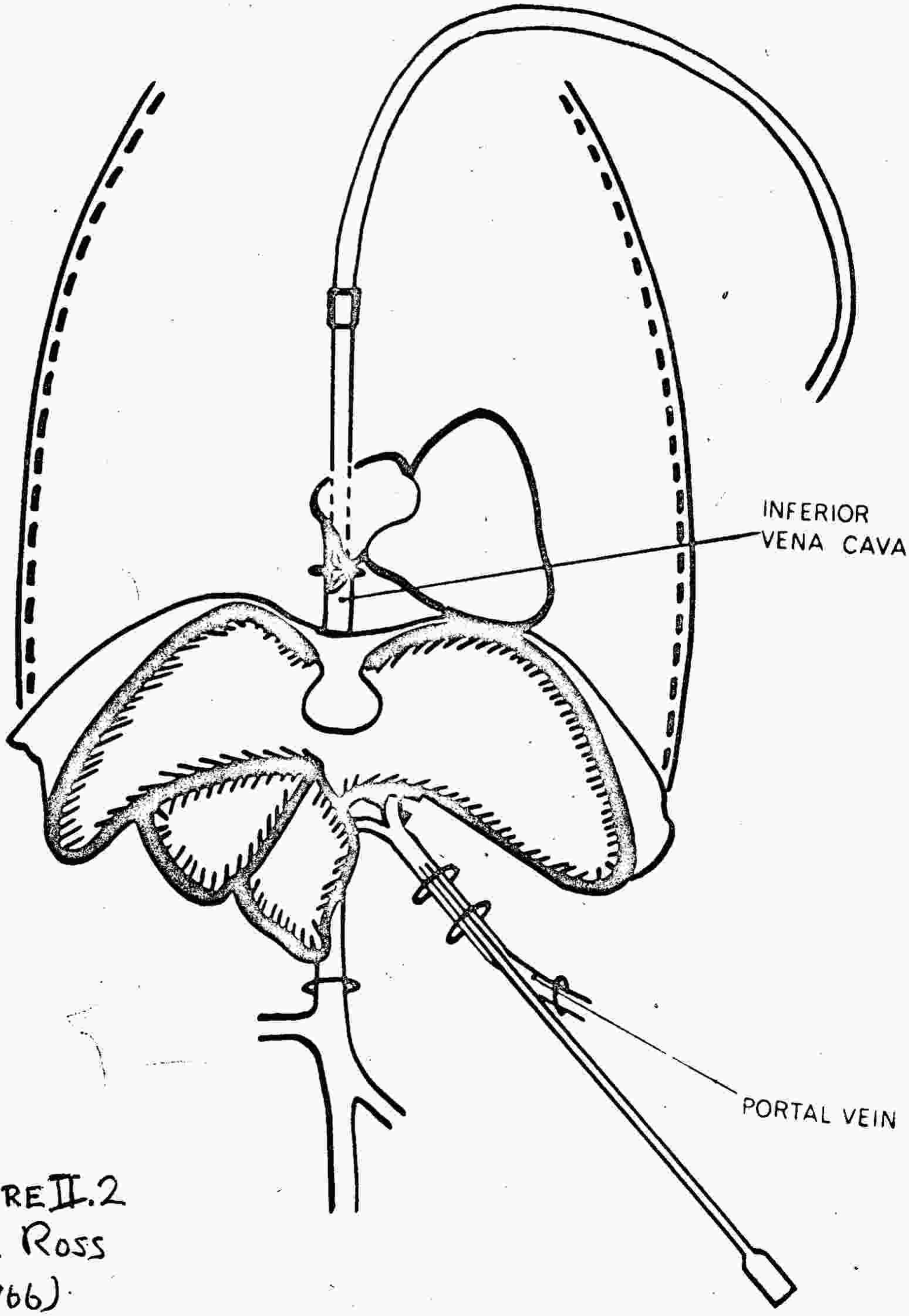


FIGURE II.2
after Ross
(1966).

the oxidation or reduction of nicotinamide-adenine dinucleotides. Extinction was measured either in a Unicam 500 or a Zeiss PMQ II spectrophotometer. The glycolytic intermediates were estimated in a recording spectrophotometer (Beckman D.U. with Gilford modifications and a Servoscribe recorder) in which the scale expansion had been set to a suitable sensitivity. ACETOACETATE and HYDROXYBUTYRATE were determined with hydroxybutyrate dehydrogenase (Williamson et al. 1962). PYRUVATE was measured with lactate dehydrogenase (Bücher et al., 1963), OXOGLUTARATE with glutamate dehydrogenase (Bergmeyer & Bernt, 1963) and OXALOACETATE with malate dehydrogenase (Hohorst & Rein, 1963). To conserve the liver extracts, acetoacetate, pyruvate and oxoglutarate were determined on the same sample by the successive addition of the appropriate dehydrogenases to the cuvettes.

MALATE (Hohorst, 1963a), LACTATE (Hohorst, 1963b) and α-GLYCEROPHOSPHATE (Hohorst, 1963c) were determined with their respective dehydrogenases. CITRATE was determined with citrate lyase (Gruber & Moellering, 1966) and GLUCOSE was estimated by the glucose oxidase method (Bergmeyer & Bernt, 1963b).

L-ALANINE was measured by the method of Williamson et al. (1967a) using purified alanine dehydrogenase.

L-ASPARTATE was measured with aspartate aminotransferase (Pfleiderer, 1963) and L-GLUTAMATE with glutamate dehydrogenase (Bernt & Bergmeyer, 1963). In the earlier experiments L-GLUTAMINE was determined as glutamate after acid hydrolysis. Samples were heated (100°) in 2N H_2SO_4 for 5 min., then cooled in ice and sufficient KOH added to make its final concentration 1N. This was then boiled for 45 min., cooled in ice and neutralised with H_2SO_4. In the later part of this work an unpublished enzymatic method of P. Lund was employed. The assay mixture was as follows:

0.5 ml. sodium acetate buffer, 0.5M, pH 5.0

0.05 ml. glutaminase (Sigma type III. 5 mg./ml., made up in distilled H_2O)

Sample + H_2O to 1 ml.

Incubation was for 1 hour at 37°. This enzymatic method yielded lower liver glutamine levels than acid hydrolysis. This may be due to hydrolysis of glutathione in the latter procedure (P. Lund, personal communication).

ATP was measured by the method of Adam (1963) and ADP and AMP in the assay of Lamprecht and Trautschold (1963). NAD and NADH were estimated by the procedure of Klingenberg (1963 a and b).

GLUCOSE-6-PHOSPHATE, GLUCOSE-1-PHOSPHATE and FRUCTOSE-

6-PHOSPHATE were measured in the same cuvette by combining the method of Bergmeyer & Klotzsch (1963) for glucose-1-phosphate with that of Hohorst (1963d) for glucose-6-phosphate and fructose-6-phosphate. FRUCTOSE-1,6-DIPHOSPHATE and TOTAL TRIOSE PHOSPHATES (dihydroxyacetone phosphate + glyceraldehyde-3-phosphate) were determined in the same cuvette by the method of Bücher & Hohorst (1963). PYRUVATE, PHOSPHOENOLPYRUVATE, 2-PHOSPHOGLYCERATE and 3-PHOSPHOGLYCERATE were determined successively on the same sample by the procedure of Czok and Eckert (1963).

ISOCITRATE was measured fluorimetrically (Beckman ratio fluorimeter) by the method of Siebert (1963) as adapted for fluorimetry by Veech (1968).

UREA was hydrolysed with urease. The assay mixture was as follows:

0.25 ml. tissue extract

0.7 ml. distilled H_2O

0.05 ml. Urease solution (1 mg./ml. Urease (Sigma type III) in phosphate buffer, 0.1M, pH 7.0)

This mixture was incubated at 37° for 1 hour and then 0.5 ml. was transferred to the outer chamber of a Conway microdiffusion unit containing 1 ml. of 0.1N HCl in the inner chamber. Diffusion was initiated by addition of 1 ml. satd. potassium metaborate to the outer chamber and

continued for 90 min. (Conway, 1962) after which time a portion (usually 0.7 ml.) of the contents of the inner chamber was made up to 3 ml. with Nessler's reagent. The colour was measured (after 10 min.) spectrophotometrically at 440 mμ.

INORGANIC PHOSPHATE was measured by the method of Berenblum & Chain (1938) as modified by Martin & Doty (1949).

AMMONIA was measured with $(NH_4)_2SO_4$-free glutamate dehydrogenase by the method of Kirsten et al. (1963). This estimation was initially very troublesome because of the non-enzymic oxidation of NADH. Florisil could not be used to prevent this interference as it removed ammonia as judged by the poor recovery of Standard Solutions. To counteract this difficulty two identical cuvettes were prepared for each sample but enzyme was added to only one. The reaction was followed until the rate of NADH oxidation became equal in the two cuvettes (about 50 min.) at which time the enzymic reaction was completed. The extinction change in the control cuvette during this time was subtracted from the extinction change in the cuvette containing enzyme and the result was used to calculate the ammonia concentration. Later in the work this procedure became unnecessary as a modification

was introduced which prevented blank NADH-oxidation without removing ammonia. This modification consisted of pipetting 2 ml. samples of the neutralised tissue extract into centrifuge tubes which contained 0.1 g. of activated charcoal (Norite), mixing the tubes by means of a vortex mixer and centrifuging at 30,000 g for 30 min. Perchloric acid blanks were treated identically. The supernatant fluid after centrifugation was used for ammonia determination. This procedure reduced the blank NADH-oxidation to negligible proportions and the recovery of known amounts of ammonia added to tissue extracts was between 86 and 93%. Norite was the only charcoal found to be effective for this assay. Other charcoals were not suitable either because they gave poor recovery of ammonia standards or gave large blank values. Special precautions were taken to ensure low ammonia blanks. Particularly important was the use of deionised water in preparing all reagents, making up the NADH solution freshly each day and ensuring that all laboratory stock bottles of ammonia solution were used in distant rooms.

Priorities in metabolite determinations. Because of their relative instability, the following metabolites were determined whenever possible on the same day as the rat was sacrificed: acetoacetate, pyruvate, oxoglutarate,

oxaloacetate, ATP, ADP, AMP and the glycolytic intermediates.

Calculation of Results and Statistical Treatment

Tissue content of metabolites is expressed as µmoles/gram fresh weight ± Standard Deviation (S.D.) and of blood as µmoles/ml. blood ± S.D. The contribution of tissue water to the dilution of extracts has been taken into account in the calculations. Water content of blood was taken to be 0.80 ml./ml. blood and of other tissues to be 0.75 ml./g. fresh wt. (Bergmeyer, 1963).

Differences between means were tested for statistical significance using Student's 't' test as described by Bailey (1959). 'P' values less than 0.05 were taken as an appropriate index of significance for this type of biological work.

CHAPTER III

METABOLISM OF AMMONIA AND RELATED INTERMEDIATES IN THE ISCHAEMIC RAT LIVER

There is no doubt that certain mammalian tissues (e.g. kidney, muscle) produce large quantities of ammonia during anoxia, but conflicting reports have appeared concerning the liver. Brown et al. (1957), estimating ammonia by microdiffusion followed by colour production with phenol-hypochlorite, found a linear increase with time elapsed before freezing the tissue. Extrapolation back to zero time indicated virtually no ammonia in the liver. Similarly, Thorn et al. (1958) also using a microdiffusion technique reported an in vivo liver content of 0.542 µmoles/g. fresh wt. which increased to 1.345 µmoles/g. after 15 min. asphyxia or to 0.85 µmoles/g. after 15 min. anoxia. In direct contrast to these reports, Reichelt et al. (1964) using an enzymatic (glutamate dehydrogenase) technique reported a content of 1.112 µmoles/g. fresh wt. in mouse liver and were unable to find any early

increase post-mortem. Thus the question of changes in the hepatic ammonia content during anoxia cannot be regarded as settled.

Evidence in favour of a greater availability of ammonia during a period of anoxia is the recent report that the alanine content of rat liver increased by over 100% within 2 min. of the liver being made ischaemic (Williamson et al., 1967a). The suggested mechanism for this rise in alanine was an increased supply of pyruvate via anaerobic glycolysis and the provision of the amino group from free ammonia via glutamate dehydrogenase and alanine aminotransferase. Support for this suggestion is the finding that there is an appreciable ammonia content in rat-liver in vivo, which is at near equilibrium with the other reactants of the glutamate dehydrogenase system (Williamson et al., 1967b). In addition, measurements of the hepatic concentrations of the reactants of alanine and aspartate aminotransferases indicate that they are close to equilibrium in vivo (Williamson et al., 1967a). Thus there are mechanisms whereby the intracellular concentration of ammonia may be regulated. However, the ammonia concentration was not measured in these experiments, and alternative though less likely explanations

for the rise in alanine are possible. For example, an increase in protein catabolism during anoxia could lead to the same result.

In view of these uncertainties it was decided to investigate in greater detail changes in metabolite patterns during the transition from the normoxic to anoxic liver with the object of obtaining more information on the origin and fate of ammonia in this situation. In addition, it was considered that the rapid perturbation of metabolic pathways during ischaemia would provide an excellent opportunity for observing the behaviour of the equilibrium systems referred to in Chapter I.

Effect of ischaemia on the content of ammonia and related metabolites in rat liver.

Portions of rat liver were freeze clamped *in vivo* (less than 10 sec.), the connecting blood vessels were then severed and the rest of the liver allowed to remain *in situ*. Further portions of the same liver were freeze clamped 2 and 5 min. later. The frozen liver was then extracted with perchloric acid as described in Chapter II and various metabolites were estimated in the deproteinised extracts. The results

are shown in Figures III.1 to III.7.* The in vivo content of glutamate was 2.35 μmoles/g. fresh wt. and did not alter significantly during ischaemia. Neither did ammonia (initial tissue content, 0.712 μmoles/g. fresh wt.), acetoacetate (0.053 μmoles/g.), aspartate (1.19 μmoles/g.), malate (0.422 μmoles/g.) or pyruvate (0.080 μmoles/g.). The fall in the content of oxoglutarate (initial tissue content, 0.165 μmoles/g. fresh wt.) and the increases in hydroxybutyrate (0.105 μmoles/g.), alanine (0.874 μmoles/g.) and lactate (0.975 μmoles/g.) were significant. The rate of lactate accumulation was 1.05 μmoles/g./min. in the first 2 min. and 0.67 μmoles/g./min. during the next 3 min. The rate of alanine accumulation was almost linear for the 5 min. period at 0.28 μmoles/g./min., confirming the experiments of Williamson et al. (1967a).

Calculation of the mitochondrial $\frac{[NAD]}{[NADH]}$ ratios from the reactants of glutamate dehydrogenase or hydroxybutyrate (as in Chapter I) shows (Figure III.3) excellent agreement at each time interval between the

---

* The results from this experiment and from all other experiments presented in graphical form in this chapter are given in tabular form in the Appendix.

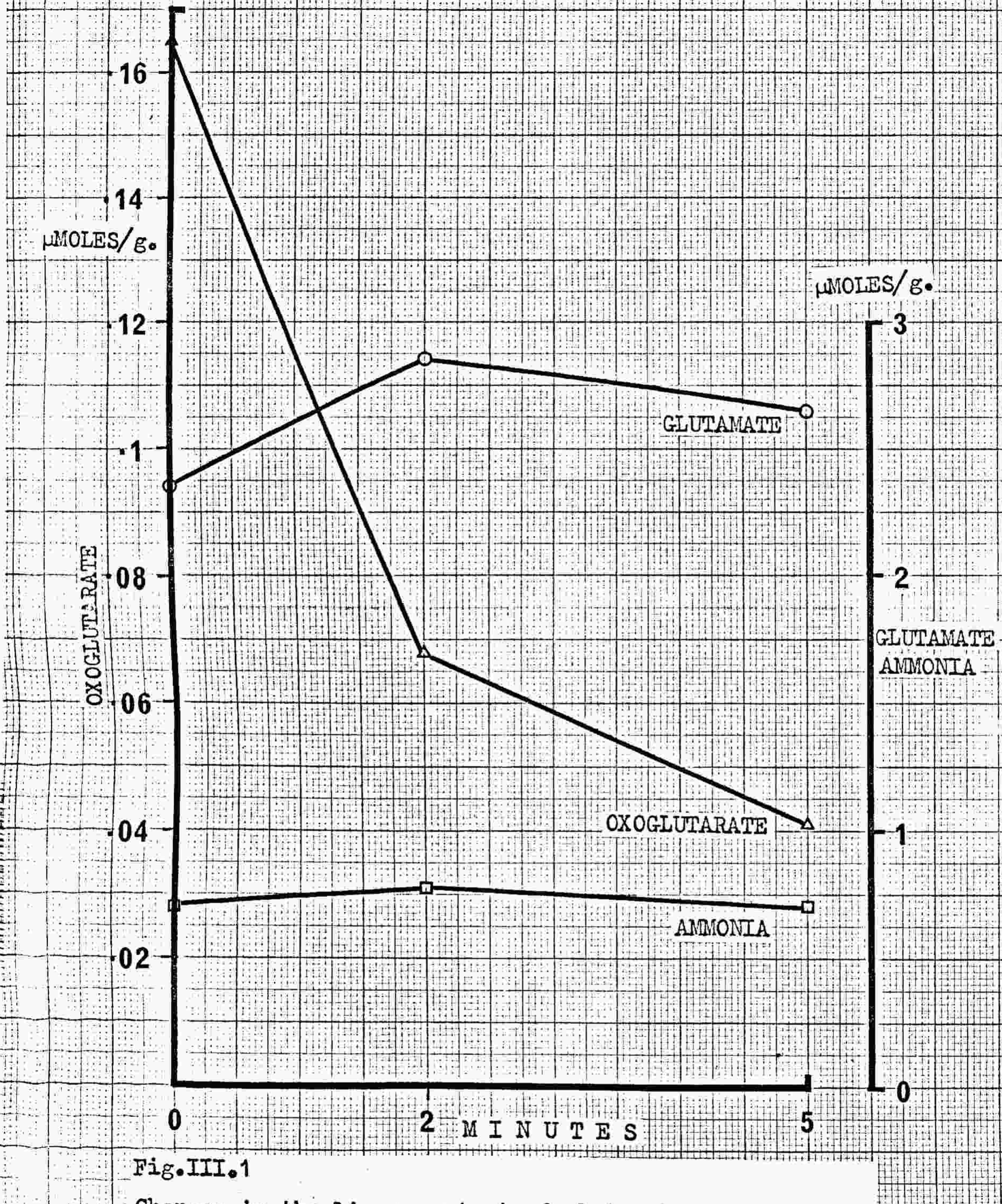


Fig.III.1

Changes in the liver content of glutamate, oxoglutarate and ammonia during ischaemia.

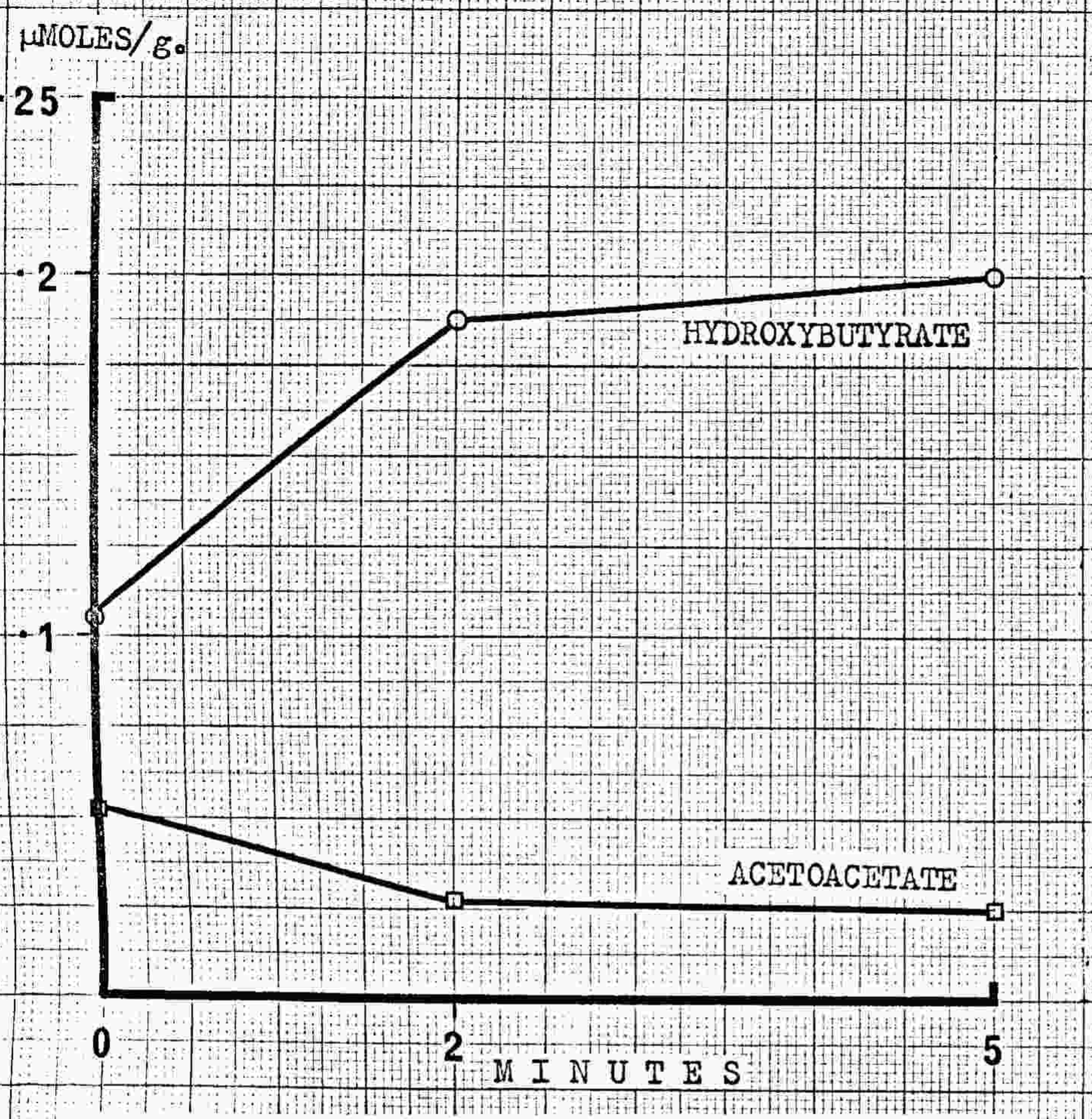


Fig.III.2

Changes in the liver content of hydroxybutyrate and acetoacetate during ischaemia.

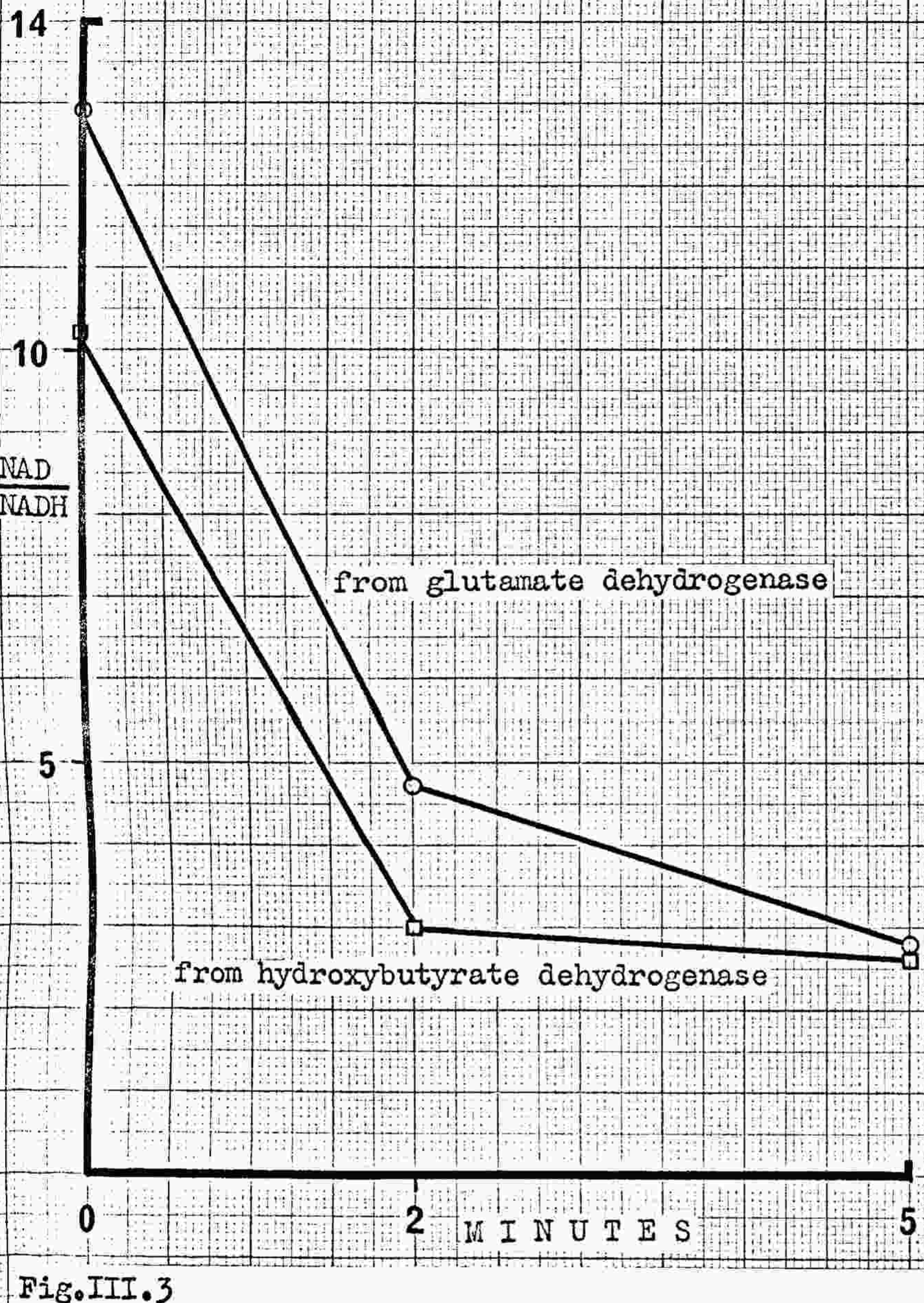


Fig.III.3

Changes, during ischaemia, in the $\frac{NAD}{NADH}$ ratio of liver mitochondria, as calculated from the glutamate dehydrogenase and hydroxybutyrate dehydrogenase systems.

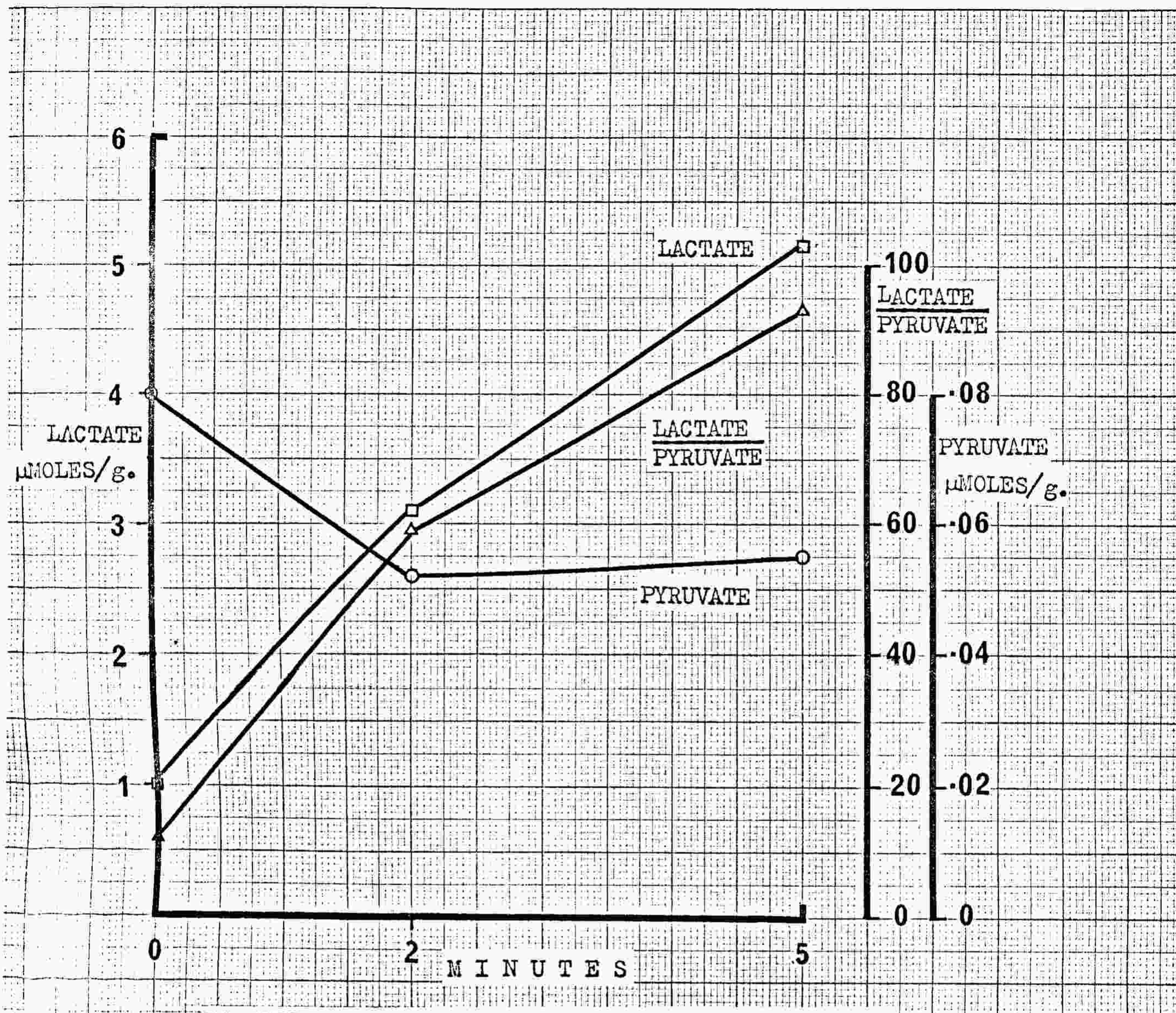


Fig.III.4

The liver content of lactate and pyruvate, and the $\frac{\text{lactate}}{\text{pyrivate}}$ ratio during ischaemia.

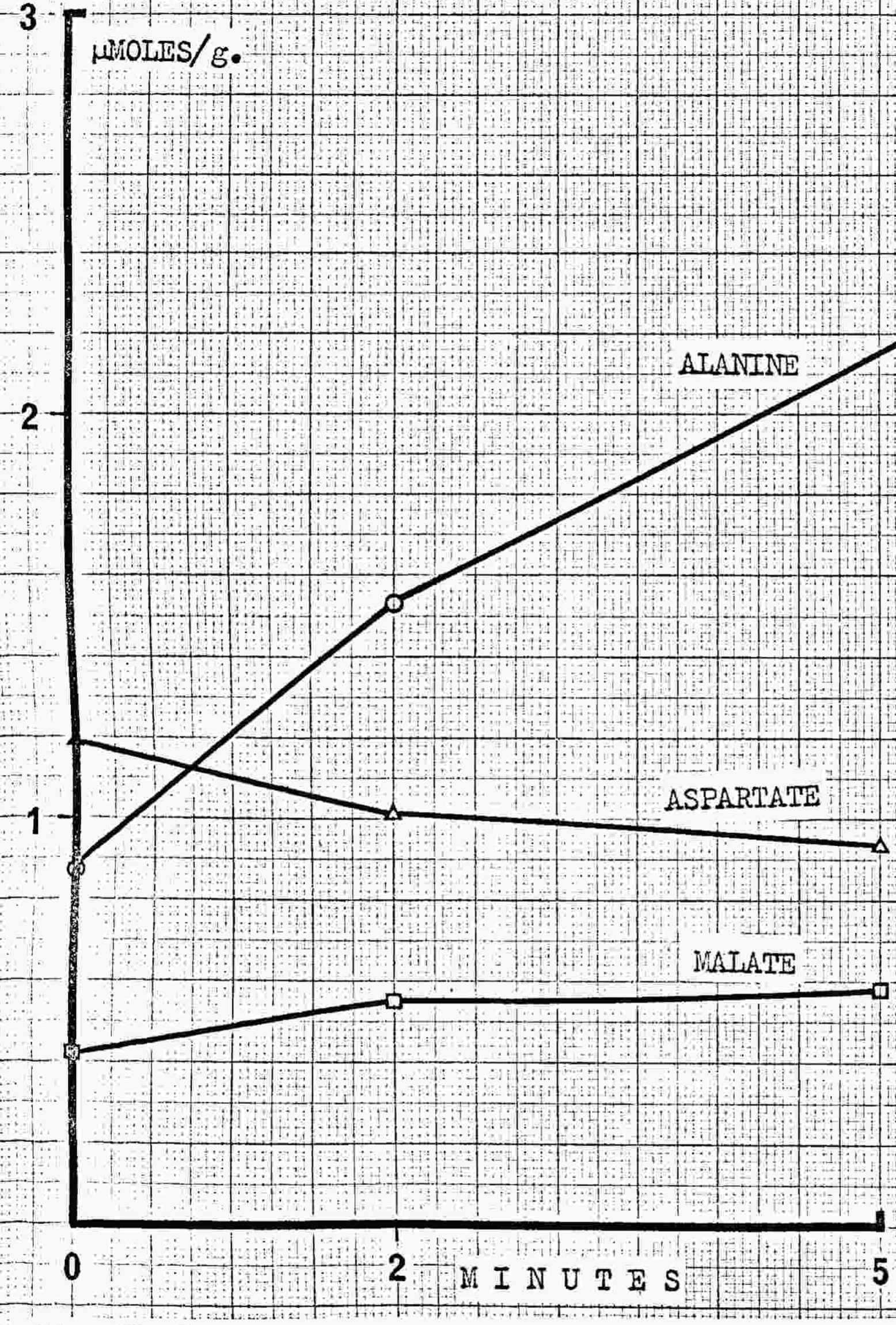


Fig.III.5

The liver contents of alanine, aspartate and malate during ischaemia in rat liver.

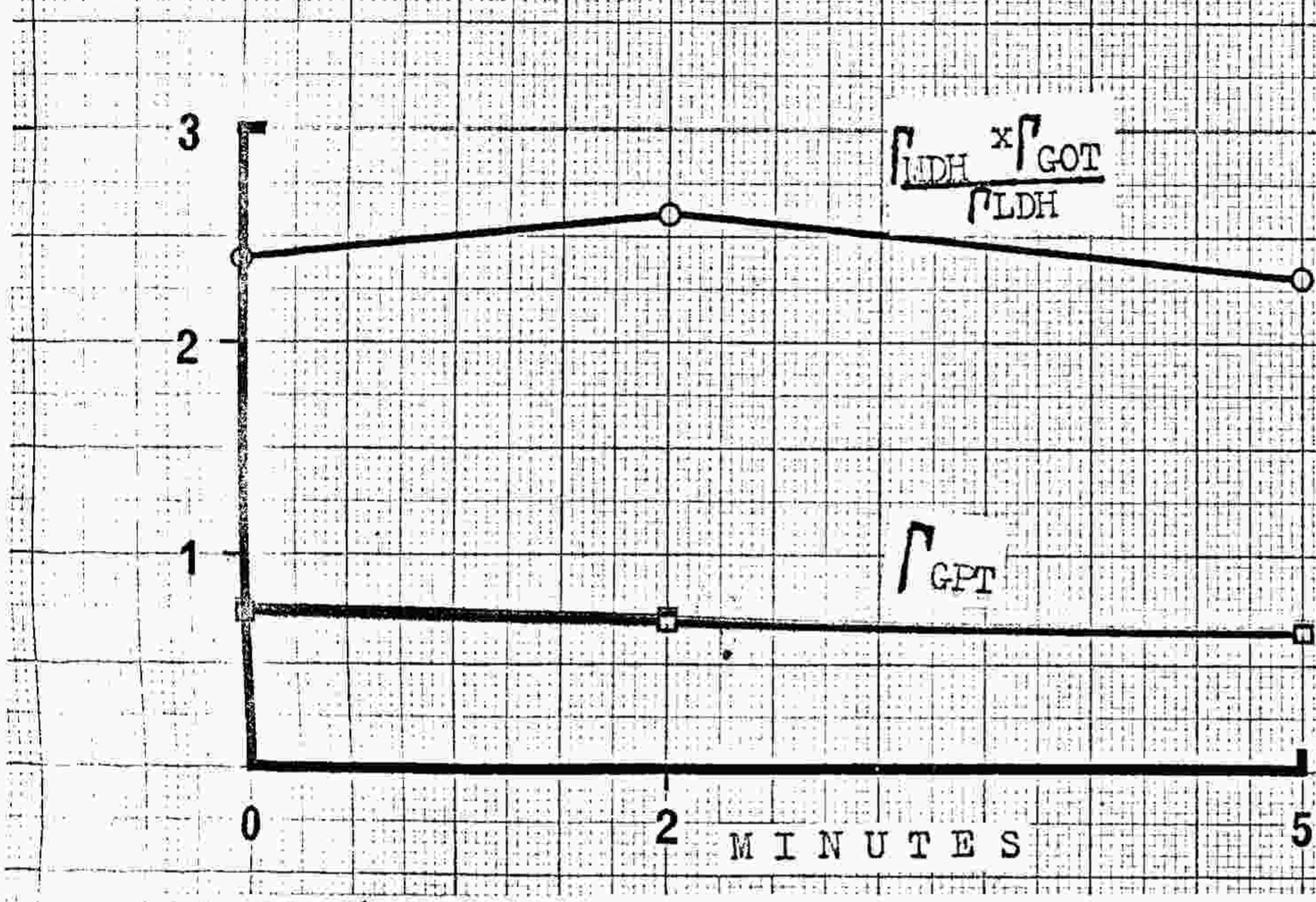


Fig.III.6

The mass action ratio for alanine aminotransferase (Γ_{GPT}) and the indirect ratio for aspartate aminotransferase $\left\{\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right\}$ during ischaemia in rat liver.

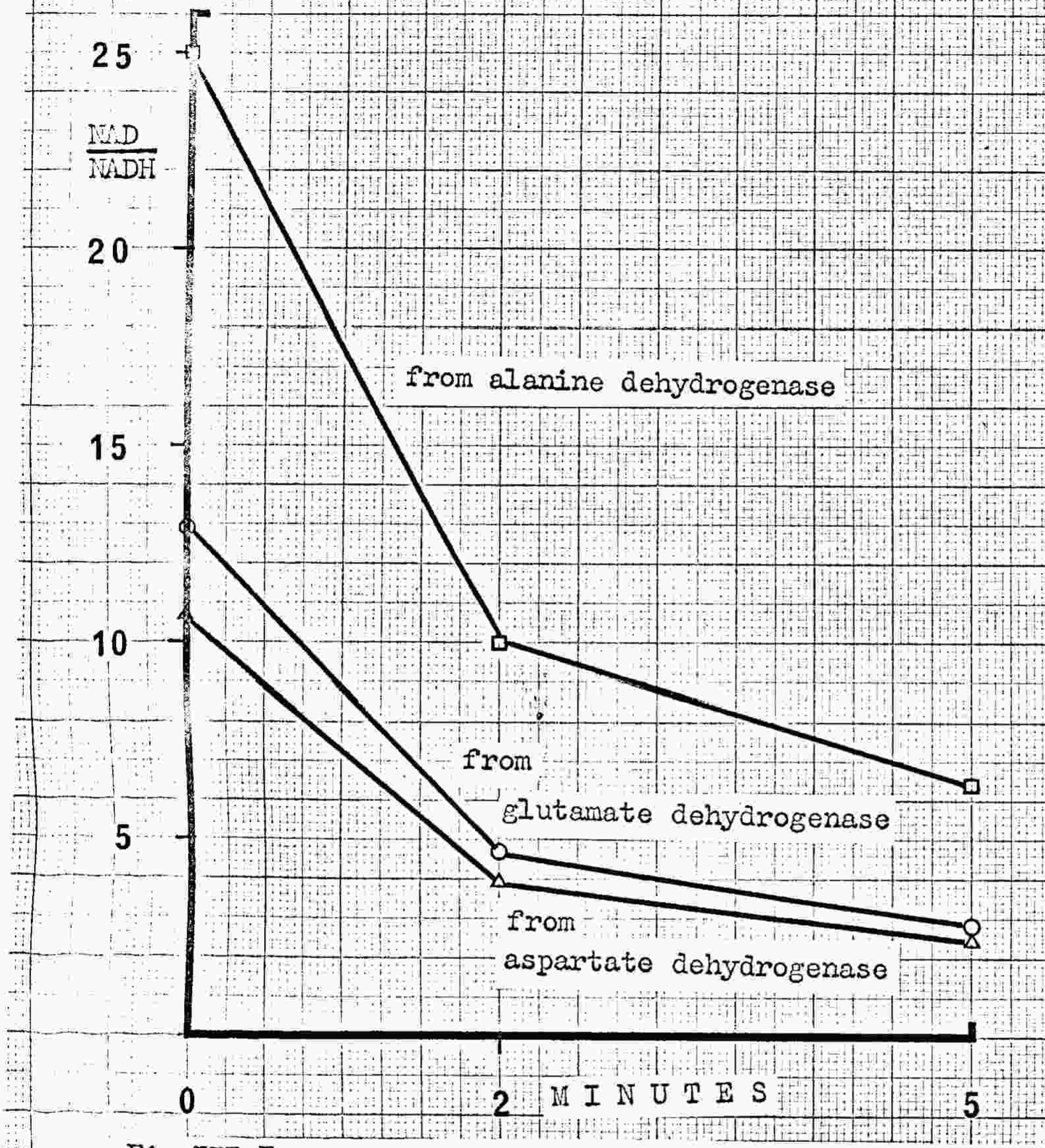


Fig.III.7

The $\frac{NAD}{NADH}$ ratio, during ischaemia, in rat liver mitochondria as calculated from three different enzyme systems.

two enzymes. Thus even during such an acute decrease in the $\frac{[NAD]}{[NADH]}$ ratio both enzymes still remain close to equilibrium. Of considerable interest is the steady value of 0.7 obtained for the alanine aminotransferase mass action ratio (Γ_{GPT}) (Figure IV.6). This is not far removed from the apparent equilibrium constant (1.48) for this system, indicating that alanine aminotransferase is still maintaining near-equilibrium during ischaemia.

Equilibrium of aspartate aminotransferase during ischaemia.

Since, in these experiments, oxaloacetate was not measured, no direct evidence can be provided as to whether aspartate aminotransferase catalyses a near-equilibrium, but indirect evidence strongly suggests that this is so. Thus if the oxaloacetate concentration is calculated (a) on the basis of aspartate aminotransferase being at equilibrium* and (b) on the basis of lactate dehydrogenase and cytoplasmic malate dehydrogenase being at equilibrium with the same pyridine nucleotide pool**, very similar values are obtained at

---

* $[Oxaloacetate] = \frac{[Aspartate]\,[Oxoglutarate]}{[Glutamate]\,K_{GOT}}$

** $[Oxaloacetate] = \frac{K_{MDH}\,[Malate]\,[Pyruvate]}{K_{LDH}\,[Lactate]}$

each time interval:

		in vivo	2 min.	5 min.
*	Oxaloacetate mμmoles/g.	8.66	2.32	1.57
**	Oxaloacetate mμmoles/g.	12.4	3.6	2.2

This may also be explained as follows. If lactate dehydrogenase, cytoplasmic malate dehydrogenase and cytoplasmic aspartate aminotransferase are all mutually at equilibrium then we can derive a composite relationship (without oxaloacetate) to describe the mutual equilibria.

$$\text{pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{NAD}^+ + \text{lactate}$$

$$\text{malate} + \text{NAD}^+ \rightleftharpoons \text{NADH} + H^+ + \text{oxaloacetate}$$

$$\underline{\text{oxaloacetate} + \text{glutamate} \rightleftharpoons \text{oxoglutarate} + \text{aspartate}}$$

Sum: pyruvate + malate + glutamate $\rightleftharpoons$ lactate + oxoglutarate + aspartate.

The apparent equilibrium constant for this composite reaction is

$$\frac{[\text{lactate}]\,[\text{oxoglutarate}]\,[\text{aspartate}]}{[\text{pyruvate}]\,[\text{glutamate}]\,[\text{malate}]} = \frac{K_{MDH} \times K_{GOT}}{K_{LDH}} = 1.65$$

The values for this expression during ischaemia are remarkably constant and close to the theoretical value (Figure IV.6). This indicates that the overall reaction is near to equilibrium. It is known that the reactants of lactate dehydrogenase and malate dehydrogenase behave as if at equilibrium during ischaemia (Hohorst et al.,

1961) - thus the third reaction, aspartate aminotransferase, must also be maintained near to equilibrium in these conditions.

Effective in vivo alanine and aspartate dehydrogenases.

An interesting point emerges from the equilibria catalyzed by glutamate dehydrogenase and the two transaminases. Glutamate and oxoglutarate are common to all three reactions so that if glutamate dehydrogenase is coupled with a transaminase, glutamate and oxoglutarate actcatalytically and do not appear in the overall equation which describes the combined reactions:

e.g. glutamate + NAD^+ + H_2O $\rightleftharpoons$ oxoglutarate + NADH + H^+ + NH_4^+

oxoglutarate + alanine $\rightleftharpoons$ glutamate + pyruvate

---

Sum: alanine + NAD^+ + H_2O $\rightleftharpoons$ pyruvate + NH_4^+ + NADH + H^+

The overall equation is that of an alanine dehydrogenase with andequilibrium constant of

$$\frac{[\text{pyruvate}][\text{NADH}][H^+][NH_4^+]}{[\text{alanine}][NAD^+][H_2O]} = \frac{K_{GluDH}}{K_{GPT}} = 2.61 \times 10^{-6}\,M \text{ at pH } 7.0$$

In the same way glutamate dehydrogenase and aspartate aminotransferase can be combined to obtain an effective aspartate dehydrogenase:

aspartate + NAD^+ + H_2O $\rightleftharpoons$ NADH + H^+ + NH_4^+ + oxaloacetate

with an equilibrium constant at pH 7.0 of 5.85×10^{-5}M. Since the constituent enzyme reactions (glutamate dehydrogenase + aminotransferase) are close to equilibrium it follows that the composite reaction is also close to equilibrium. Hence the liver content of alanine, pyruvate and ammonia or aspartate, oxaloacetate and ammonia may be used to arrive at an estimate of the mitochondrial NAD/NADH ratio (Figure III.7). (For calculation purposes the oxaloacetate concentration used was the mean of the two values obtained on page 35). The fact that the mitochondrial redox state as measured on the basis of 'alanine dehydrogenase' or 'aspartate dehydrogenase' is so similar to that calculated from glutamate dehydrogenase and also that they move in the same direction to similar extents during ischaemia is good evidence that alanine and aspartate are linked to the mitochondrial pyridine nucleotides by a mechanism equivalent to a reversible 'equilibrium' alanine dehydrogenase and aspartate dehydrogenase.

Equilibrium of isocitrate dehydrogenase and malic enzyme during ischaemia.

During the course of these ischaemic experiments

the opportunity was taken to study the behaviour of the cytoplasmic NADP-linked enzymes during ischaemia. Veech (1968) has shown that malic enzyme and isocitrate dehydrogenase are in equilibrium with the same pool of NADP-NADPH and that thus the ratios $\frac{[Malate]}{[Pyruvate]\,[CO_2]}$ and $\frac{[Isocitrate]}{[Oxoglutarate]\,[CO_2]}$ can be used to calculate the cytoplasmic $\frac{[NADP]}{[NADPH]}$ ratio.

Figure III.8 shows the levels of the relevant metabolites, both <u>in vivo</u> and after 2 min. ischaemia, while Figure III.9 illustrates the alterations in the ratios $\frac{[Malate]}{[Pyruvate]}$ and $\frac{[Isocitrate]}{[Oxoglutarate]}$. The changes in oxoglutarate, pyruvate and malate were similar to those in Figure II.1, 4, 5. Isocitrate fell to exactly 50% of the initial value. There was an excellent agreement between the $\frac{[NADP]}{[NADPH]}$ ratio as calculated from the malic enzyme (0.0069) and from isocitrate dehydrogenase (0.0073) in the <u>in vivo</u> condition. It was not possible to calculate the $\frac{[NADP]}{[NADPH]}$ ratio during ischaemia because the CO_2 concentration has certainly altered and is not amenable to direct measurement in this situation. Nevertheless the ischaemic movements of the $\frac{[malate]}{[pyruvate]}$ ratio and the $\frac{[isocitrate]}{[oxoglutarate]}$ ratio in the same direction and to very similar extents suggests that near equilibrium is maintained by these two enzymes. Further calculation justified this

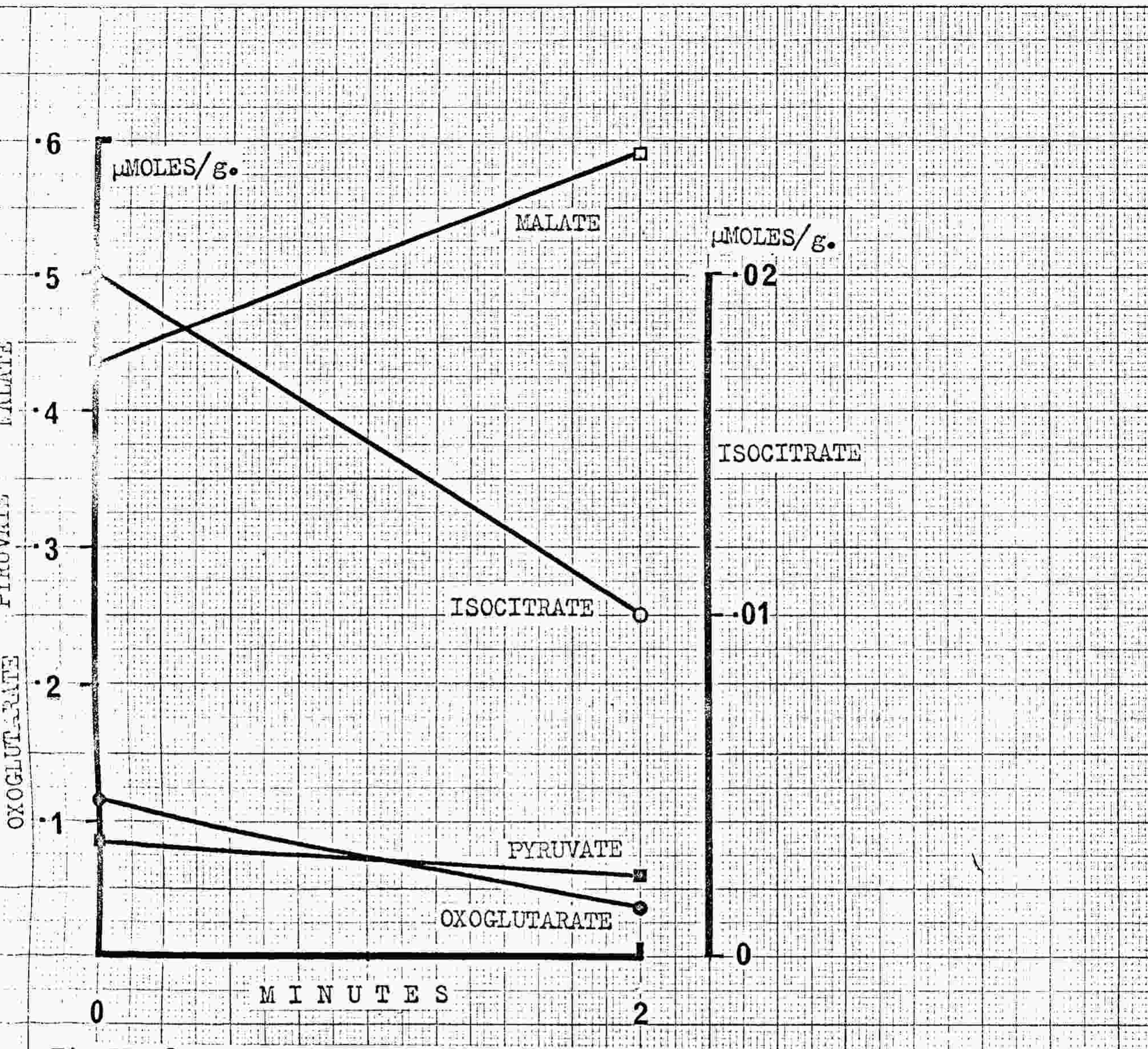


Fig.III.8

The liver content of malate, isocitrate, pyruvate and oxoglutarate during ischaemia.

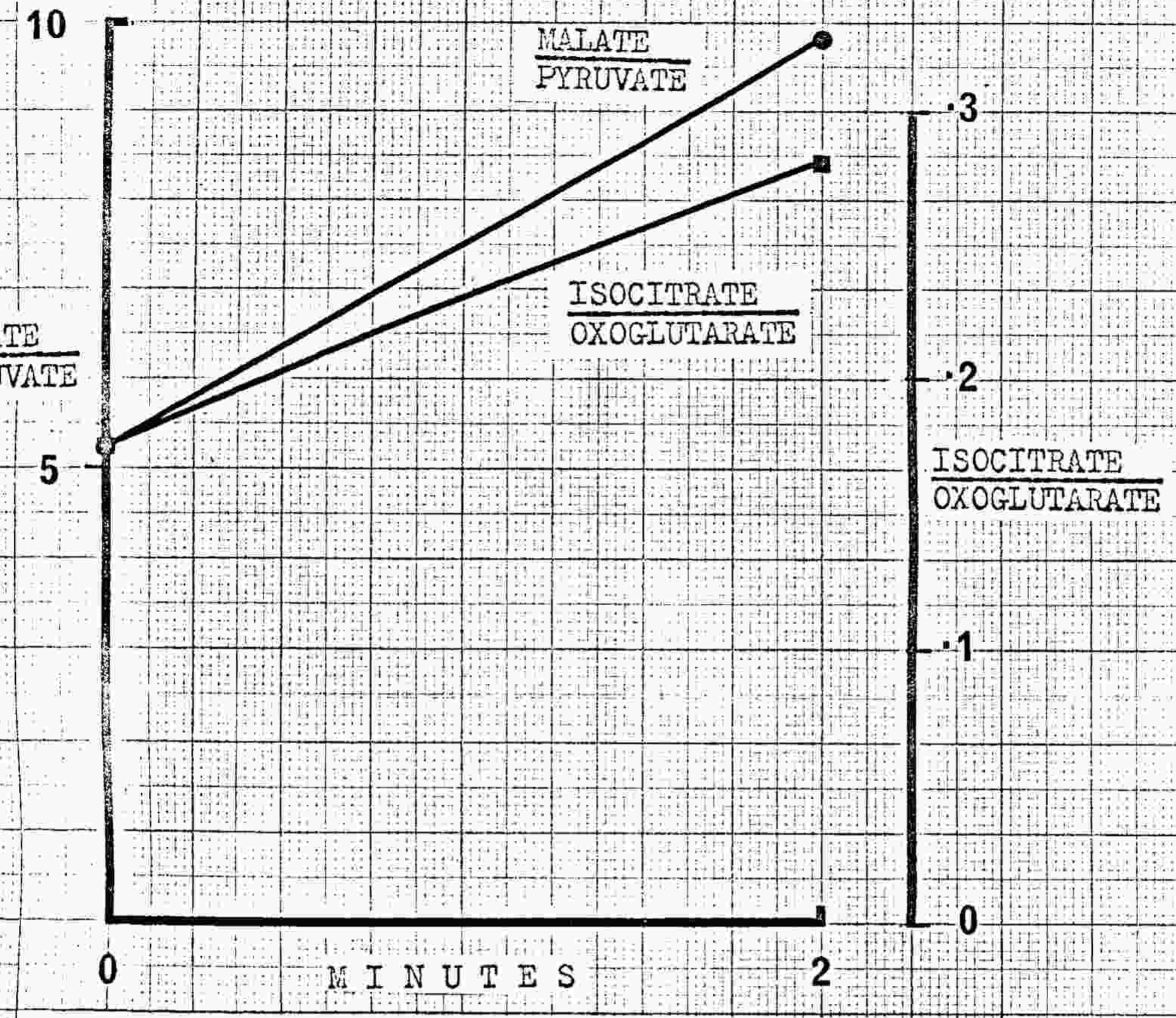


Fig.III.9

The ratios $\frac{\text{malate}}{\text{pyruvate}}$ and $\frac{\text{isocitrate}}{\text{oxoglutarate}}$ in rat liver during ischaemia.

conclusion:

$$K_{ICDH} = \frac{[OXOGLUTARATE]\,[CO_2]\,[NADPH]}{[ISOCITRATE]\,[NADP]}$$

$$\frac{[ISOCITRATE]}{[OXOGLUTARATE]} = \frac{[NADPH]\,[CO_2]}{[NADP] \times K_{ICDH}}$$

$$K_{M.E.} = \frac{[PYRUVATE]\,[CO_2]\,[NADPH]}{[MALATE]\,[NADP]}$$

$$\frac{[MALATE]}{[PYRUVATE]} = \frac{[NADPH]\,[CO_2]}{[NADP] \times K_{M.E.}}$$

If both enzymes are at equilibrium with the same nucleotide pool then:

$$\frac{[MALATE]\,[OXOGLUTARATE]}{[PYRUVATE]\,[ISOCITRATE]} = \frac{K_{ICDH}}{K_{M.E.}} = 27.5$$

The <u>in vivo</u> value for this expression is 30.1 and after 2 min. ischaemia (despite large changes in the liver content of some of the individual metabolites) it is 35.2. Thus these two enzymes tend to remain quite close to equilibrium even during ischaemia.

<u>Mechanism for the ischaemic synthesis of alanine - the effect of cycloserine</u>.

Although there is no apparent change in the ammonia level during ischaemia it is likely that a considerable formation of ammonia occurs, as evidenced by the 2-3 fold increase in the alanine level (Figure III.5). The present explanation for this increase is a mechanism involving

the concerted action of glutamate dehydrogenase and alanine aminotransferase to bring about the reductive amination of pyruvate (arising via anaerobic glycolysis) by free ammonia. Since the ammonia level does not in fact rise and pyruvate actually falls, if this mechanism holds the mass-action 'driving-force' for the synthesis of alanine must come from the reduction of the mitochondrial pyridine nucleotide system. To obtain further evidence for the involvement of alanine aminotransferase and glutamate dehydrogenase in this accumulation of alanine during ischaemia, attempts were made to specifically inhibit these enzymes <u>in vivo</u>.

L-Cycloserine is a powerful inhibitor of alanine aminotransferase (Braunstein et al., 1961-63) and there is some evidence that it is effective <u>in vivo</u> (Otto, 1965) in rats. Therefore L-cycloserine was injected intraperitoneally into fed rats which were killed 60 min. later. Portions of their livers were freeze-clamped immediately and further portions 2 min. and 5 min. later as in the previous experiments. The results from this experiment (Figures IV.10 to IV.13) are consistent with an <u>in vivo</u> inhibition of alanine aminotransferase. Four criteria may be taken to support this:

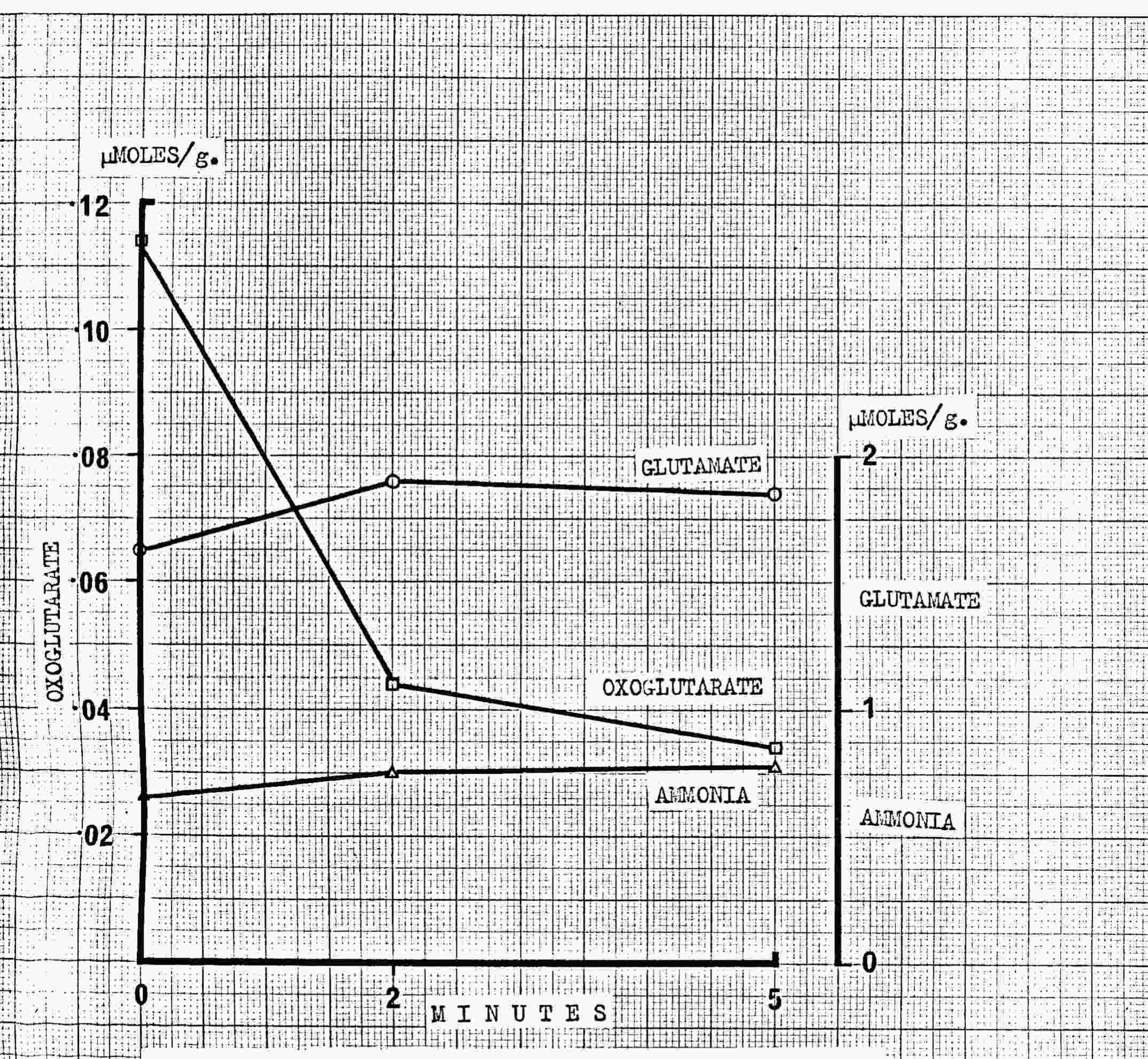


Fig.III.10

The contents of glutamate, ammonia and oxoglutarate, during ischaemia, in the livers of rats that had been injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

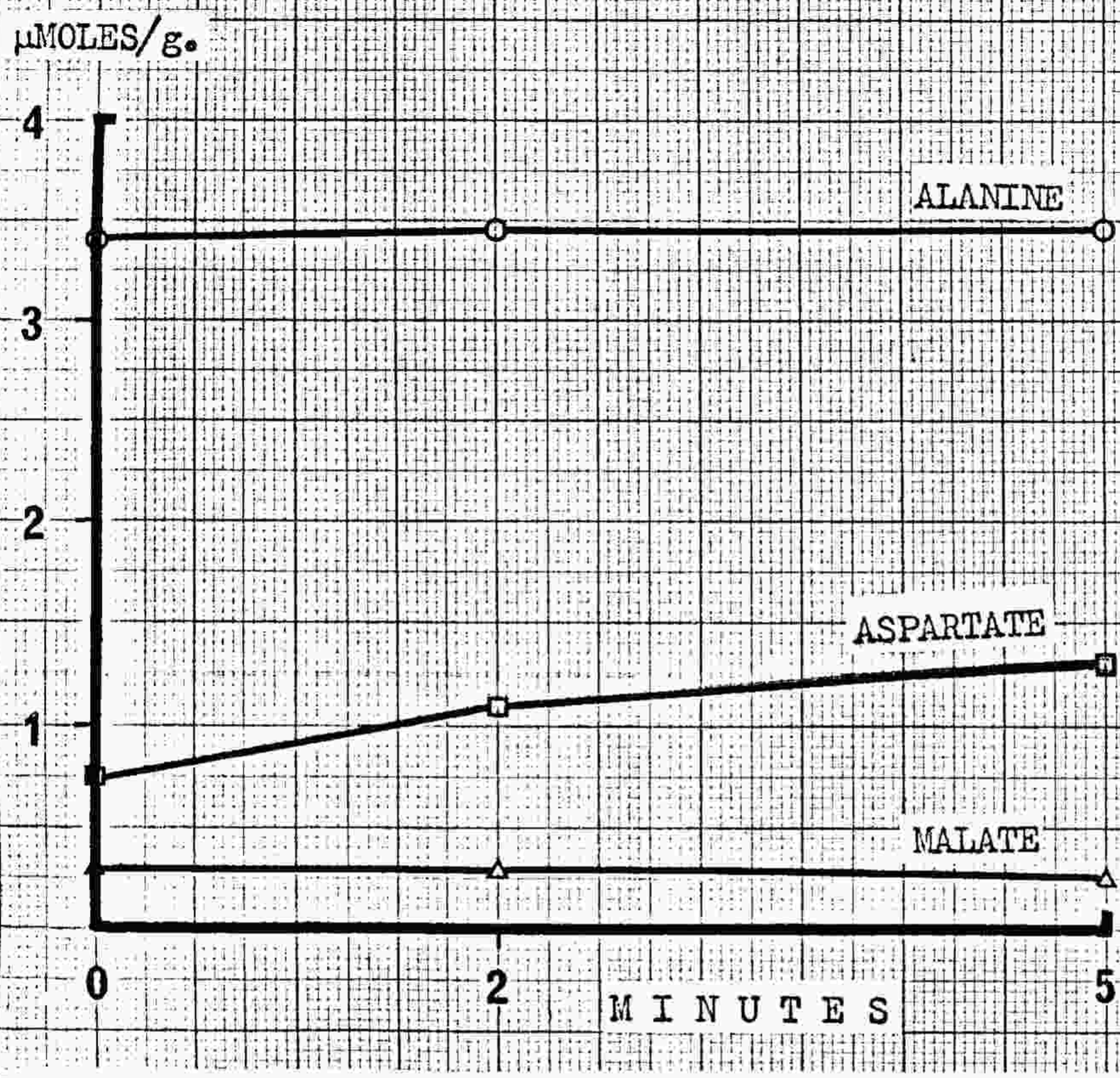


Fig.III.11

The contents of alanine, aspartate and malate, during ischaemia, in livers from rats that had been injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

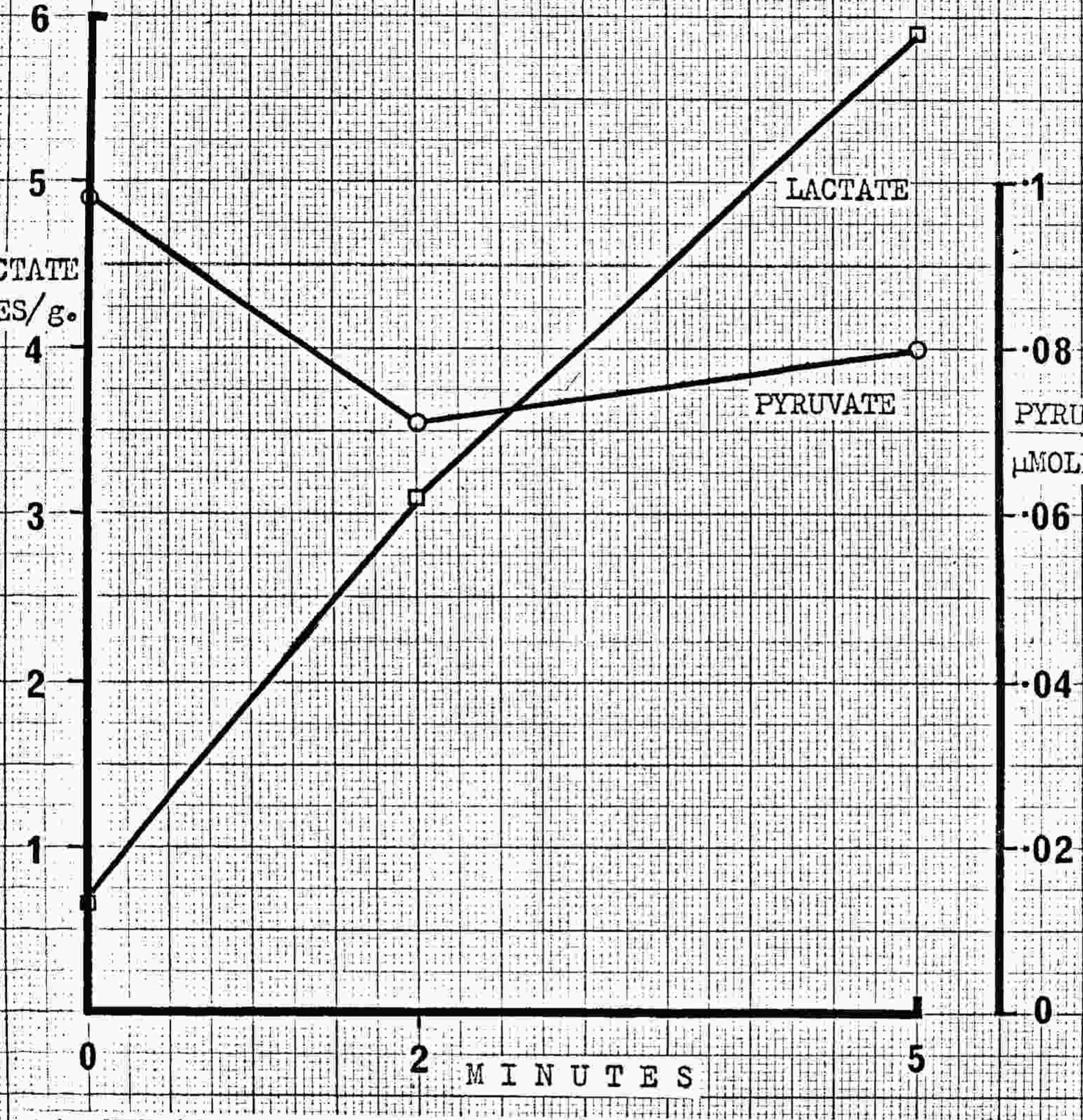


Fig.III.12

The contents of lactate and pyruvate, during ischaemia, in the livers of rats that had been injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.)

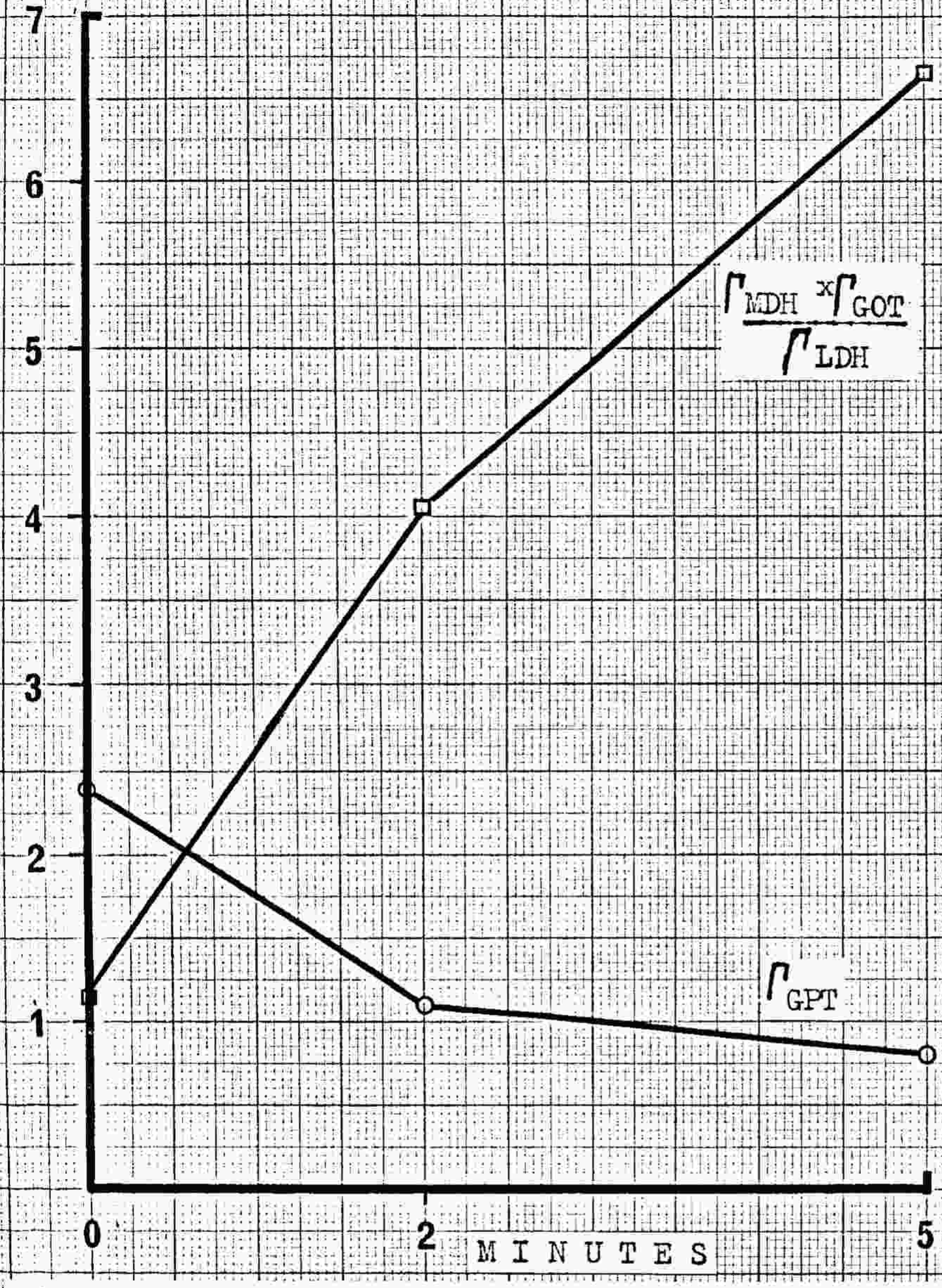


Fig.III.13

The mass action ratio for alanine aminotransferase (Γ_{GPT}) and the indirect ratio for aspartate aminotransferase $\left\{\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right\}$ during ischaemia in liver from rats that had been injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

(a) the steady alanine level in the cycloserine-treated rats during ischaemia (Figure III.11) in contrast to the increase observed in control rats (Figure III.5);

(b) the elevated (4-fold) alanine level (3.40 µmoles/g.) in comparison with that measured in the control experiment (0.874 µmoles/g.);

(c) the erratic changes in the alanine aminotransferase mass action ratio (Figure III.13) in contrast to the constant value obtained in normal rats (Figure III.6);

(d) <u>in vitro</u> assay of the alanine aminotransferase activity of the livers of these cycloserine-treated rats demonstrated a 70-75% inhibition.

The failure of hepatic alanine to rise in the cycloserine-treated rats positively implicates alanine aminotransferase in its formation in the normal rats, and excludes the possibility that it arises from increased protein catabolism.

Although the alanine aminotransferase was unable to remove ammonia in this situation there was still no significant increase in the liver ammonia content. There was, however, almost a 2-fold increase in the aspartate content, demonstrating that aspartate aminotransferase can take over, to a certain extent, the role of alanine

aminotransferase in the removal of ammonia. The amount of nitrogen incorporated into aspartate (0.6 μmoles/g./5 min.) was less than half of that incorporated into alanine (1.4 μmoles/g./5 min.) in the control experiment. Since ammonia accumulated in neither experiment there must either be some other ammonia-binding mechanism operative in the ischaemic L-cycloserine-treated rat liver or else there is a smaller release of ammonia in this situation.

The other metabolites behaved qualitatively during ischaemia as they did in the control experiment (Figures III.10 to III.12) except for malate which showed a tendency to fall as opposed to the non-cycloserine treated experiment when it was elevated. This probably reflects, in the first case, the decrease in aspartate during ischaemia and in the present experiment the rise in the aspartate level. The composite expression $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ also appears to go out of equilibrium during ischaemia.

<u>An attempt to inhibit glutamate dehydrogenase in vivo.</u>

5-Bromofuroic acid is a powerful <u>in vitro</u> inhibitor of glutamate dehydrogenase (Baker et al., 1960; Caughey et al., 1957) and was therefore employed in an

attempt to inhibit the enzyme in vivo. A fed rat was injected intraperitoneally with 1 ml. of an aqueous 5-bromofuroate solution (5 mg./ml.) and 40 min. later the rat was killed and a portion of its liver freeze-clamped immediately. A further portion was freeze-clamped 5 min. later. Alanine was measured in both tissue samples and was found to rise normally - from 1.18 μmoles/g. fresh wt. at time zero to 2.51 μmoles/g. after 5 min. ischaemia. Thus, either glutamate dehydrogenase is not involved in the ischaemic synthesis of alanine in rat liver, or 5-bromofuroate is not an effective in vivo inhibitor. The latter possibility is thought to be much more likely.

The origin of the ammonia in ischaemia.

The ammonia arising during ischaemia is likely to be derived in two ways:

(a) decreased availability of ATP will lower the activity of the urea cycle with consequent diversion of ammonia to non-energy requiring pathways (e.g. formation of alanine);

(b) increased catabolism of certain nitrogen-containing metabolites.

It is extremely difficult to assess the contribution

of source (a). In the case of source (b), some of the nitrogen-containing compounds which can give rise to ammonia are amenable to quantitative measurement. These include glutamine which can be deaminated by glutaminase to ammonia and glutamate, and the adenine nucleotides, since AMP can be deaminated by adenylic acid deaminase. Consequently these metabolites were measured during ischaemia (Figure III.14) to see whether a decrease in their content could explain the source of ammonia. There were small falls in the levels of both glutamine and total adenine nucleotides - although only the latter was significant. It is therefore difficult to evaluate with any confidence the extent of their contribution to the ammonia incorporated into alanine during ischaemia.

Figure III.15 shows a remarkable constancy in the adenylate kinase mass action ratio during ischaemia. This is treated in detail in the Discussion to this chapter.

<u>A possible effect of ether on the relative amounts of adenine nucleotides in the liver</u>.

The <u>in vivo</u> $\frac{ATP}{ADP}$ ratio reported in Figure III.14 is 2.15. Many authors have obtained values of over 3. The

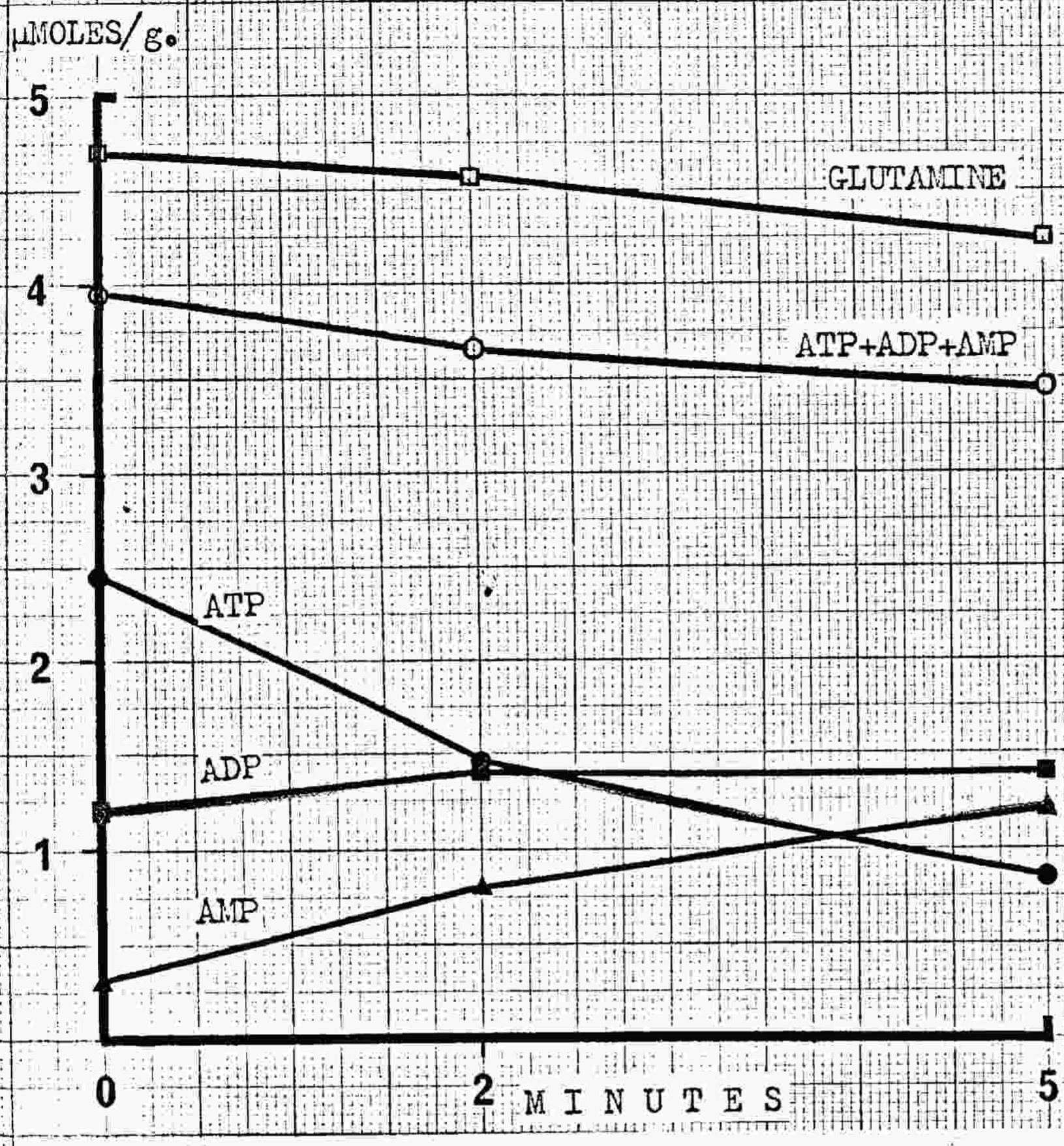


Fig.III.14

The contents of glutamine and the adenine nucleotides in rat liver during ischaemia.

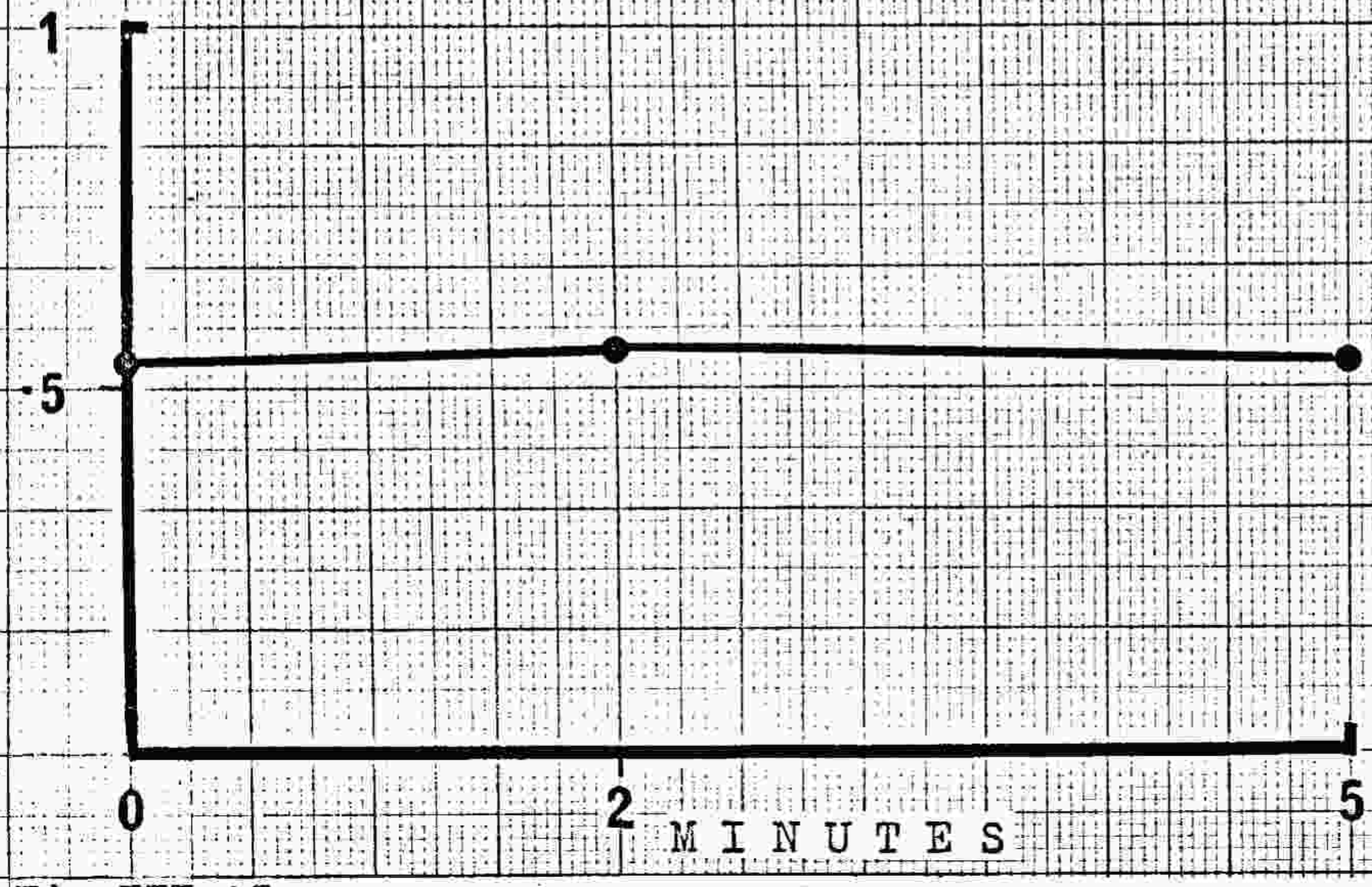


Fig.III.15

The mass action ratio for adenylate kinase during ischaemia in rat liver.

following is a selection:

Reference	ATP	ADP	AMP	$\frac{ATP}{ADP}$
Hohorst et al. (1959)	2.90	0.90	-	3.2
Underwood (1965)	2.77	0.64	0.09	4.3
Start & Newsholme (1968)	2.45	0.76	0.13	3.2

All these results were obtained using ether as an anaesthetic so that the lower $\frac{ATP}{ADP}$ ratio recorded in this thesis could be the result of either the absence of ether anaesthesia or the possible delay in the freezing of the liver relative to the in situ methods of the other authors. These possibilities were investigated by the following experiment: one group of rats (group (a)) were killed as usual by cervical dislocation and their livers freeze-clamped as rapidly as possible; group (b) were anaesthetised using an ether-air mixture as described by Start & Newsholme (1968) but were killed by cervical dislocation so that they are comparable to group (a) in all but the ether anaesthesia; group (c) were anaesthetised with ether-air and their abdomens were then carefully opened and the livers freeze-clamped in situ. This last group is comparable to the experiments of the cited authors.

The results of this experiment (Table III.1) show an

Table III.1 Adenine nucleotides in rat liver after sacrifice by different procedures

Rats (male; 180-200 g.) were killed (for details see text) and their livers freeze-clamped. Results are expressed as μmoles/g. fresh wt. ± S.D. There were 4 animals in each group.

Group	ATP	ADP	AMP	$\frac{ATP}{ADP}$	Total adenine nucleotides
(a) Killed by cervical dislocation	2.54±0.325	1.22 ±0.230	0.303± 0.075	2.15±0.776	4.06
(b) Ether anaesthesia + cervical dislocation	2.73±0.295	1.04 ±0.214	0.217±0.057	2.71±0.799	3.99
(c) Ether anaesthesia + liver frozen in situ	2.86±0.434	0.974±0.096	0.166±0.050*	2.95±0.567	4.00

* significantly different from group (a); 0.05>P

$\frac{ATP}{ADP}$ ratio of 2.15 in group (a) and 2.95 in group (c), thus showing that there is a real difference between the two methods of killing the rat. Group (b) also had a high ratio (2.71) suggesting that it is ether *per se* which is responsible for this rather than freezing *in situ*. Nevertheless, as none of the ratio differences were significant it is difficult to assess their importance. However, there was a significant difference between the AMP levels in group (a) and group (c), the latter value being only 54% of the group (a) level. Krebs (1964) has pointed out that if the adenylate kinase equilibrium holds the AMP concentration will change far more (proportionately) than the $\frac{ATP}{ADP}$ ratio. Thus the lowered AMP content in group (c) may signify that the non-significant elevation in $\frac{ATP}{ADP}$ is, in fact, real.

Amino acid metabolism during ischaemia in starved rats.

Williamson et al. (1967a) have shown that the hepatic content of certain gluconeogenic amino acids decreases in situations where glucose is being rapidly synthesised by the liver. In particular, 48 hours starvation reduced the alanine content to one-third of the fed value. It was therefore of interest to compare

the ischaemic metabolism of some of these amino acids in the starved rat with that occurring in the normal fed rat. Figures III.16 and III.17 illustrate ischaemic experiments on starved rats and on starved, L-cycloserine injected rats respectively. The results are quite similar to the experiments with fed rats. There was an increase in alanine levels (from 0.378 μmoles/g. at time zero to 1.77 μmoles/g. at 5 min.) while glutamate and aspertate remained fairly constant (Figure III.16). In the L-cycloserine-injected, starved rats (Figure III.17), the alanine content of the liver remained quite steady at almost 4 μmoles/g., while aspertate increased from 0.294 μmoles/g. at time zero to 1.02 μmoles/g. after 5 min. ischaemia.

Ischaemia in Brain and Kidney.

Experiments were carried out to ascertain whether tissues other than the liver could remove ammonia by the sequential action of glutamate dehydrogenase and alanine aminotransferase. Brain and kidney were chosen because ammonia metabolism is important in both these organs. Brain is known to be susceptible to ammonia toxicity, while the excretion of ammonia by the renal tubule is a valuable mechanism for the conservation of

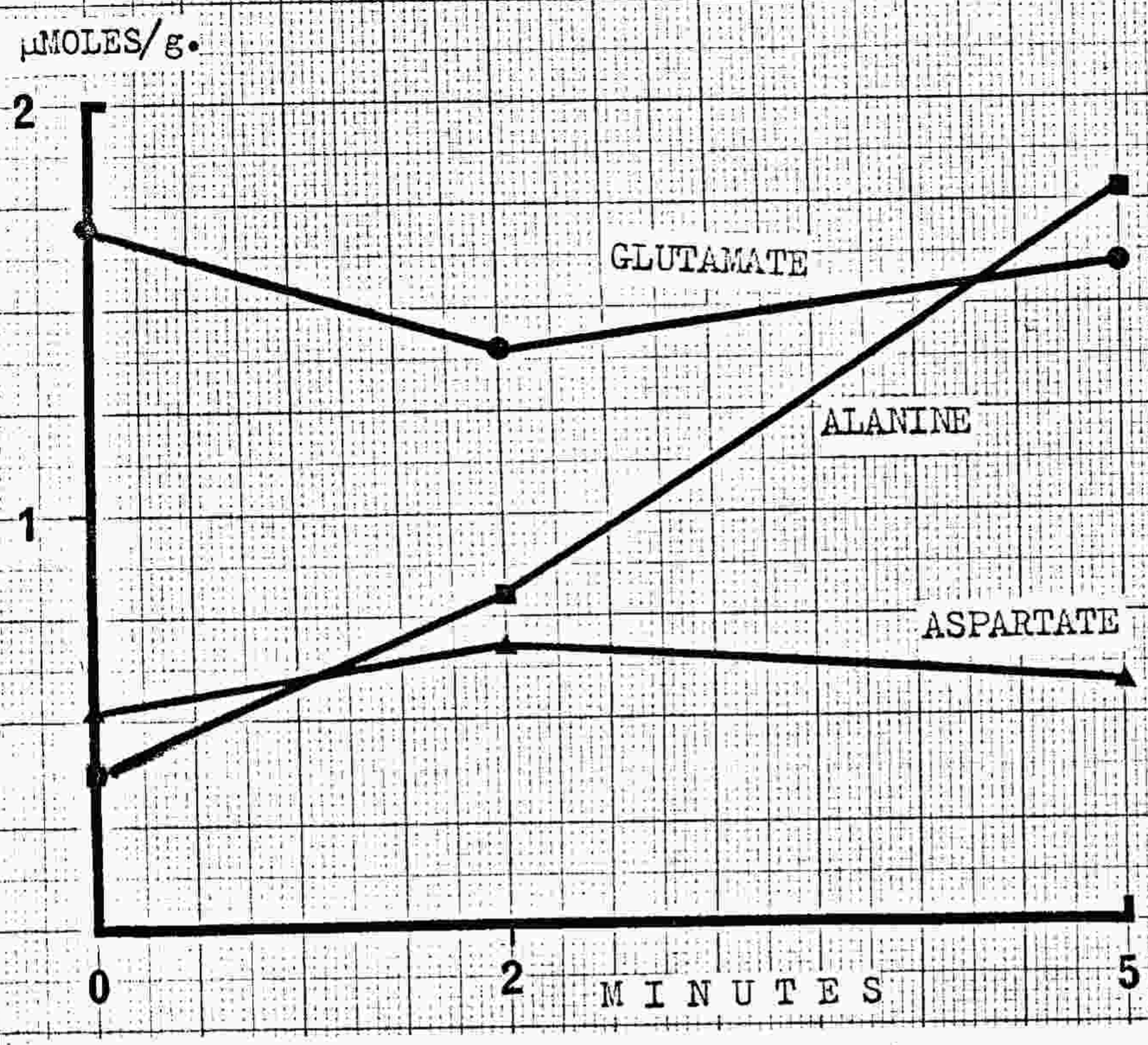


Fig.III.16

The contents of alanine, aspartate and glutamate , during ischaemia, in liver from 48-hour fasted rats.

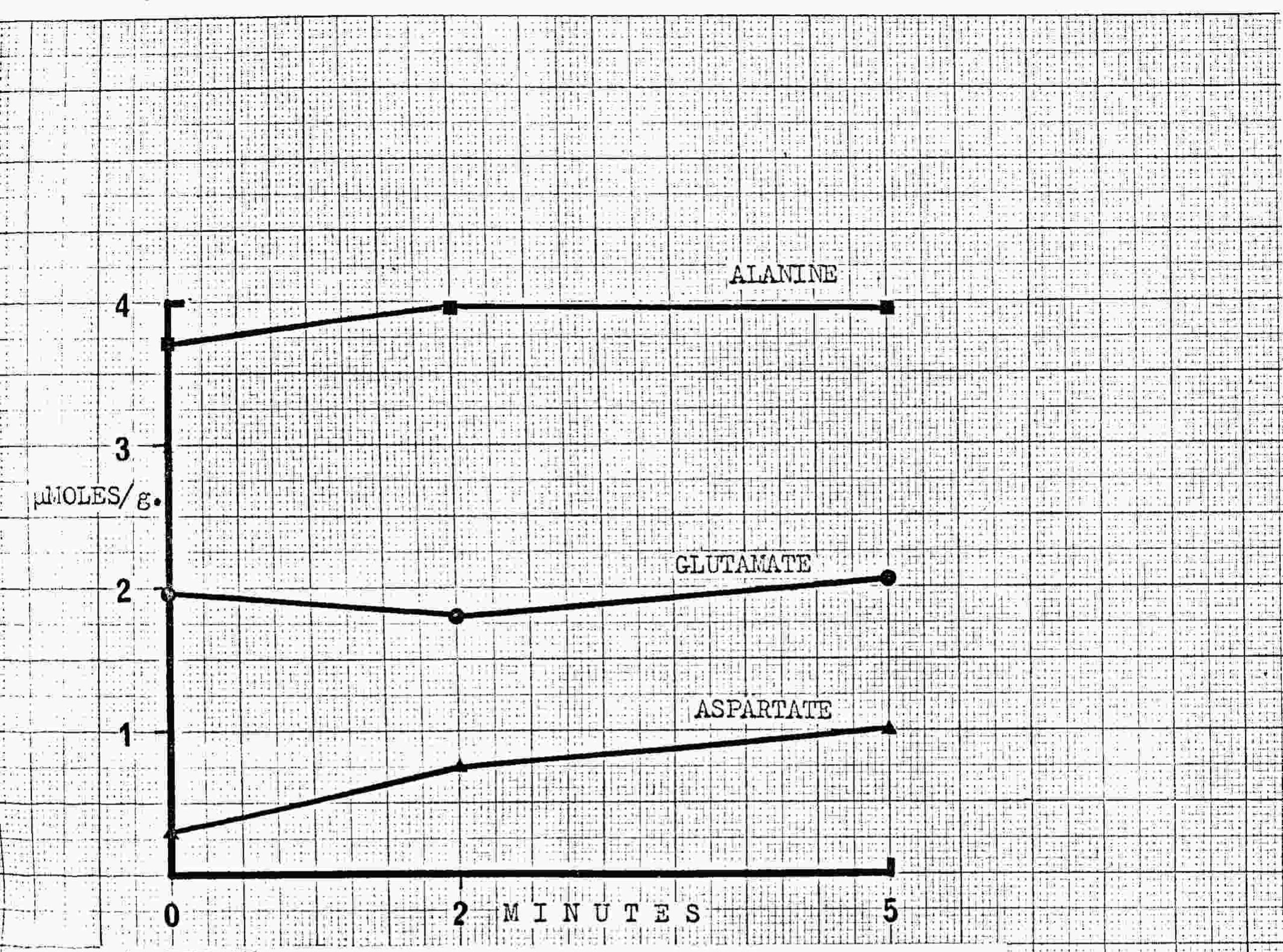


Fig.III.17

The contents of alanine, aspartate and glutamate, during ischaemia, in liver from 48-hour fasted rats that had been injected (ip) 60 min. previously with L-cycloserine (25 mg. / kg. body wt.).

sodium and potassium. Furthermore, both tissues have sufficient activity of the relevant enzymes (Williamson et al., 1967b; Awapara & Searle, 1952) for this pathway to be quantitatively important. Therefore various metabolites related to ammonia metabolism were measured in these organs both <u>in vivo</u> and after ischaemia. Tables III.2 and III.3 contain the results of these experiments.

It is possible that the freezing of the whole head was not rapid enough to prevent the brain becoming slightly anoxic. This is especially apparent if one compares the high $\frac{\text{Lactate}}{\text{Pyruvate}}$ ratio (37.1) and low $\frac{\text{ATP}}{\text{ADP}}$ ratio (1.63) with those obtained by Schmahl et al. (1965) (12.8 and 5.2 respectively) using a specialised technique for cooling the brain of anaesthetised cats <u>in situ</u>, or with those obtained by Lowry's group (Lowry et al., 1964; Goldberg et al., 1966) on mouse brain (24.8 and 2.57 respectively) - the mouse being small enough to be very rapidly frozen. Nevertheless it can be seen that there is an accumulation of ammonia in ischaemia (0.271 µmoles/g./2 min.). There is also a small elevation in the alanine level (0.193 µmoles/g./2 min.) suggesting that the glutamate dehydrogenase/aminotransferase mechanism may be operative to a small extent in brain. The mass action ratio for alanine aminotransferase was very far

Table III.2

Some metabolites in whole brain, in vivo and after ischaemia

Rats (male; 180-200 g.) were decapitated, and their heads were either plunged into liquid nitrogen (*in vivo* group), or allowed to stand at room temperature for 2 min. (ischaemic group) before freezing. For other details see Chapter II. Results are expressed as μmoles/g. fresh wt. and are the means of three experiments.

	In vivo	2 min. ischaemia
Ammonia	0.340	0.611
Glutamate	10.4	9.80
Aspartate	2.34	2.57
Alanine	0.570	0.763
Lactate	4.19	9.15
Pyruvate	0.113	0.031
Malate	0.310	0.195
Oxoglutarate	0.047	0.017
ATP	1.05	0.107
ADP	0.644	0.506
AMP	0.363	1.55
$\frac{\text{Lactate}}{\text{Pyruvate}}$	37.1	295
$\frac{\text{ATP}}{\text{ADP}}$	1.63	0.069
$\frac{\text{ADP}}{\text{AMP}}$	0.023	0.043

Table III.3.

Some metabolites in kidney, in vivo and after ischaemia

Rats (male; 180-200 g.) were killed and their kidneys freeze-clamped either immediately (in vivo) or after severing the blood vessels (ischaemic). Results are expressed in μmoles/g. fresh wt. This table represents a typical experiment.

	In vivo	3 min. ischaemia
Ammonia	0.600	1.45
Glutamate	3.08	4.82
Glutamine	2.74	1.59
Alanine	0.129	0.195

from equilibrium but it is difficult to interpret this because of the known compartmentation of glutamate in brain tissue (Berl & Waelsch, 1962). There was no appreciable change in the tissue content of glutamate and aspartate. The total adenine nucleotides (ATP + ADP + AMP) was very constant indicating that these cannot be the source of the ammonia.

Considerable accumulation of ammonia occurred in the ischaemic kidney, which seemed to be largely at the expense of glutamine. The amount of alanine formation was very small, so that the glutamate dehydrogenase aminotransferase systems appear not to be operative at all in the kidney.

Discussion

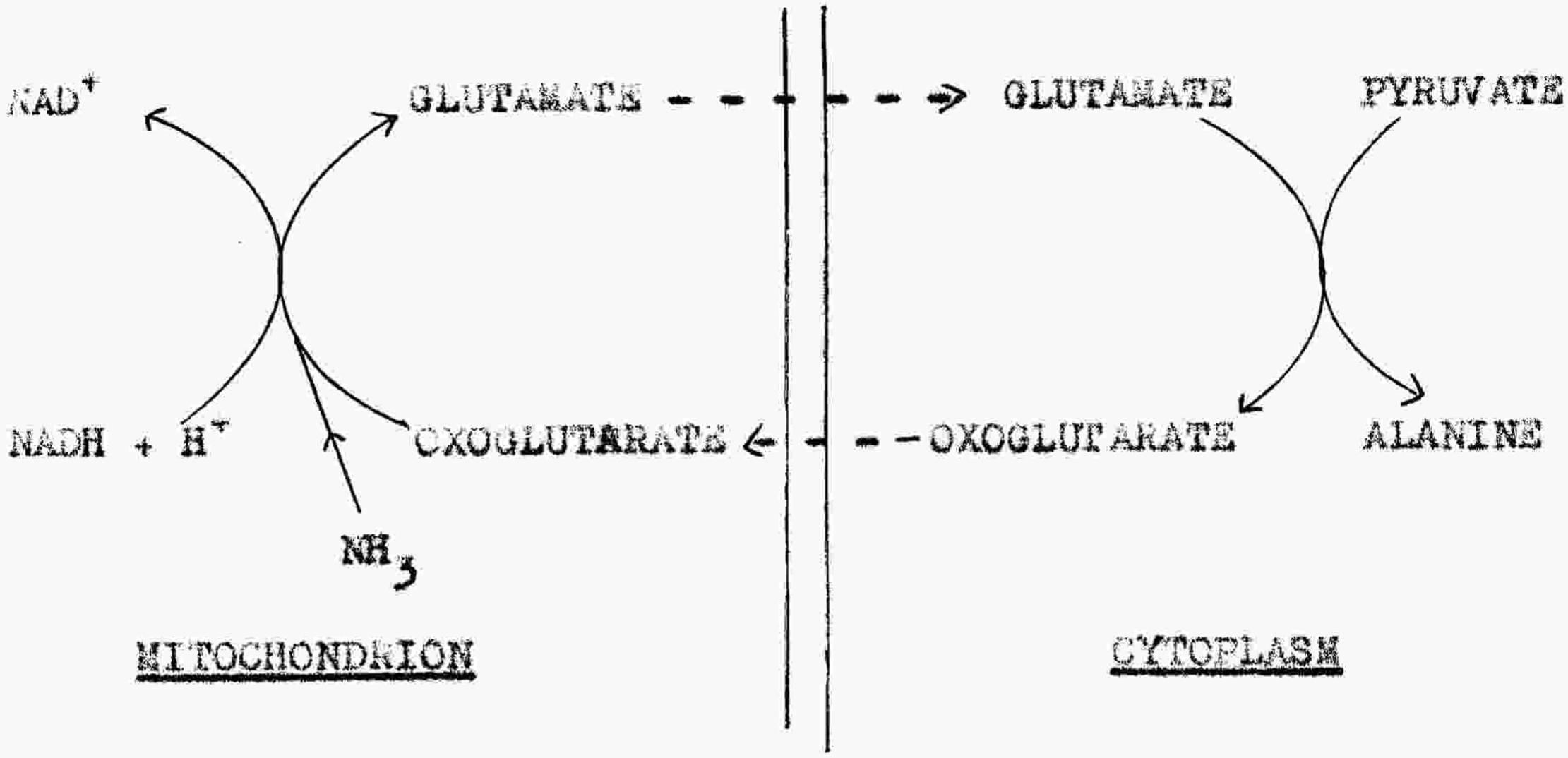
The experiments in this chapter were partly designed to test whether certain enzyme-catalysed equilibria which appear to hold *in vivo* are still maintained during the acute changes which are initiated by the lack of oxygen. It is quite possible that when intermediates are measured in freeze-clamped tissue the overall contents may indicate an equilibrium though no real functional equilibrium exists. The uneven compartmentation of some metabolites could easily bring

about such a situation. However, if after rapid changes in the tissue contents of the individual metabolites the overall equilibrium still appears to be maintained then a much stronger case can be made out for it being functional. Using these criteria many enzymes were found to catalyse near-equilibrium reactions. Glutamate dehydrogenase and hydroxybutyrate dehydrogenase behaved as if at equilibrium with the same pool of pyridine nucleotides; direct measurement of the reactants of alanine aminotransferase indicated that this enzyme also catalysed a near-equilibrium while indirect measurements suggested a similar result for aspartate aminotransferase. Malic enzyme and the cytoplasmic isocitrate dehydrogenase maintained equilibrium during ischaemia while the adenine nucleotides appeared to be strictly controlled by the adenylate kinase equilibrium.

<u>The subcellular site of alanine and aspartate syntheses</u>.

Since glutamate dehydrogenase is exclusively located in the mitochondrion the initial step of either alanine or aspartate synthesis must take place there. The site of transamination is more equivocal because of the occurrence of both alanine and aspartate aminotransferases in the cytoplasm and in the mitochondrion (Katanuma et al., 1966). If the synthesis of alanine occurs in the

cytoplasm it must involve the passage of glutamate from mitochondrion to cytoplasm and the return of oxoglutarate from cytoplasm to mitochondrion, as follows:



Alternatively the alanine synthesis could occur exclusively in the mitochondrion, but if this is the case the alanine must diffuse out and enter into equilibrium with the cytoplasmic alanine aminotransferase. This is so because, as already mentioned, the measured liver contents of glutamate, oxoglutarate, pyruvate and alanine are close to the alanine aminotransferase equilibrium but since the same pyruvate content is known to be in equilibrium with lactate dehydrogenase (an exclusively cytoplasmic enzyme) then the measured overall aminotransferase equilibrium must be operative in the

cytoplasm. The synthesis of aspartate during ischaemia, when alanine aminotransferase is inhibited, poses many problems. Firstly the composite expression $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ tended to go out of equilibrium; secondly it is difficult to visualise a suitable source of oxaloacetate during ischaemia; thirdly, the mechanism by which the cell changes from alanine to aspartate synthesis when alanine aminotransferase is inhibited is not immediately apparent. It is possible that the oxaloacetate is supplied via pyruvate carboxylase:

$$\text{pyruvate} + CO_2 + ATP \longrightarrow \text{oxaloacetate} + ADP + Pi$$

This requires the provision of ATP in an anoxic situation. However, Figure III.14 shows that the fall in ATP during ischaemia is not as precipitate as might be expected. This, no doubt, is due to anaerobic glycolysis; the ATP so formed could perhaps be translocated into the mitochondrion by the mechanism of Klingenberg & Pfaff (1966). At present, however, no satisfactory explanations are available for the disequilibrium of the expression $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ or for the mechanism for the switch to aspartate synthesis.

The behaviour of the nucleotides NAD and NADH during ischaemia.

From the data in Figures III.3, III.4 it can be seen that $\frac{[NAD]}{[NADH]}$ cyto as measured by the $\frac{[lactate]}{[pyruvate]}$ ratio decreased from 738 to 152 after 2 min. ischaemia. During the same period $\frac{[NAD]}{[NADH]}$ mito, as measured by the $\frac{[3\text{-hydroxybutyrate}]}{[acetoacetate]}$ ratio, decreased from 10.2 to 3.02. Measurements of the total nucleotides during this ischaemic period gives the following results (μmoles/g. fresh wt.):

	Initial value	2 min. ischaemia
NAD	0.83	0.67
NADH	0.16	0.19
$\frac{NAD}{NADH}$	5.2	3.5

It is immediately apparent that the measured ratio of $\frac{[NAD]}{[NADH]}$ is quite similar to that calculated for the mitochondrion but not at all like the cytoplasmic ratio. Mayor, Veloso & Williamson (1968) have evidence for the proposition that the nucleotides measured in the whole liver are largely representative of the mitochondrion rather than the cytoplasm. However, a closer examination of these figures reveals an anomaly. If the cytoplasmic $\frac{[NAD]}{[NADH]}$ ratio is 738 and the mitochondrial ratio is 10.2 then, if there are no other pools of pyridine nucleotides

in the liver cell, the measured ratio of $\frac{[NAD]}{[NADH]}$ should be somewhere between 736 and 10.2. In fact it is 5.2. There are three possible explanations for this enomaly:

(a) the existence of a third pool of pyridine nucleotides with a ratio of $\frac{[NAD]}{[NADH]}$ less than 5.2;

(b) the binding of a large proportion of the NADH so that it was not available to take part in the reactions catalysed by lactate dehydrogenase or hydroxybutyrate dehydrogenase;

(c) if the intramitochondrial pH were more alkaline than pH 7.0 then the calculated $\frac{[NAD]}{[NADH]}$ ratio for a given $\frac{[hydroxybutyrate]}{[acetoacetate]}$ ratio would be lowered. An elevation to pH 7.3 would result in a $\frac{[NAD]}{[NADH]}$ ratio of 5.13 from a $\frac{[hydroxybutyrate]}{[acetoacetate]}$ ratio of 1.98 (Figure IV.2).

At present there are no means of directly establishing which of the above explanations, if any, are correct. The

<u>The adenylate kinase equilibrium during ischaemia</u>.

Adenylate kinase catalyses the reaction

$$ATP + AMP \rightleftharpoons 2ADP$$

The apparent equilibrium constant measured for this reaction varies between 0.4 and 0.9 depending on the Mg^{2+} concentration (Gevers, 1966). That a value of about

0.5 is consistently maintained during ischaemia suggests that adenylate kinase controls the relative amounts of the adenine nucleotides in this situation. This constancy is in contrast to a report by Chance et al. (1965) who rendered an anaesthetised (ether + urethane) rat's liver anoxic by clamping off the hepatic blood supply. They found the adenylate kinase mass action ratio to fluctuate between 0.37 and 2.0. Since the periods of anoxia used by these authors were shorter (Maximum = 60 sec.) than those used in the present study, the two sets of results are not necessarily incompatible.

It is now established that adenylate kinase is associated with the outer mitochondrial membrane (Chappell & Crofts, 1965; Bräiczka et al., 1966). Since adenine nucleotides can passively penetrate this membrane but can only penetrate the inner memorane by means of a specific atractylate sensitive translocase (Klingenberg & Pfaff, 1966) it follows that adenylate kinase reacts with the cytoplasmic adenine nucleotide pool, not the mitochondrial one. Thus it is the cytoplasmic adenine nucleotides whose relative concentrations are controlled by adenylate kinase both <u>in vivo</u> and during ischaemia. Mitochondria occupy about 18.6% of the cell volume in rat liver (Lehninger, 1964) and

the concentration of total adenine nucleotides in mitochondria is about 6mM (Klingenberg & Pfaff, 1966). The liver content of these nucleotides is 4 μmoles/g. fresh wt. (Figure III.14) but since 1 g. rat liver contains 0.75 ml. water this liver content can be corrected to an overall 'concentration' of 5.33mM. From these figures it can be calculated that 79% of the total cellular adenine nucleotides is extramitochondrial. Thus changes in the extramitochondrial adenine nucleotides could easily mask changes in the mitochondrial nucleotides so that their relative concentrations within the mitochondrion may easily be quite different from the measured overall content.

CHAPTER IV

RESPONSE OF RAT-LIVER METABOLITES TO AN AMMONIA LOAD

The conclusions arrived at in the previous chapter were that glutamate dehydrogenase, alanine aminotransferase and aspartate aminotransferase are close to equilibrium in the liver and are mutually interconnected by common pools of glutamate and 2-oxoglutarate. Furthermore, that glutamate dehydrogenase and 3-hydroxybutyrate dehydrogenase are close to equilibrium and are interconnected by common pools of NAD and NADH. These observations were for the *in vivo* and ischaemic liver. The experiments in this chapter will be concerned with whether these relationships are still functional *in vivo* when the animal is presented with an ammonia load. The provision of this load might be expected to greatly increase the flux through some of these enzymes and since it is not enzyme activity alone that determines whether near equilibrium is maintained but enzyme activity in relation to the flux catalysed by the enzyme this type of

experiment should provide a test of whether these equilibria still hold under extreme conditions. In addition, since it is likely that an ammonia load would substantially alter the liver content of many metabolites, these experiments should serve to establish whether the equilibria are functional or whether they are simply a fortuitous agreement between the tissue contents of the relevant metabolites and the equilibrium constants of the particular enzymes.

If the equilibria observed during ischaemia are functional, several predictions may be made. Firstly, the glutamate dehydrogenase-aminotransferase system could remove some of the ammonia load as alanine and aspartate, provided adequate supplies of the corresponding keto acids (pyruvate and oxaloacetate) were available. Secondly, since ammonia is an oxidant with respect to the mitochondrial pyridine nucleotides, an increase in its concentration should result in an increase in the ratio $\frac{[NAD]}{[NADH]}$ which in turn should manifest itself as a decrease in the ratio $\frac{[3\text{-hydroxybutyrate}]}{[acetoacetate]}$.

Administration of an Ammonia Load to Fed Rats

In these experiments sufficient of a 1M solution of NH_4Cl (neutralised to pH 7.0 with NaOH) was injected

intraperitoneally so that the final dose was 2.5 mMoles/kg. body weight. This amount of ammonia was administered in all subsequent experiments, unless otherwise stated. Preliminary experiments showed that this quantity of ammonia gave large effects while at the same time being below the lethal dosage. None of the rats thus injected ever manifested symptoms of distress (e.g. drowsiness, convulsions, etc.). The chloride of ammonium was chosen because other salts (e.g. ammonium lactate, ammonium acetate) might give ambiguous results due to further metabolism of the anions. The rats were killed at various time intervals after injection and their livers were freeze-clamped immediately, ground up and extracted with perchloric acid as described in Chapter II.

It was thought advisable to first check the effects of injection per se on hepatic metabolites; therefore fed rats were injected intraperitoneally with 0.9% (w/v) NaCl (0.5 ml./200 g. body wt.) and killed at intervals afterwards. The results demonstrate the importance of performing this control experiment (Table IV.1). The increase in alanine content was significant at 2 min. The increase in lactate was not significant but is considered to be a real increase as similar effects were observed in later saline injection experiments on starved

Table IV.1 Metabolites in rat liver after saline injection

Rats (male; 180-200 g.) were injected (i.p.) with 0.9 NaCl (2.5 ml./kg. body wt.), killed at the times indicated and their livers freeze-clamped. Control animals (0 min.) were killed without injection. For other details, see Chapter II. Results are expressed as μmoles/g. fresh wt. ± S.D. There were 5 rats in each group.

	0 min.	2 min.	5 min.
Alanine	1.06 ±0.232	1.66 ±0.117*	1.37 ±0.286
Lactate	0.867±0.273	1.20 ±0.403	1.06 ±0.212
Pyruvate	0.075±0.030	0.104±0.054	0.113±0.062
Glutamate	1.95 ±0.282	1.97 ±0.637	1.93 ±0.313
Oxogluterate	0.155±0.054	0.122±0.043	0.174±0.041
Lactate / Pyruvate	11.5	11.5	9.40
GLT	1.12	0.985	1.09

* Significantly different from 0 min. level $P<0.0025$

rats. At 5 min. after injection the alanine and lactate values were not appreciably different from the non-injected rats. None of the other metabolites measured (malate, 3-hydroxybutyrate, acetoacetate, glutamine, aspartate and urea) changed significantly after saline injection (up to 25 min.).

The changes observed in the liver after administration of ammonia are quite complex (Figures IV.1 to IV.5)*. The response of some amino acids and urea are shown in Figure IV.1. Alanine and aspartate increased about 3-fold to a peak at 5 min. and thereafter slowly returned to the initial levels at about 20 to 25 min. The change in glutamate was biphasic; initially it fell at 2 min. by 0.8 μmoles/gram and then slowly increased to a peak at 15 min., returning to the zero time value at 25 min. There was a rise in the glutamine content at 2 min. (0.8 μmoles/g.) which is similar in magnitude to the decrease in glutamate at this time, suggesting that glutamine synthesis may be responsible for the disappearance of glutamate. The liver content of glutamine returned to

---

*These results, and all results presented as figures in this chapter, are given in tabular form in the appendix.

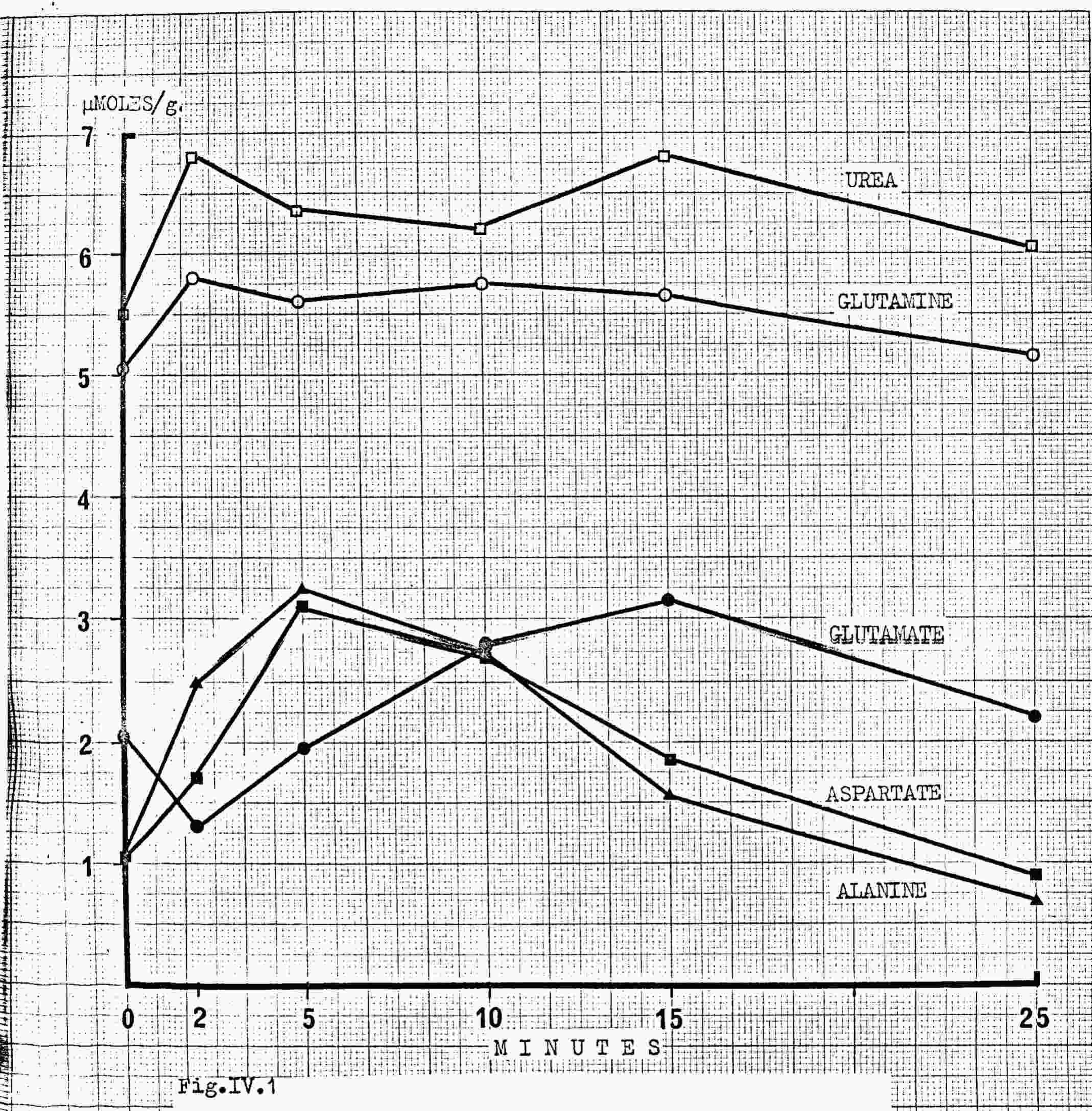


Fig.IV.1

Liver content of urea, glutamine, glutamate, alanine and aspartate after administration of ammonium chloride (ip) to fed rats.

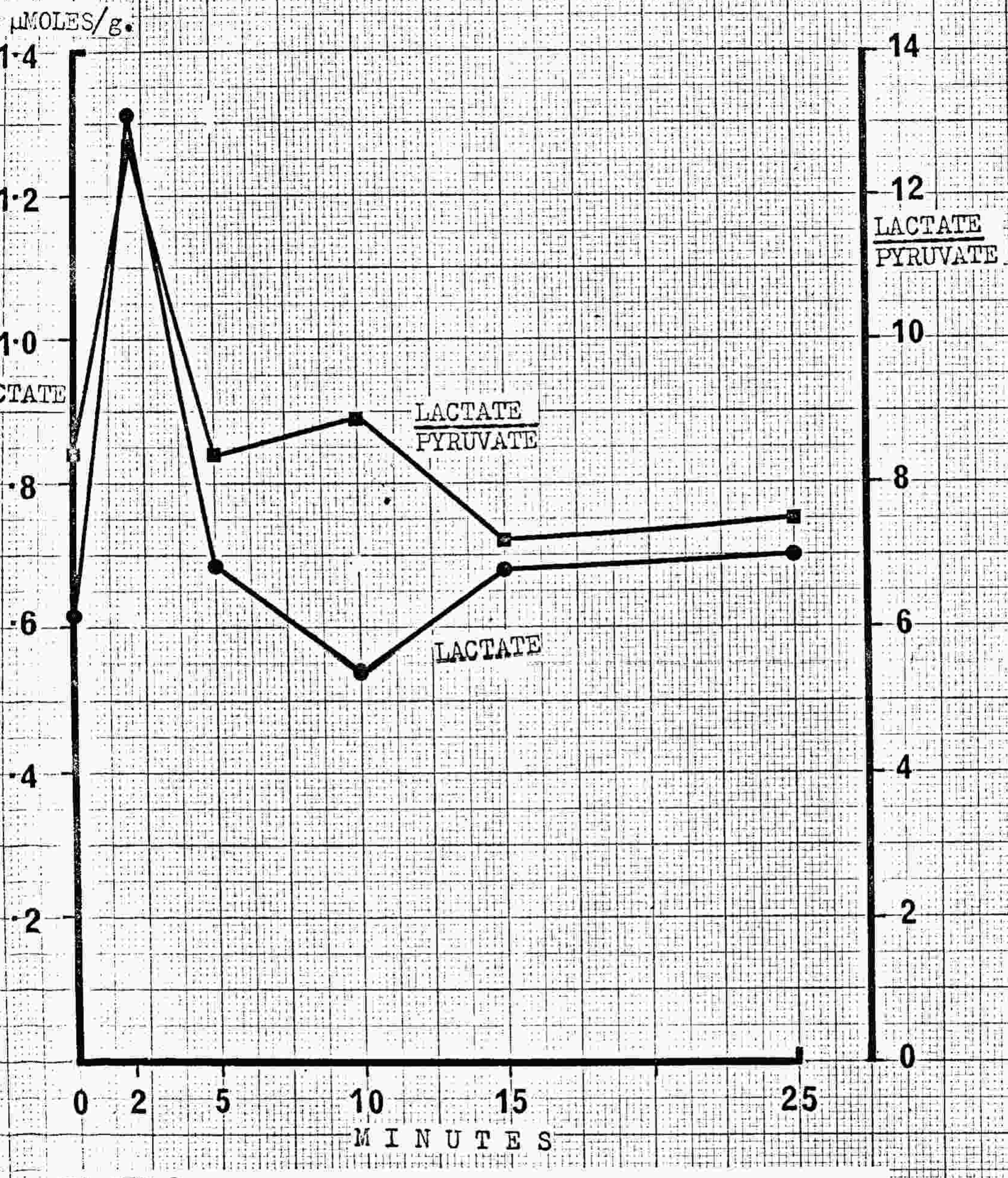


Fig.IV.2

Liver content of lactate and the $\frac{\text{lactate}}{\text{pyruvate}}$ ratio after administration of ammonium chloride to fed rats.

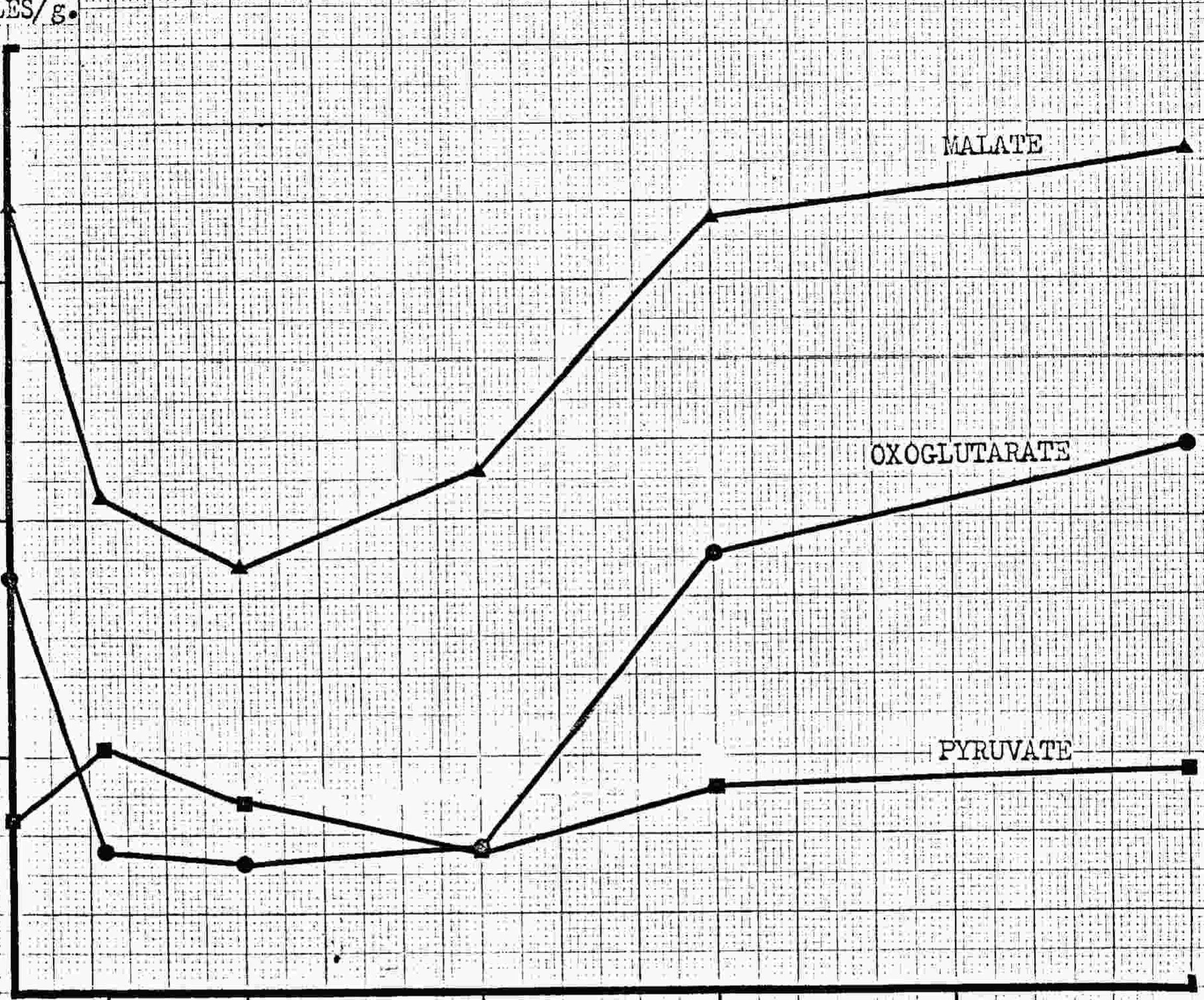


Fig.IV.3

Liver content of malate, oxoglutarate and pyruvate after administration of ammonium chloride to fed rats.

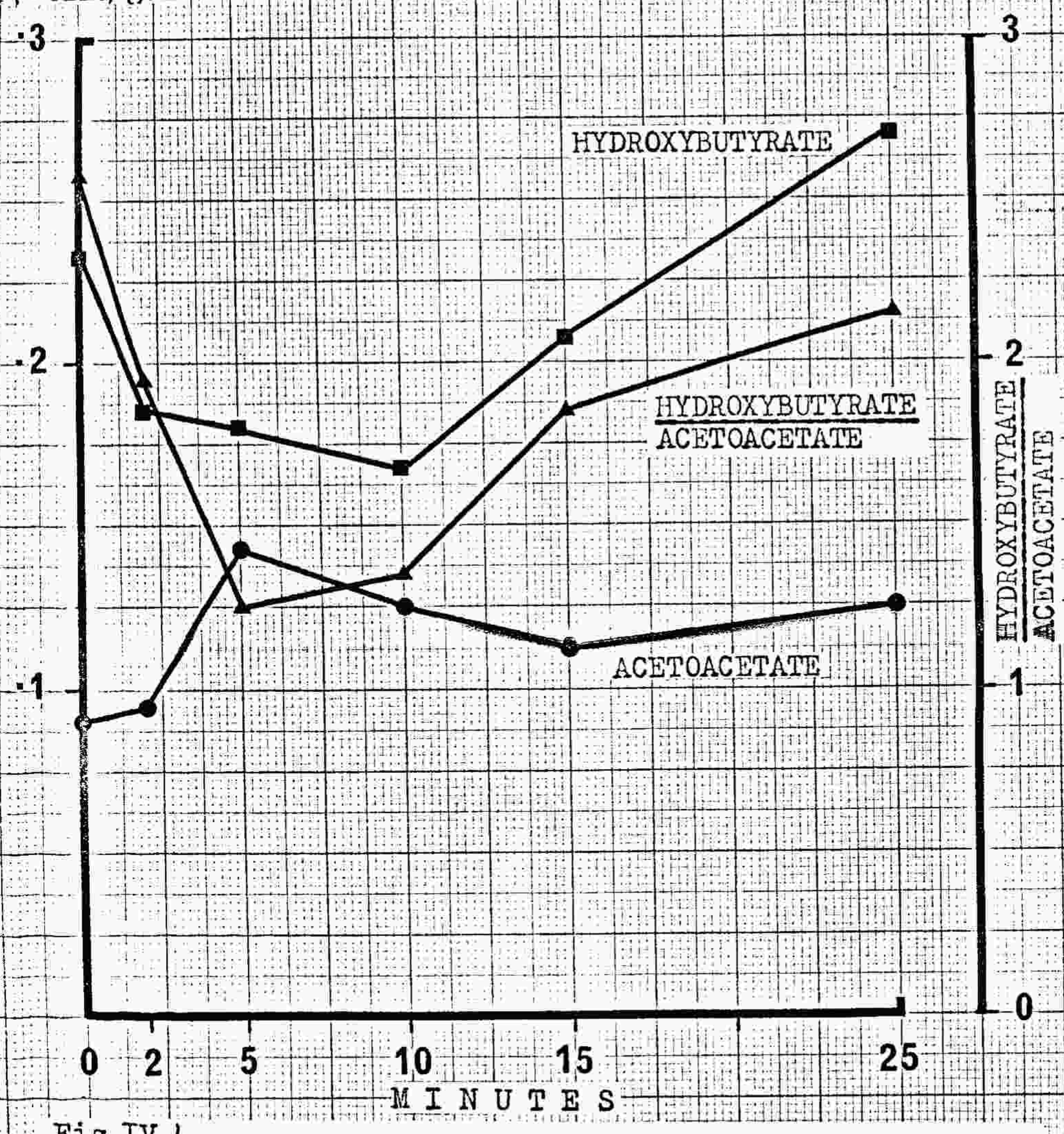


Fig.IV.4

Liver content of acetoacetate and hydroxybutyrate and the hepatic $\frac{\text{hydroxybutyrate}}{\text{acetoacetate}}$ ratio after administration of ammonium chloride to fed rats.

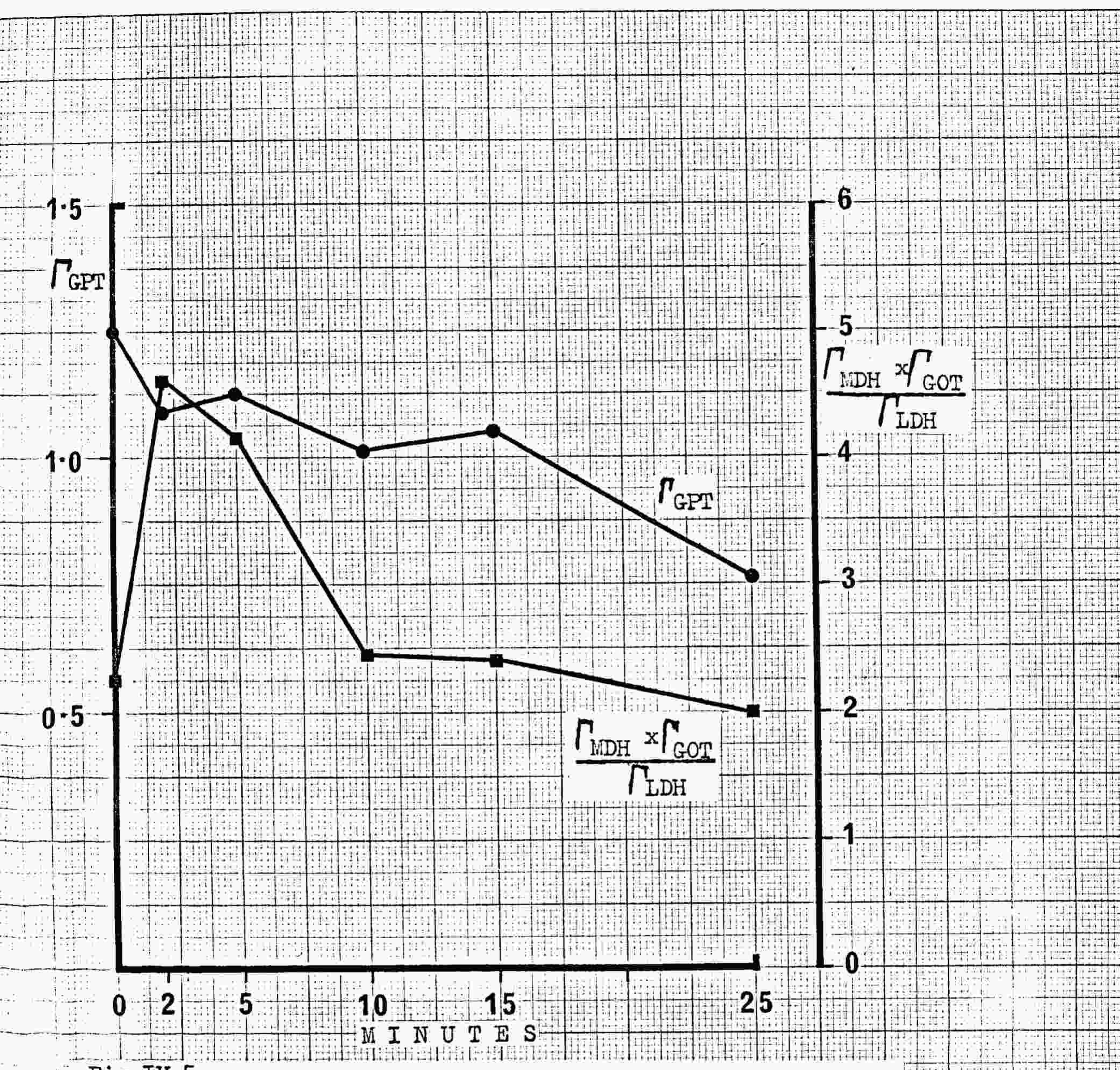
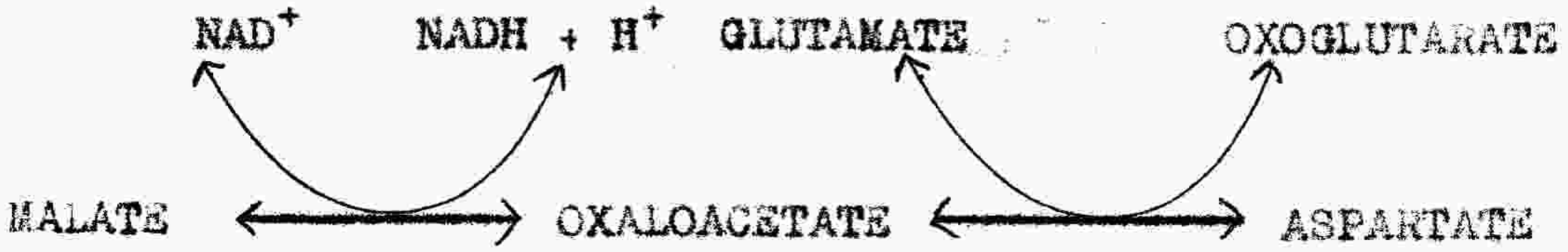


Fig.IV.5

Changes in the mass action ratio for alanine aminotransferase (Γ_{GPT}) and the indirect mass action ratio for aspartate aminotransferase $\left(\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right)$ after administration of ammonium chloride to fed rats.

the initial level by 25 min. There was also a rise (1.3 μmoles/g.) at 2 min. in the liver urea content which probably does not nearly represent the full extent of the increase in urea synthesis because of its easy permeability through the liver plasma membrane (Hems et al., 1968) and its excretion by the kidney.

The changes in lactate (Figure IV.2) and pyruvate (Figure IV.2) were similar to those observed after saline injection (Table IV.1) so that no special effect of ammonia need be invoked to explain the transient increases in these metabolites. The liver content of malate (Figure IV.3) initially decreased after ammonia (5 min.) and thereafter returned to normal (initial) values at 15 and 25 min. This is in the opposite direction to the changes in aspartate (Figure IV.1) and is probably indicative of a sharing of a common cytoplasmic pool of oxaloacetate by both aspartate aminotransferase and malate dehydrogenase as illustrated by the following scheme:



The tissue content of 2-oxoglutarate fell sharply after ammonia injection (Figure IV.3). This can be interpreted in terms of the mass action changes brought about via glutamate dehydrogenase. The sudden increase in the intracellular ammonia concentration would drive the reaction towards glutamate synthesis thus lowering the oxoglutarate concentration. This would also increase the ratio $\frac{[NAD]}{[NADH]}$ and thus decrease the ratio $\frac{[3\text{-hydroxybutyrate}]}{[acetoacetate]}$ if the equilibrium between glutamate dehydrogenase and hydroxybutyrate dehydrogenase was operative under these conditions.

These predictions were in fact borne out (Figure IV.4). Within minutes of the ammonia challenge there was a fall in 3-hydroxybutyrate and a rise in acetoacetate resulting in a sharp fall in the ratio $\frac{[3\text{-hydroxybutyrate}]}{[acetoacetate]}$ which slowly returned to control values at about 25 min. This experiment extends to fed rats the original finding of Williamson et al. (1967b) who recorded a similar decrease in this ratio after ammonia injection to starved rats.

The sum of 3-hydroxybutyrate and acetoacetate remained quite constant at about 0.3 μmoles/g. during these experiments. Hecknagel and Potter (1951) found an increased rate of ketogenesis in liver slices and homogenates on addition of 10-20 mM NH_4Cl and showed that this was

probably due to an inhibition of citric acid cycle activity through removal of cycle intermediates via the reductive amination of 2-oxoglutarate. No increase in the rate of ketogenesis was apparent in these in vivo experiments.

The mass action ratio for alanine aminotransferase ($\Gamma_{G.T}$) was remarkably constant (Figure IV.5) both during the anabolic and catabolic phases. The expression $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ which was derived in Chapter III to describe mutual equilibrium between the reactants of cytoplasmic aspartate aminotransferase, lactate dehydrogenase and cytoplasmic malate dehydrogenase (theoretically = 1.65) was perturbed during the phase of aspartate synthesis (2 min. and 5 min.) but had returned to control values by 10 min.

To check whether the ammonia load seriously interfered with the energy metabolism of the liver, hepatic adenine nucleotides were measured (Table IV.2). Ammonia did not appear to appreciably alter the hepatic content of the adenine nucleotides at any time. This would appear to make an in vivo inhibition of the citric acid cycle by ammonia, such as that reported by Recknagel & Potter (1951) in vitro, unlikely.

Table IV.2 Effect of injection of NH_4Cl on the adenine nucleotides in rat liver

Rats (male; 180-200 g.) were injected (i.p.) with 2.5 mMoles NH_4Cl/kg. body wt., killed at the times indicated and their livers freeze-clamped. The control group (0 min.) were not injected. For other details see Chapter II. Results are expressed as μmoles/g. fresh wt. There were 2 rats in each group.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
ATP	2.69	2.75	2.62	2.44	2.47	2.67
ADP	1.07	1.33	1.21	1.37	1.24	1.24
AMP	0.216	0.197	0.213	0.254	0.250	0.187

Concentrations of metabolites in blood after an ammonia load

An overall picture of these responses to ammonia necessitates the measurement of various metabolites in the blood. Such measurements might be expected to give information on the extent to which changes in the concentrations of metabolites in the liver are mirrored by similar changes in blood. Therefore blood was taken from the abdominal aorta of ether - anaesthetised rats after ammonia injection, at a time (5-10 min.) when maximal changes occurred in the liver. The results are shown in Table IV.3. The ammonia concentration in blood from control rats was extremely low - below the limits of detection of the method - but it was greatly elevated after ammonia administration. The concentrations of alanine, aspartate and glutamate remained constant suggesting that none of these amino acids was released from the liver after ammonia injection. A significant increase in the level of glutamine was observed - this will be dealt with more fully in the Discussion.

Although there was no appreciable change in the sum of 3-hydroxybutyrate + acetoacetate there were changes in the individual ketone bodies. The level of 3-hydroxybutyrate fell slightly while that of acetoacetate was significantly elevated; thus there was a large

Table IV.3

Effect of injection of NH_4Cl on the concentrations of some metabolites in arterial blood

Male rats, about 300 g. wt. were used. The control rats were not injected; the experimental group received NH_4Cl (2.5 mMoles/kg. body wt.) intraperitoneally and blood was taken 5-10 min. afterwards. For other details see Chapter II. Results are in μMoles/ml. blood ± S.D. There were at least 5 animals in each group.

	Control group	NH_4Cl injected group	P
Ammonia	<0.020	0.679±0.166	
Alanine	0.369±0.039	0.376±0.067	
Aspartate	0.026±0.012	0.024±0.003	
Glutamate	0.091±0.006	0.089±0.012	
Glutamine	0.556±0.044	0.615±0.039	<0.05
Pyruvate	0.123±0.040	0.101±0.024	
Oxoglutarate	0.042±0.008	0.035±0.004	
Hydroxybutyrate	0.101±0.035	0.090±0.019	
Acetoacetate	0.027±0.018	0.050±0.016	<0.05
Hydroxybutyrate / Acetoacetate	3.74	1.80	
Γ_{GPT}	1.38	1.46	

decrease in the ratio $\frac{[\text{3-hydroxybutyrate}]}{[\text{acetoacetate}]}$. Since whole blood contains no 3-hydroxybutyrate dehydrogenase the decrease in ratio must reflect a decrease within a tissue - this is almost certainly the liver as it possesses the highest activity of both glutamate dehydrogenase and 3-hydroxybutyrate dehydrogenase (Williamson et al., 1967b) and because the ratio within the liver decreased similarly after an ammonia load (Figure IV.4).

The value for the mass action ratio of alanine aminotransferase was strikingly close to the apparent equilibrium constant (1.48) for this enzyme. Whether this is fortuitous or actually represents an equilibrium catalysed by the small amount of enzyme in normal blood (7.3 mU/ml. in human serum (Schmidt & Schmidt, 1967)) is not known.

Effect of pre-injection with L-cycloserine on the response of the liver to an ammonia load

To obtain more concrete evidence on the role of alanine aminotransferase in the synthesis of alanine after NH_4Cl injection the ammonia loading experiments were repeated with rats which had been pre-injected with L-cycloserine. This compound has been shown in Chapter III to be an effective *in vivo* inhibitor of alanine

aminotransferase. Rats were injected intraperitoneally with an aqueous solution (5 mg./ml.) of L-cycloserine (25 mg./kg. body wt.). One hour later half of the rats received an ammonia load. The other half (controls) received saline to establish what changes might be due to the trauma of injection.

Increases in lactate and pyruvate (Table IV.4) similar to those found (Table IV.1) on injection of NaCl to normal rats occurred 2 min. after the injection of saline to L-cycloserine-treated rats. There was a large decrease in oxoglutarate and a smaller fall in glutamate, 2 min. after injection. No changes in other metabolites (aspartate, urea, malate, 3-hydroxybutyrate, acetoacetate) were found in this period and no changes whatever in any metabolite later than 5 min. post-injection.

When cycloserine-treated rats were injected with NH_4Cl (Figures IV.6 to IV.10) many of the responses were qualitatively very similar to those from normal rats (Figures IV.1 to IV.5). Glutamate (Figure IV.6) initially fell and then rose to a peak at 10-15 min. before returning to the initial level at 25 min. Oxoglutarate (Figure IV.7) fell sharply but then recovered to 'overshoot' the initial value at 25 min. Lactate and pyruvate (Figures IV.8 and IV.7) showed a transient rise at 2 min. similar to that

Table IV.4 Effect of saline injection on L-cycloserine treated rats

Rats (male; 180–200 g.) were injected (i.p.) with L-cycloserine (25 mg./kg. body wt.) and 60 min. later were injected (i.p.) with 0.9% NaCl (2.5 ml./kg. body wt.). They were then killed at the times indicated and their livers freeze-clamped. Control animals (0 min.) were not injected with saline. For other details see Chapter II. Results are expressed as μMoles/g. fresh wt. ± S.D. There were 5 animals in each group.

	0 min.	2 min.	5 min.
Alanine	2.86 ±0.471	3.41 ±0.852	3.01 ±0.334
Lactate	0.740±0.270	1.68 ±0.932*	0.750±0.493
Pyruvate	0.071±0.026	0.111±0.060	0.063±0.018
Glutamate	1.69 ±0.182	1.23 ±0.145**	1.42 ±0.047
Oxoglutarate	0.168±0.045	0.095±0.044**	0.146±0.049
$\frac{\text{Lactate}}{\text{Pyruvate}}$	10.4	15.1	11.9
Γ_{GPT}	4.00	2.48	4.91

* significantly different from 0 min. level $P<0.05$

** " " " " " " $P<0.025$

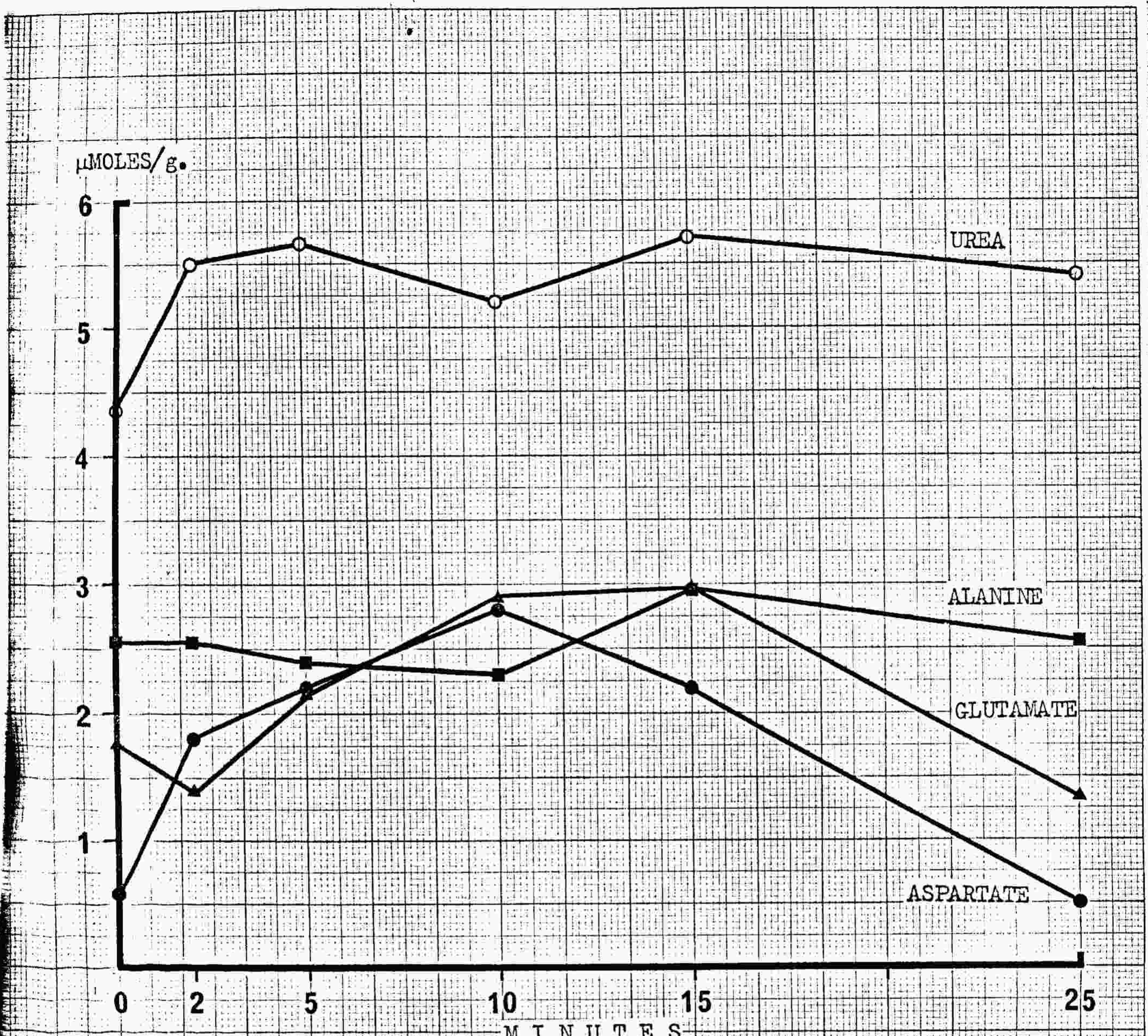


Fig.IV.6

Liver content of urea, glutamate, alanine and aspartate after administration of ammonium chloride to fed rats injected (ip) 60 min. previously with L-cycloserine (25 mg. / kg. body wt.)

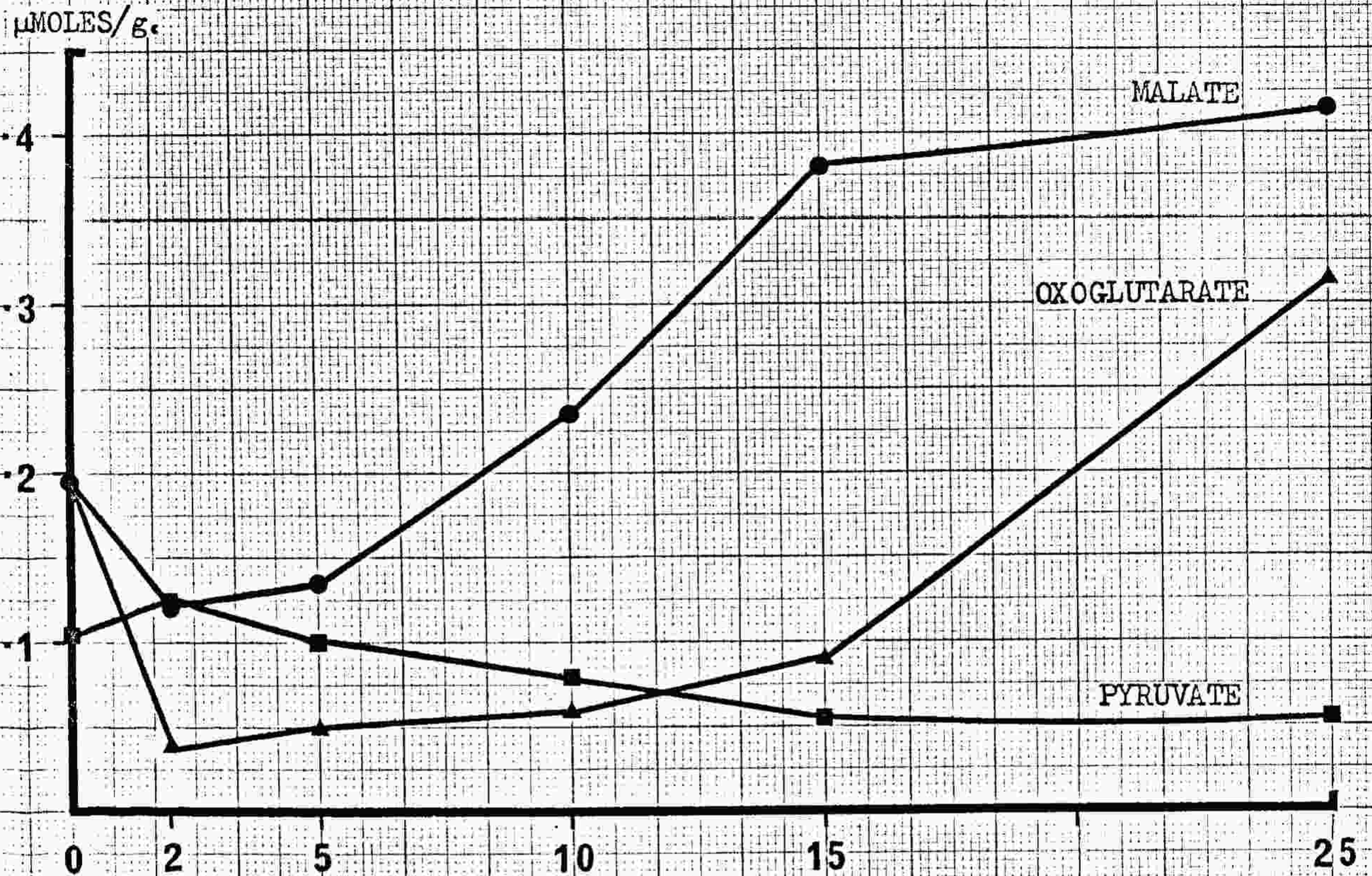


Fig.IV.7

Liver content of malate, oxoglutatate and pyruvate after administration of ammonium chloride to fed rats injected (ip) 60 min. previously with L-cycloserine (25mg./kg. body wt.).

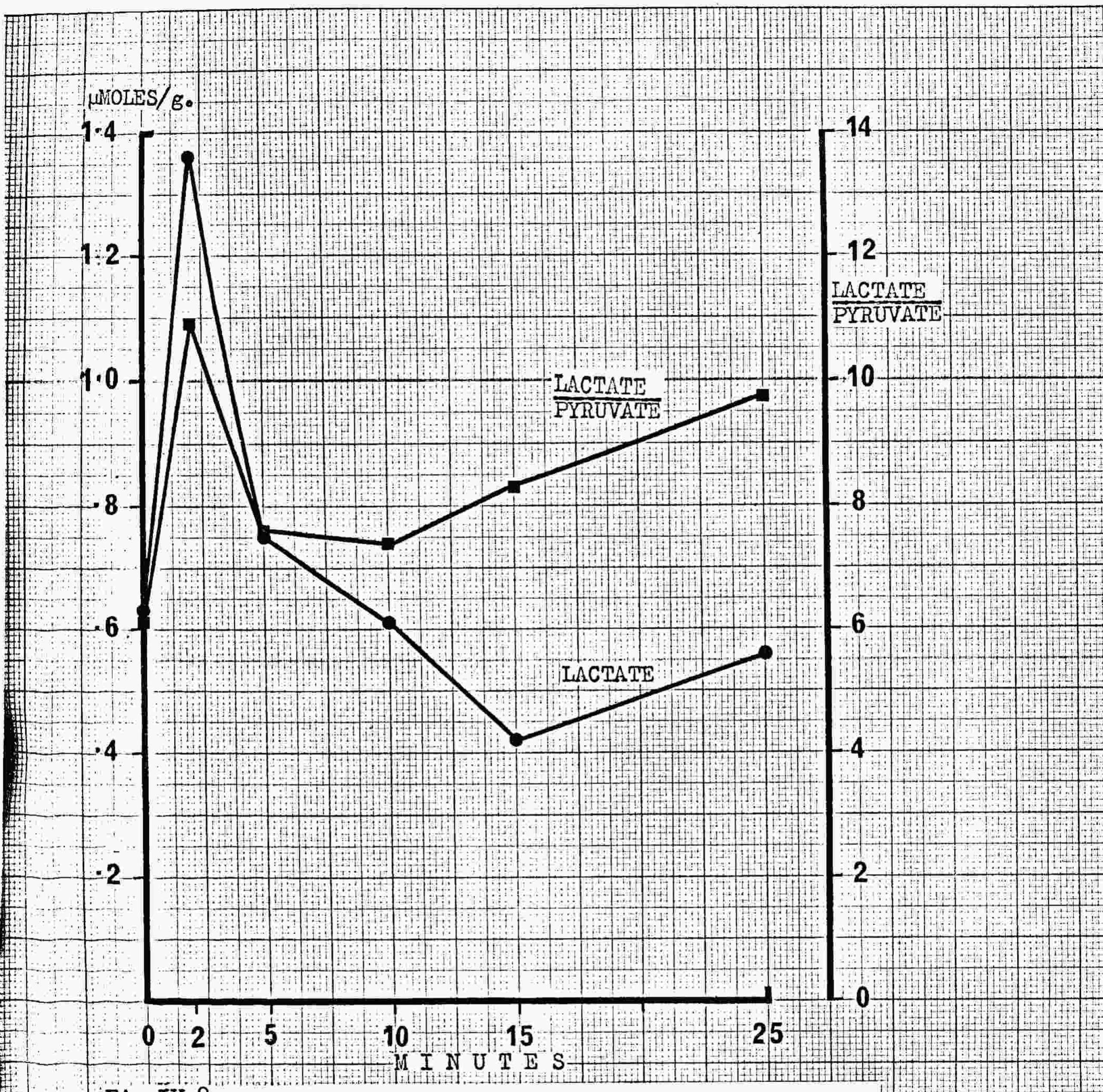


Fig.IV.8

Liver content of lactate and the hepatic $\frac{\text{lactate}}{\text{pyruvate}}$ ratio after administration of ammonium chloride to fed rats injected 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

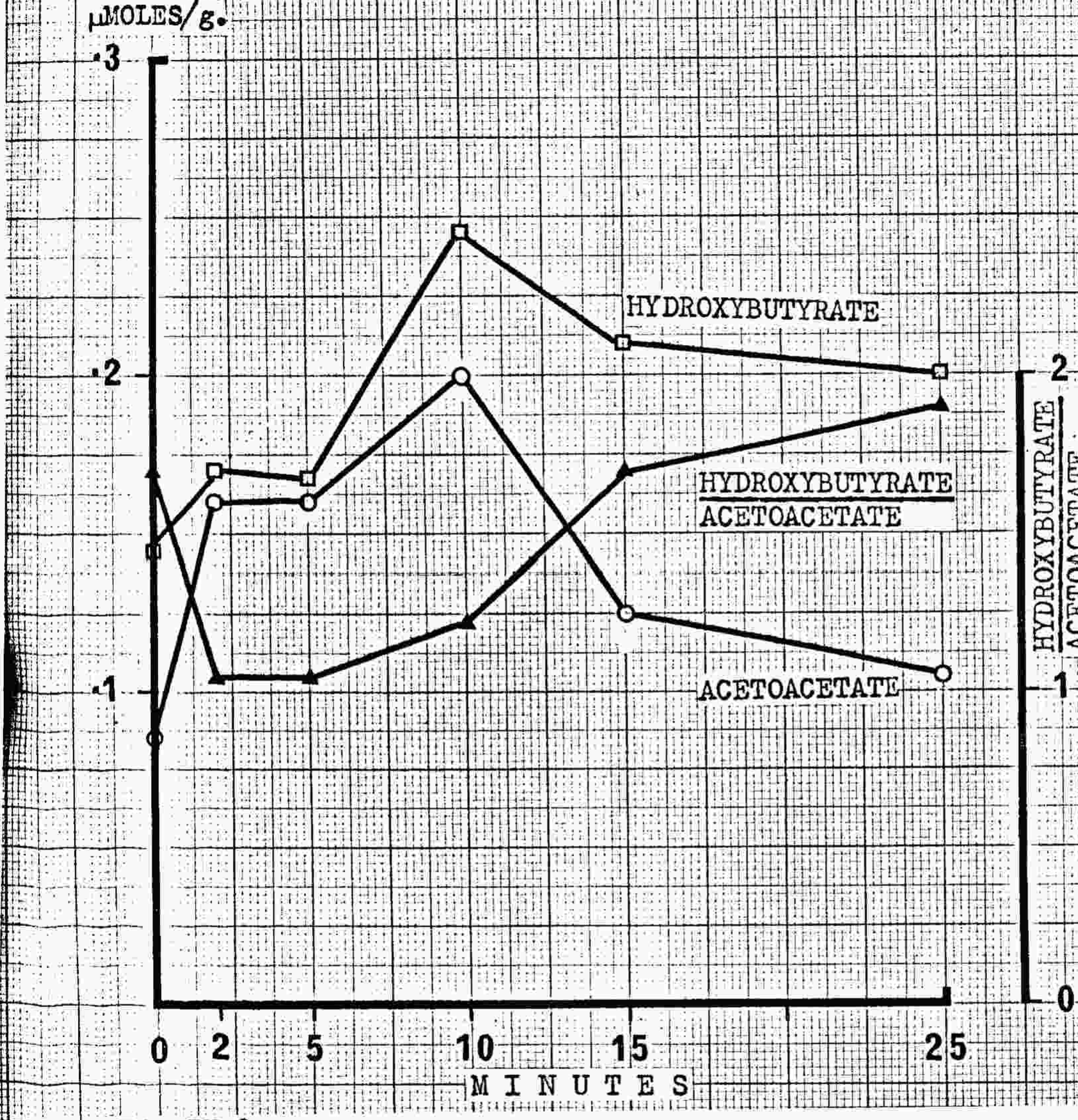


Fig.IV.9

Liver content of acetoacetate and hydroxybutyrate and the hepatic $\frac{\text{hydroxybutyrate}}{\text{acetoacetate}}$ ratio after ammonium chloride administration to fed rats injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

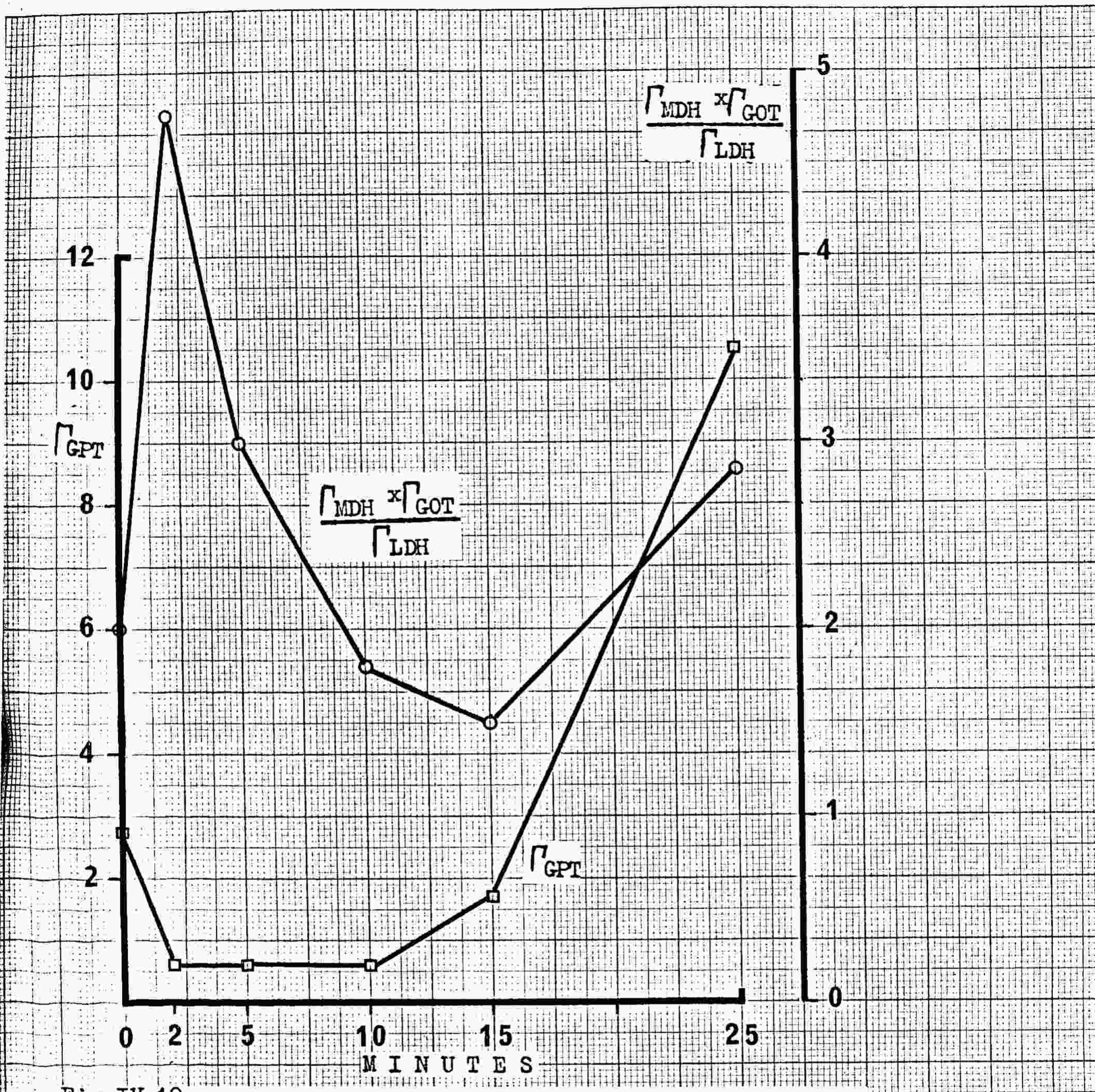


Fig.IV.10

Changes in the mass action ratio for alanine aminotransferase (Γ_{GPT}) and in the indirect ratio for aspartate aminotransferase $\left\{\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right\}$ after ammonium chloride administration to fed rats injected (ip) 60 min. previously with L-cycloserine (25 mg./ kg. body wt.).

observed in saline injected animals (Table IV.4). There was a rapid increase in urea levels (Figure IV.6) which only slowly began to return to initial levels at 25 min. The liver content of acetoacetate (Figure IV.9) was increased and, while the changes in 3-hydroxybutyrate were less regular, there was a definite decrease in the ratio $\frac{[\text{3-hydroxybutyrate}]}{[\text{acetoacetate}]}$ which returned to normal at 15 min.

However, there are important differences between these present experiments and those without L-cycloserine. The tissue content of malate (Figure IV.7) decreased rapidly as in Figure IV.3 but recovered and 'overshot' to a level (0.413 µmoles/g.) which is more than double the initial value and significantly different from it. Even more striking is the behaviour of alanine (Figure IV.6) which does not vary in content at all in contrast to its synthesis in Figure IV.1. Furthermore GPT fluctuated in an uncontrolled fashion often far from the equilibrium position (1.48). This is in accordance with the <u>in vivo</u> inhibition of this enzyme, while the lack of net synthesis of alanine supports the view that alanine aminotransferase was involved in the accumulation of alanine recorded in the original experiment (Figure IV.1).

The changes in aspartate levels (Figure IV.6) were

quite similar to those in the experiment with normal rats except that a plateau is attained between 5 and 15 min. rather than a peak at 5 min. The amount of aspartate accumulating in the first two minutes was double that synthesised in the normal rats (1.20 μmoles/g. as against 0.64 μmoles/g.). In addition, if the area under the aspartate curve in Figure IV.6 is measured and compared to that in Figure IV.1 it is found to be 40% greater (32.6 μmole minutes/g. as opposed to 23.2 μmole minutes/g.). Thus on inhibition of alanine aminotransferase the accumulation of aspartate becomes quantitatively more important in the removal of an ammonia load.

<u>Effect of pre-injection of L-arginine on the response of the liver to an ammonia load</u>

The elevation of alanine and aspartate following an ammonia load is best explained by assuming that the quantity of ammonia injected temporarily over-extended the capacity of the urea cycle so that the intracellular concentration of ammonia became elevated and caused amino acid synthesis by direct mass-action effects. The urea cycle could conceivably be rate-limiting for two main reasons:

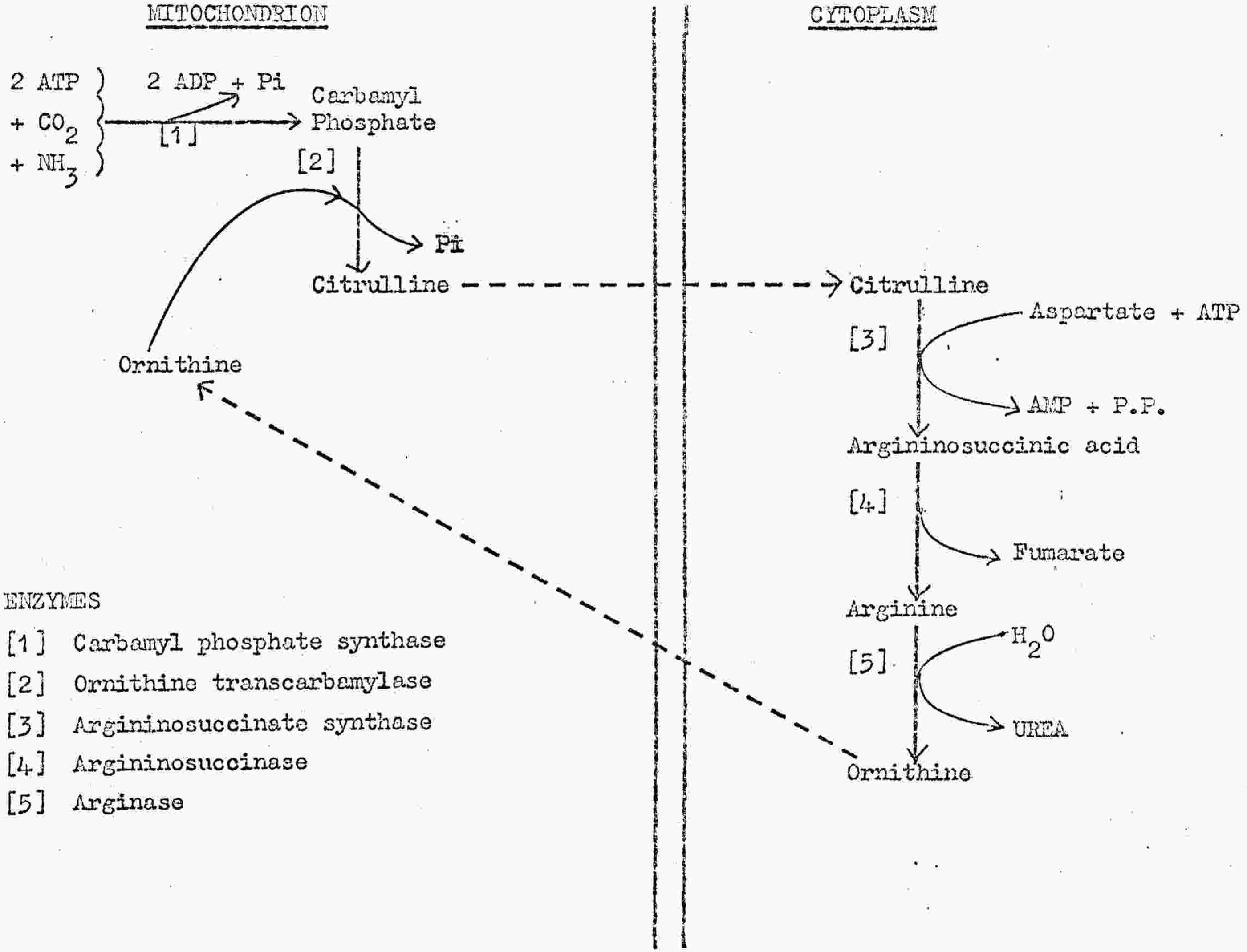
(a) insufficient activity of the cycle enzymes, which could be caused by,

(i) too low a concentration of the respective enzyme proteins,

(ii) too low a concentration of cycle intermediates to saturate fully the existing amounts of the enzymes;

(b) because of the subcellular distribution of the urea cycle enzymes (citrulline synthesis is a mitochondrial process, Grisolia & Cohen (1953), Charles et al. (1967); the synthesis of arginine from citrulline is a cytoplasmic process, Ratner (1954); arginase is exclusively located in the cytoplasm, Rosenthal et al. (1956)) there must be a constant penetration of the inner mitochondrial membrane by citrulline (out) and ornithine (in), (see Scheme 69a). This membrane penetration could also be rate-limiting.

Possibility (a)(ii) can be experimentally tested by artificially elevating the concentrations of the cycle intermediates and then presenting an ammonia load; if the intermediates were rate-limiting an enhanced synthesis of urea and consequently a smaller net synthesis of alanine and aspartate would occur. Greenstein et al. (1956) have shown that L-arginine has a protective effect



ENZYMES

[1] Carbamyl phosphate synthase

[2] Ornithine transcarbamylase

[3] Argininosuccinate synthase

[4] Argininosuccinase

[5] Arginase

on rats which have been given what is normally a lethal dose of ammonium acetate and this has been interpreted as indicating an increase in the activity of the urea cycle. The effect of L-arginine on the synthesis of alanine and aspartate was examined in the next series of experiments. L-Arginine (3 mMoles/kg. body wt.) was administered to fed rats, followed 60 min. later by the usual ammonia load. The rats were killed at various intervals afterwards and their livers freeze-clamped and treated as in Chapter III. It can be seen (Figure IV 11) that the changes in the amino acids alanine, aspartate and glutamate were qualitatively similar to those in normal rats (Figure IV.1) but they were much smaller and more transient. Alanine and aspartate reached maximal levels by 2 min. (instead of 5 min.) and had returned to initial values at 10 min. (instead of 15-25 min.). This is illustrated more clearly by Figure IV 12 which plots the sum (glutamate + alanine + aspartate) after an ammonia load both in normal rats (data from Figure IV.1) and L-arginine-injected rats (data from Figure IV.11). Much less ammonia was stored in these amino acids (and for a much shorter period) in the L-arginine treated rats. This strongly suggests that the maximal activity of the urea cycle <u>in vivo</u> is limited by the concentration

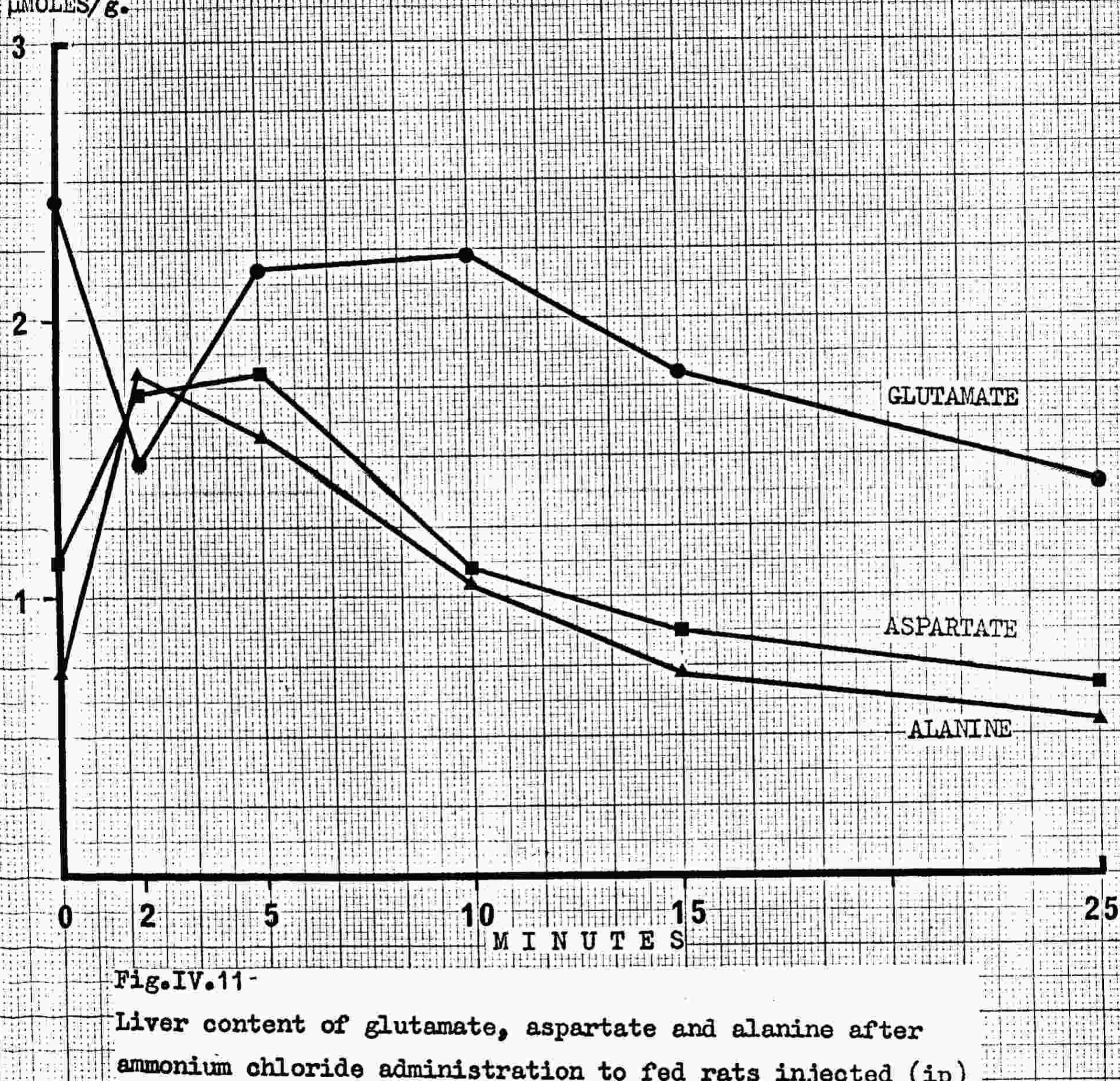


Fig.IV.11-

Liver content of glutamate, aspartate and alanine after ammonium chloride administration to fed rats injected (ip) 60 min. previously with L-arginine (3 mMoles/kg. body wt.).

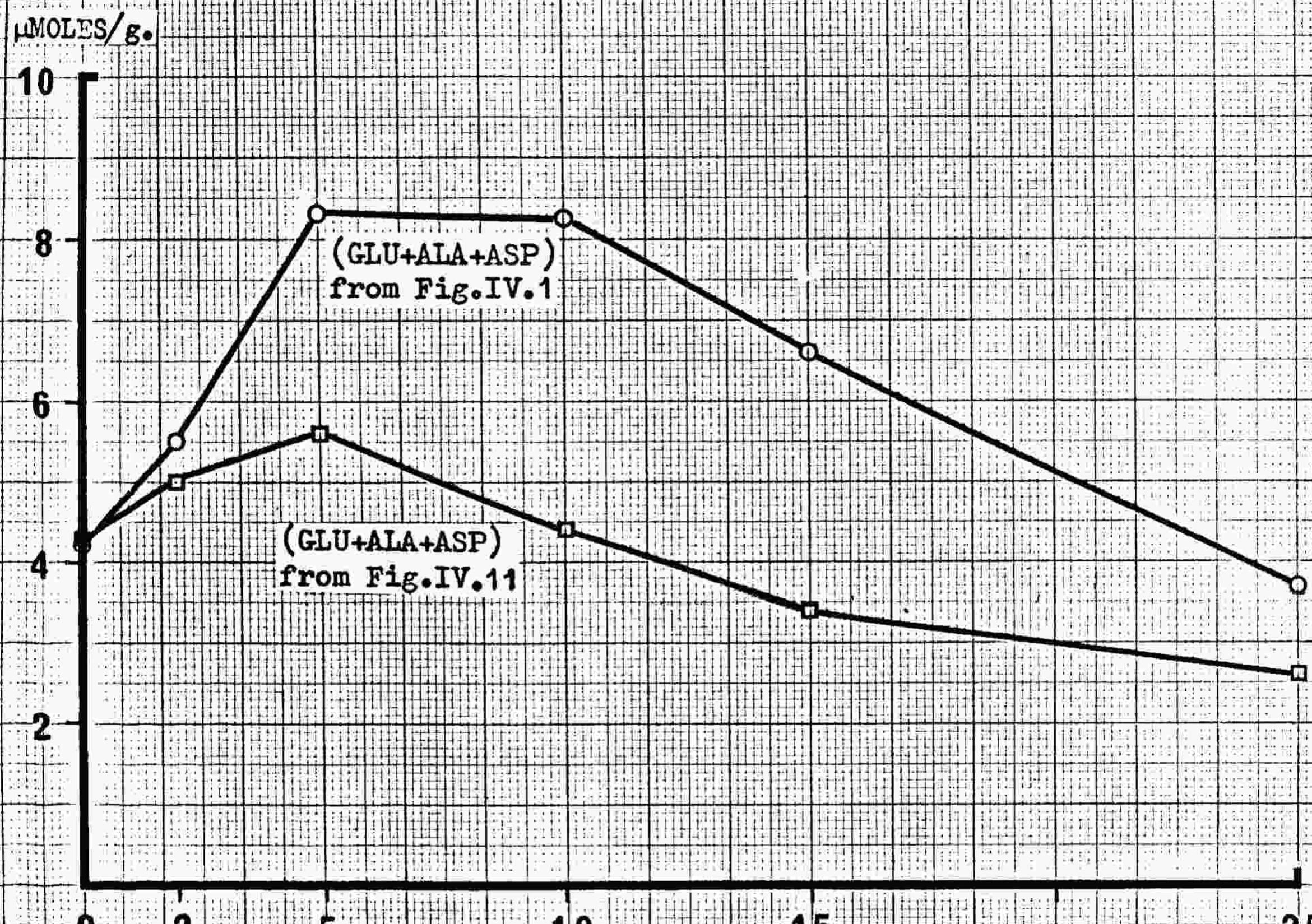


Fig.IV.12

Liver content of (glutamate+alanine+aspartate) after ammonium chloride administration to normal fed rats (data from Fig.IV.1) or to L-arginine preinjected rats (data from Fig.IV.11).

of the cycle intermediates rather than by the amount of the individual enzymes.

Administration of an ammonia load to starved rats

Hepatic metabolism is completely re-orientated during starvation. Fatty acids instead of carbohydrate become the major fuel of respiration and the synthetic activity of the liver is directed to the formation of ketone bodies and glucose, while lipid synthesis is completely suppressed. These changes are accompanied by alterations in the tissue content of many metabolites and enzymes, including some of those involved in ammonia metabolism. For example, the utilization of amino acids for hepatic gluconeogenesis is increased; the activities of alanine and aspartate aminotransferases increase (Knox and Greengard, 1965) as do the activities of the urea cycle enzymes (Schimke, 1963). It was therefore of great interest to study the response of the starved rat to an ammonia load and to compare it with the fed rat. Accordingly, ammonia was administered to rats which had been starved for 48 hours (Figures IV.13 to IV.17). The behaviour of glutamine, aspartate, lactate, pyruvate, 3-hydroxybutyrate and acetoacetate was extremely similar

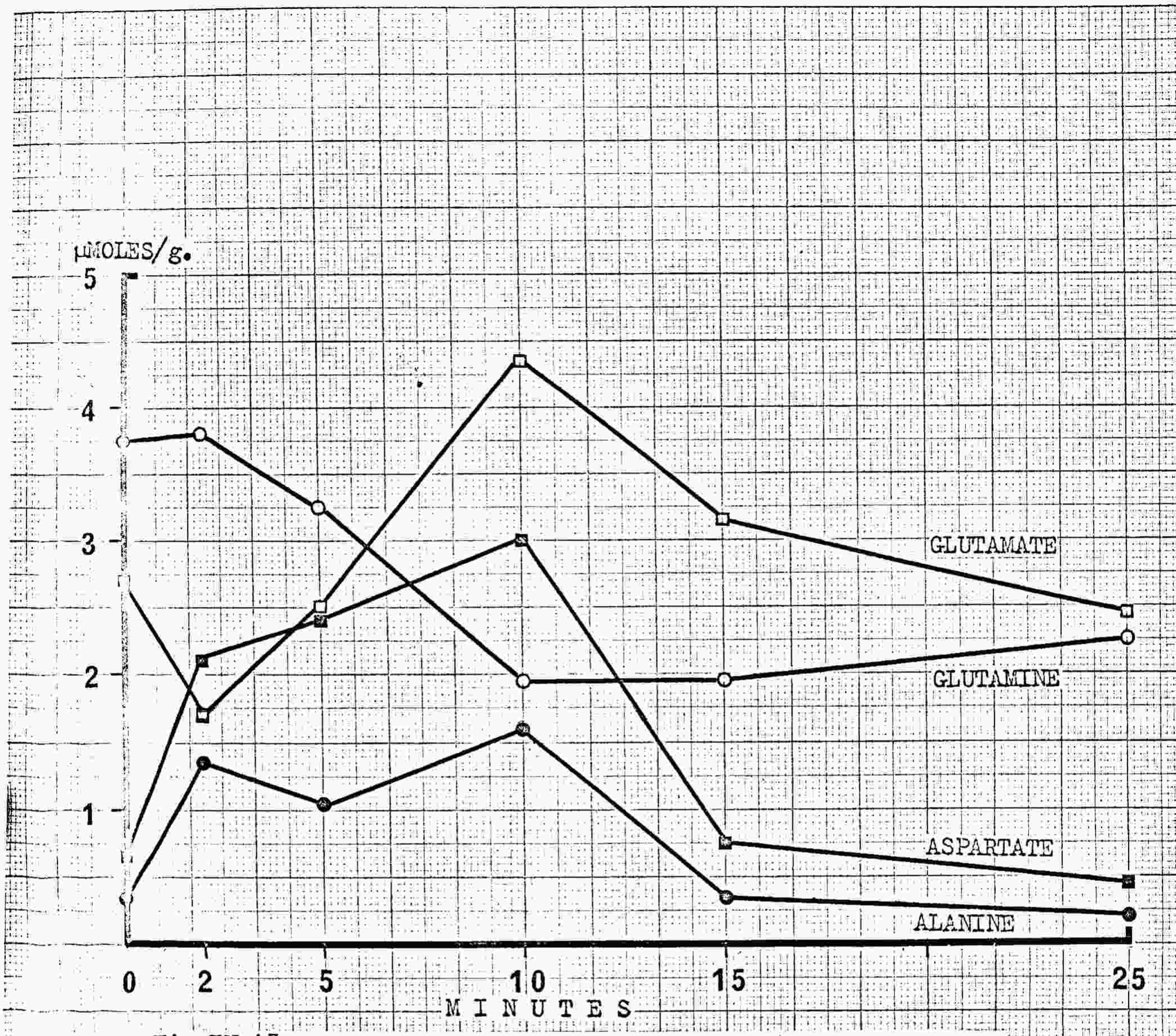


Fig.IV.13

Liver contents of glutamate, glutamine, aspartate and alanine after administration of ammonium chloride to 48-hour fasted rats.

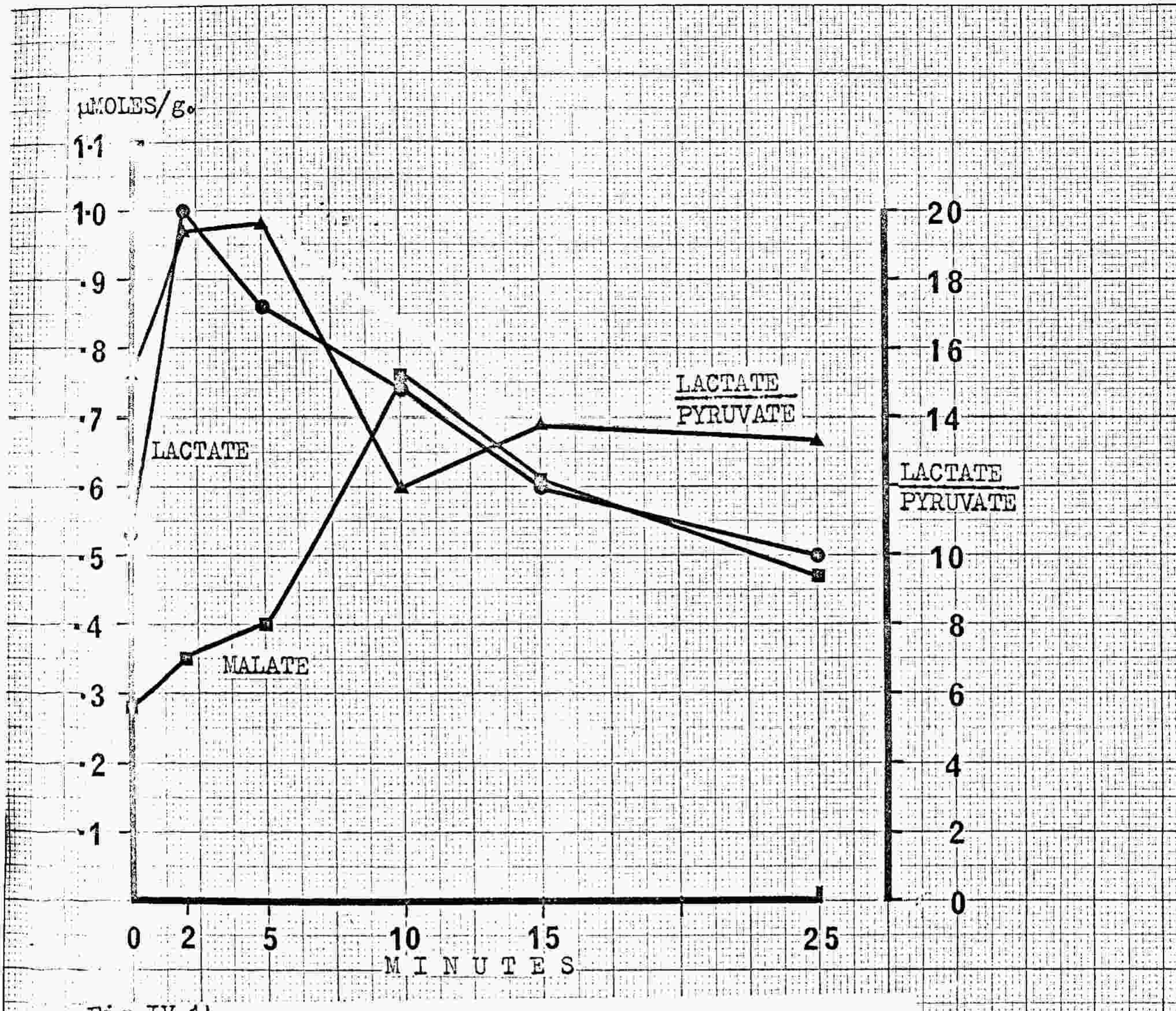


Fig.IV.14

Liver content of lactate and malate and the hepatic $\frac{\text{lactate}}{\text{pyruvate}}$ ratio after administration of ammonium chloride to 48-hour fasted rats.

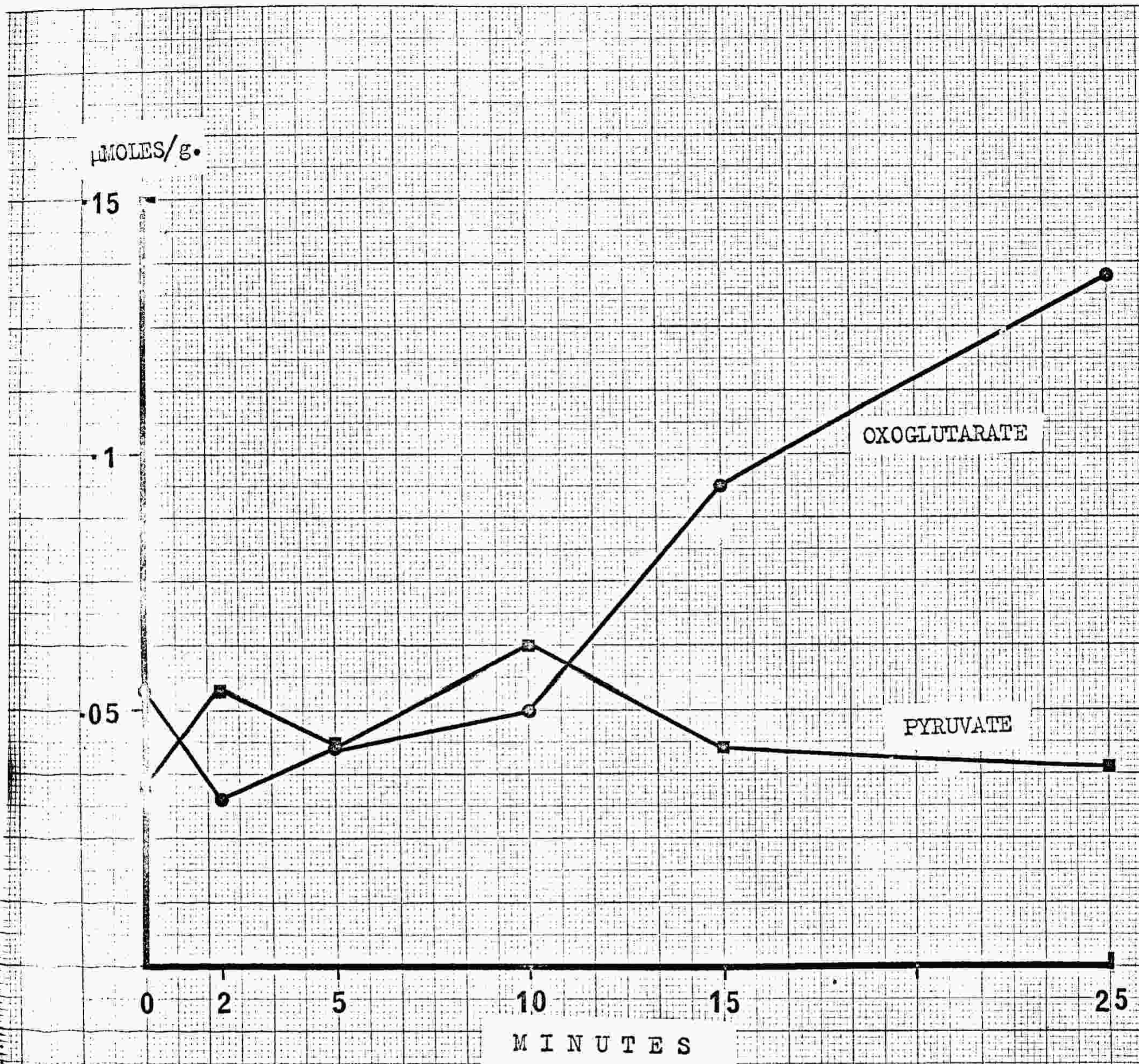


Fig. IV.15.

Liver content of oxoglutarate and pyruvate after administration of ammonium chloride to 48-hour fasted rats.

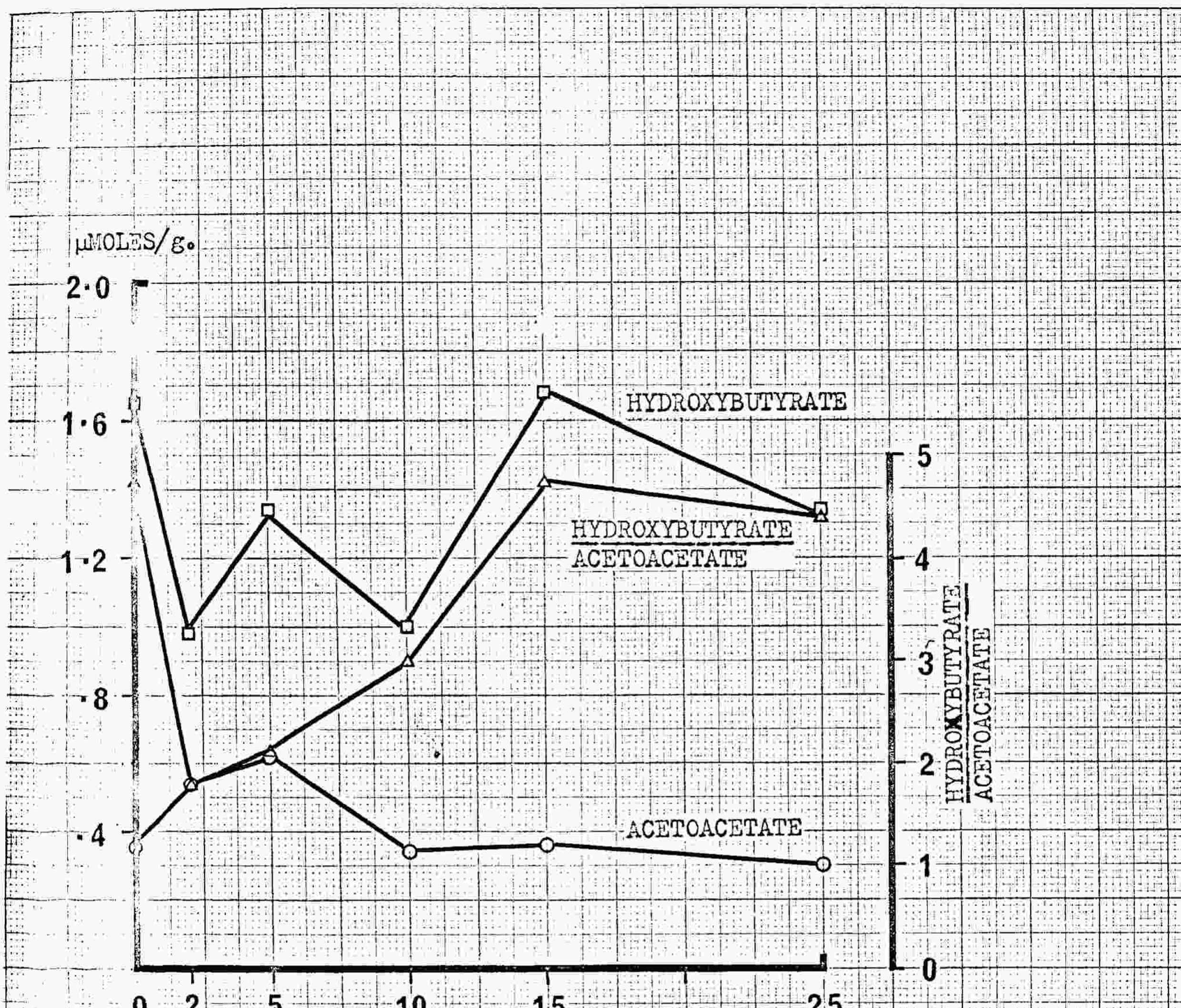


Fig.IV.16

Liver content of hydroxybutyrate and acetoacetate and the hepatic $\frac{\text{hydroxybutyrate}}{\text{acetoacetate}}$ ratio after administration of ammonium chloride to 48-hour fasted rats.

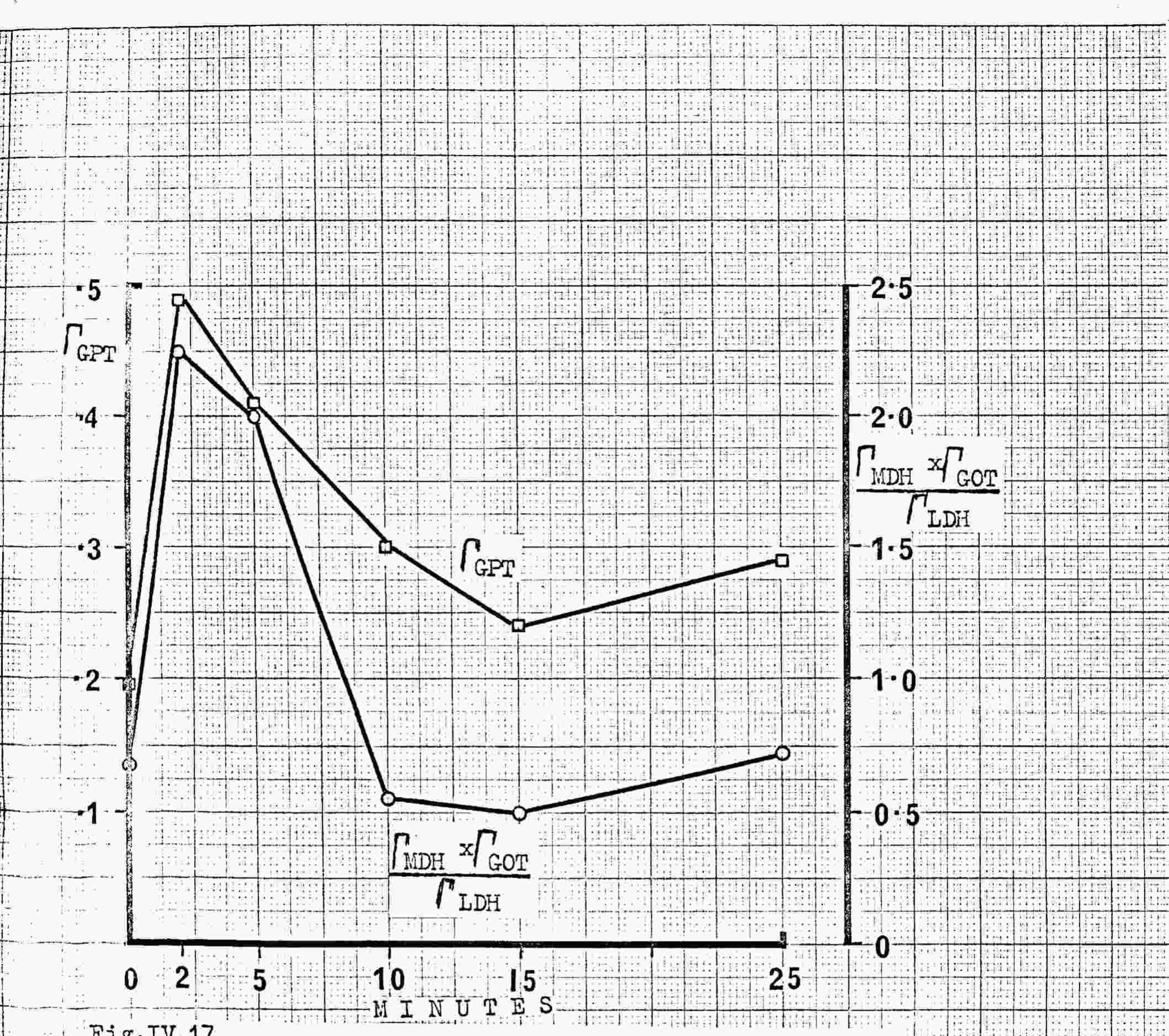


Fig.IV.17

The mass action ratio for alanine aminotransferase (Γ_{GPT}) and the indirect ratio for aspartate aminotransferase $\left(\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right)$ after ammonium chloride administration to 48-hour fasted rats.

to their behaviour in fed rat (Figures IV.1 to IV.4) and so requires no separate exposition here. There are, however, four striking differences between the response of the starved rat and that of the fed rat to an ammonia load:

(a) the behaviour of alanine and GPT,

(b) the fall in glutamine,

(c) the increase in malate levels,

(d) the elevation of oxoglutarate at 25 min.

The initial alanine level (Figure IV.3) was quite low relative to that in the fed animal and the amount formed after ammonia injection was only about 50% of that in fed animals. Furthermore, the mass action ratio for alanine aminotransferase was low (0.194) relative to the equilibrium constant (1.48) or the value in fed rats (about 1.0).

Glutamine (Figure IV.13) showed no tendency to rise, contrary to its behaviour in fed rats, but actually fell to about 50% of the initial level after 10 min. and remained low even at 25 min. Malate (Figure IV.14) increased 2½-fold to a peak at 10 min. and then slowly began to return towards initial values. Oxoglutarate (Figure IV.15) decreased immediately on injection as in fed rats but on recovery 'overshot' to a level 2½ times

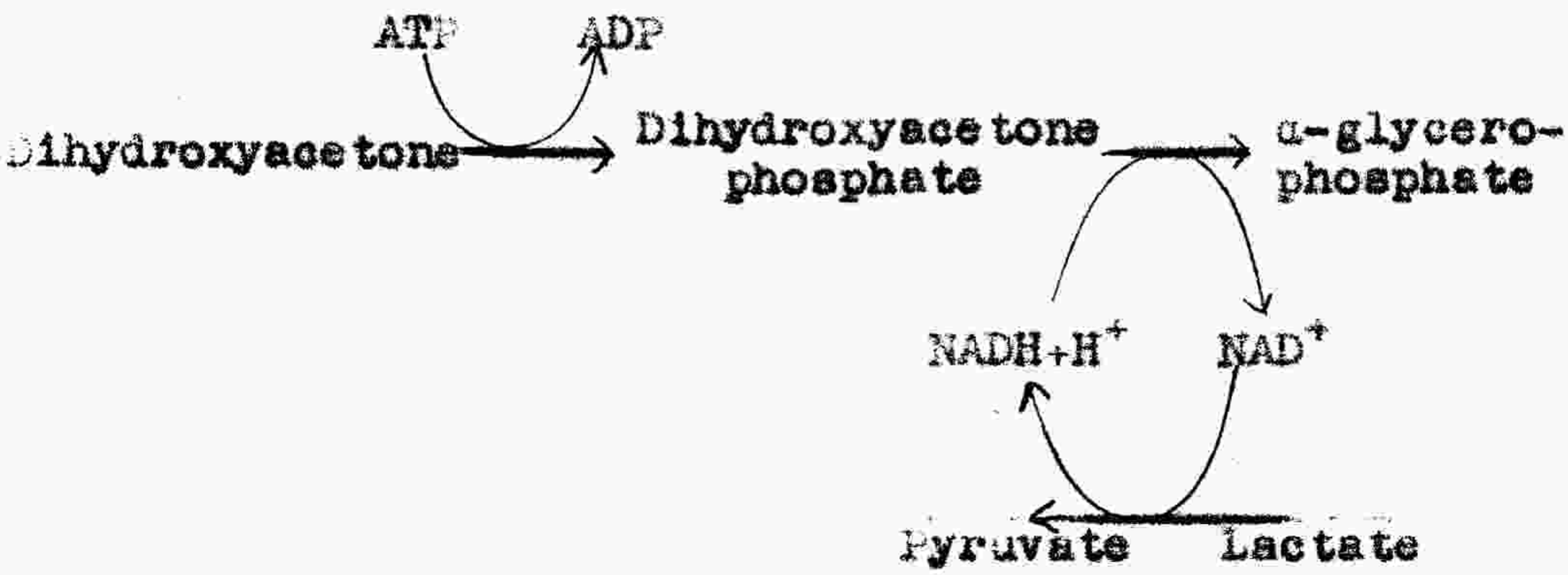
the initial value.

Some of these effects will be examined in more detail in the next experiments.

<u>The effect of elevating the hepatic pyruvate concentration of starved rats on the amount of alanine formed after an ammonia load</u>.

The small formation of alanine in starved rats after an ammonia load and the apparent disequilibrium of the alanine aminotransferase system (Figures IV.13; IV.17) could possibly be explained by the low pyruvate levels in these livers. Hopper & Segal (1962) have made a detailed kinetic analysis of rat liver alanine aminotransferase and from their results it can be deduced that at a glutamate concentration of 2.05 mM (fed level, Figure IV.1) the Km for pyruvate is 0.083 mM - quite close to the <u>in vivo</u> pyruvate level (0.073 µmoles/g.) in these rats. At a glutamate concentration of 2.67 mM (starved level, Figure IV.13) the Km for pyruvate is 0.095 mM which is almost 3-times the <u>in vivo</u> pyruvate level (0.035 µmoles/g.) in starved rats. Thus the functional <u>in vivo</u> activity of the enzyme (as opposed to the total activity which is commonly assayed at near optimal substrate concentrations) could well be decreased in starvation because of the low

pyruvate levels. This hypothesis can be tested if a means of rapidly increasing the hepatic pyruvate concentration were available. Williamson (1967) showed that an intramuscular injection of dihydroxyacetone to starved rats increased the liver contents of lactate and pyruvate; the mechanism for this elevation is presumably as follows: dihydroxyacetone is converted to dihydroxyacetone phosphate in rat liver by glycerokinase and ATP (Bublitz & Kennedy, 1954), the triose phosphate provides an increased flux of carbon to the glycolytic/gluconeogenic pathway thus elevating the concentration of lactate and pyruvate and lowering the lactate/pyruvate ratio through the mediation of α-glycerophosphate dehydrogenase as in the following scheme:



Therefore starved rats (48 hours) were injected intravenously with dihydroxyacetone (1 ml. of 40% (w/v)/200 g.

body wt.) and ten minutes later were given the usual ammonia load. Comparison of the initial (zero time) levels in this experiment (Figures IV.18 to IV.21) with those for starved rats (Figures IV.13 to IV.16) confirms the effects of dihydroxyacetone observed by Williamson (1967). There were large increases (greater than 2-fold) in the hepatic levels of oxoglutarate, lactate and pyruvate and a large decrease (by about 50%) in the concentration of ketone bodies. In addition, the alanine level in the dihydroxyacetone-treated rats increased about 6-fold and the alanine aminotransferase mass action ratio returned to values much closer to the apparent equilibrium (1.48). On injection of ammonium chloride many of the changes were similar to those observed previously for fed or starved rats (decrease in ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$, decrease in oxoglutarate, elevation of lactate) and need not be enlarged upon here. The changes in the amino acids (Figure IV.21), however, merit special attention. The variations in the liver contents of glutamate and aspartate were very similar to those observed in starved rats (Figure IV.13) except that the amount of aspartate formed was much smaller. The synthesis of alanine (Figure IV.21) was much greater (peak of 7.5 μmoles/g. at 10 min.) than in starved rats (Figure IV.13) peak of 1.6

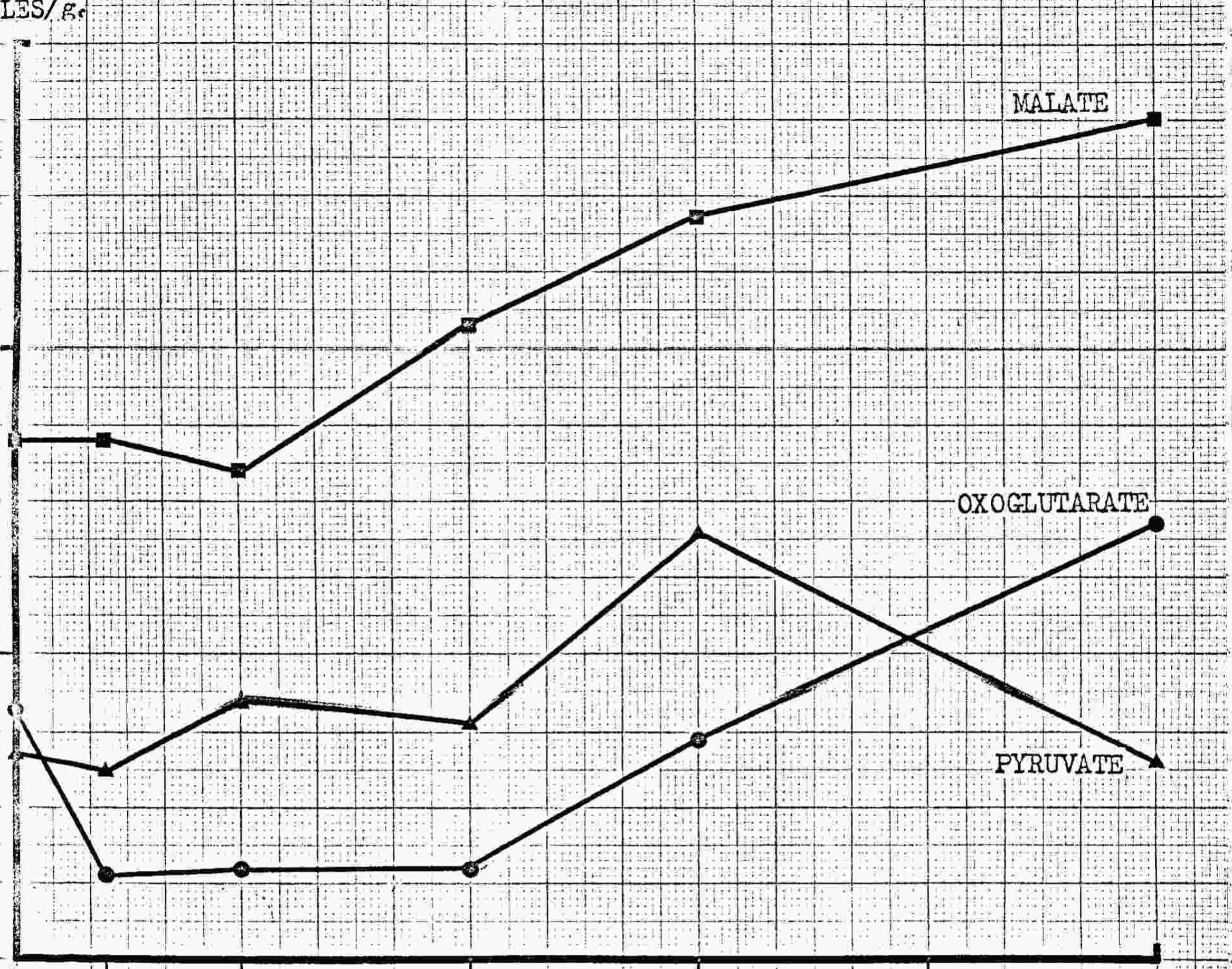


Fig.IV.18

Liver content of malate, oxoglutarate and pyruvate after ammonium chloride administration to 48-hour fasted rats injected (im) 10 min. previously with dihydroxyacetone (2g./kg. body wt.).

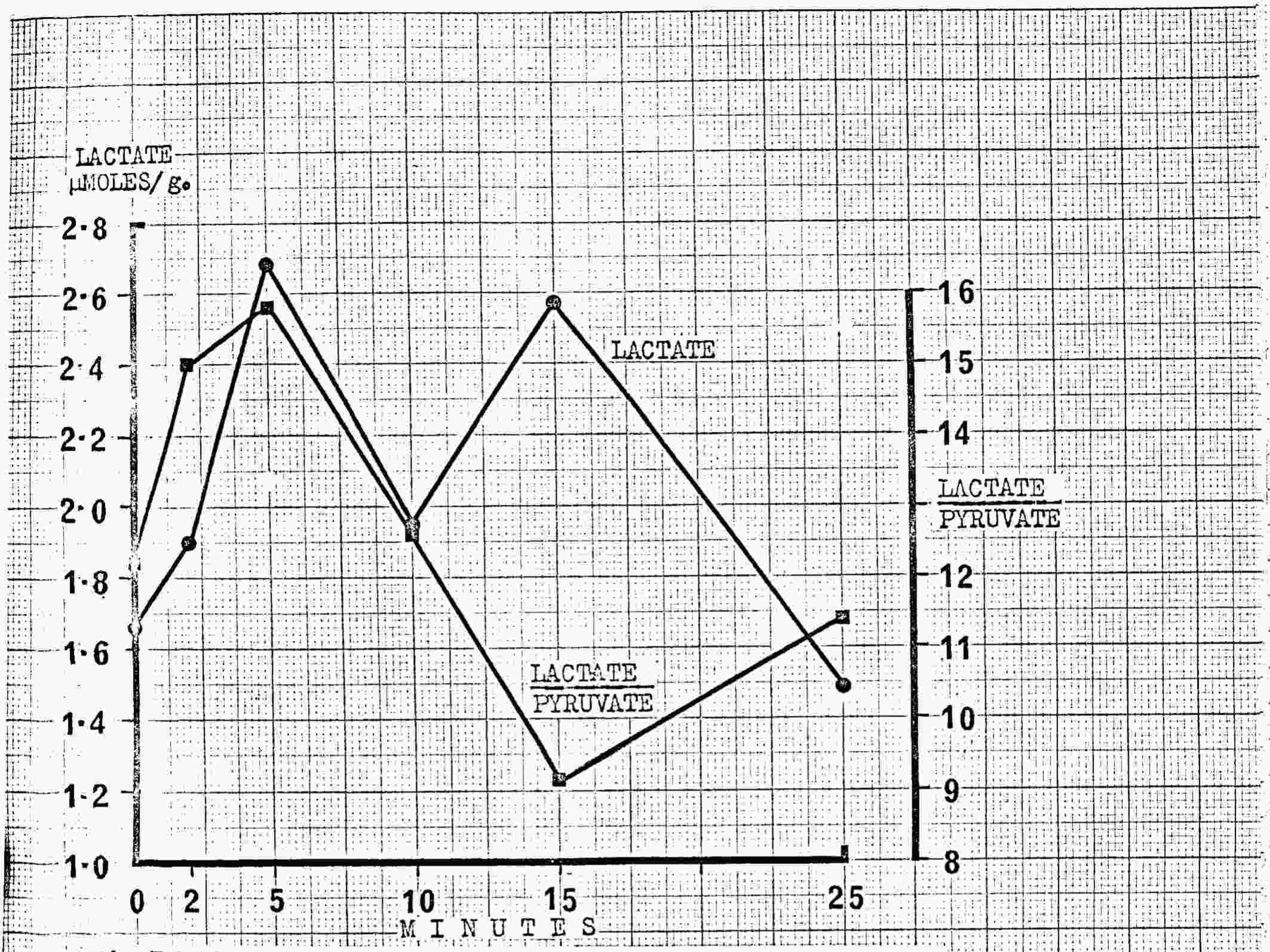


Fig.IV.19

Liver content of lactate and the hepatic $\frac{\text{lactate}}{\text{pyruvate}}$ ratio after ammonium chloride administration to 48-hour fasted rats injected 10 min. previously with dihydroxyacetone (2g./kg. body wt.).

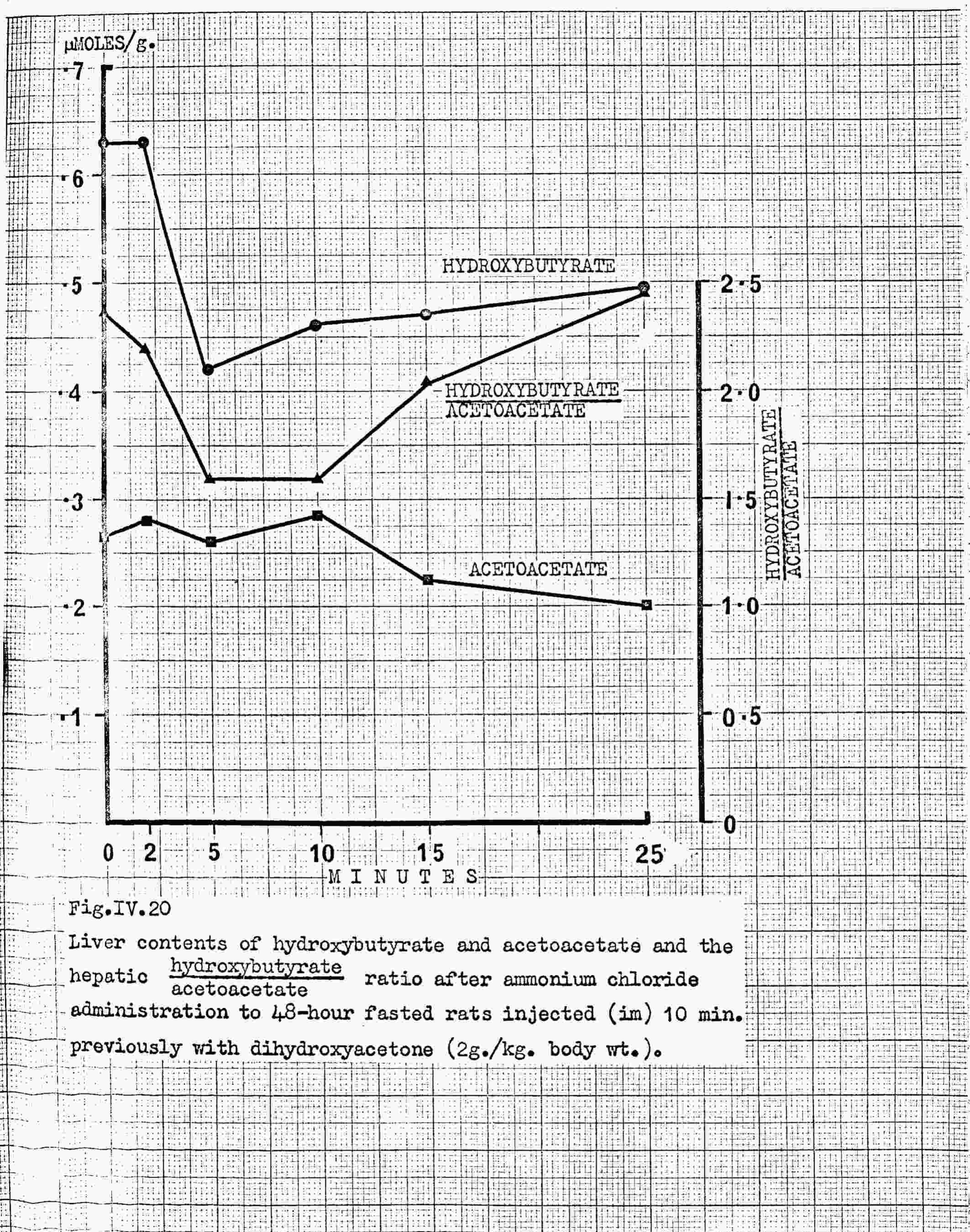


Fig.IV.20

Liver contents of hydroxybutyrate and acetoacetate and the hepatic $\frac{\text{hydroxybutyrate}}{\text{acetoacetate}}$ ratio after ammonium chloride administration to 48-hour fasted rats injected (im) 10 min. previously with dihydroxyacetone (2g./kg. body wt.).

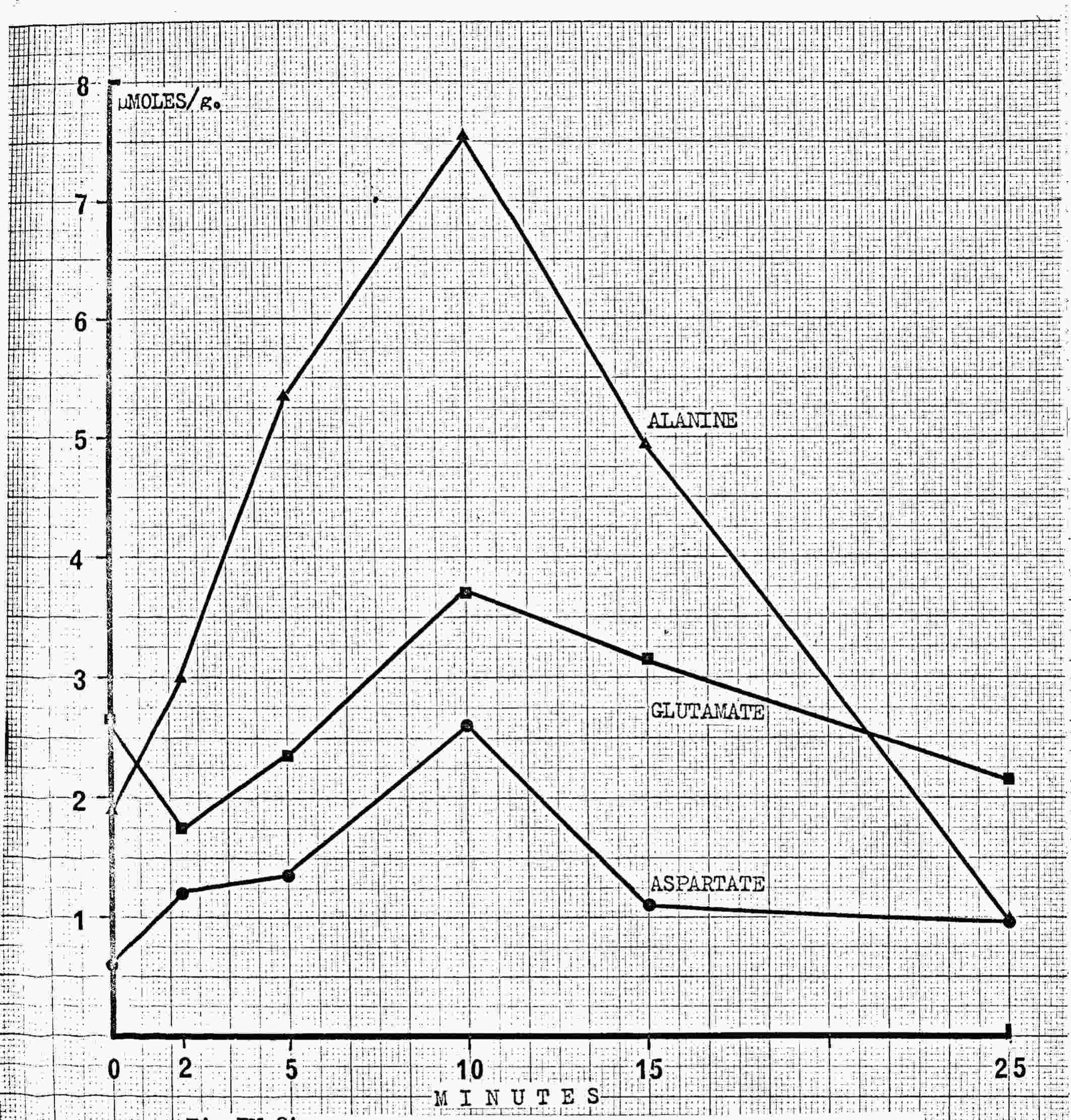


Fig.IV.21

Liver content of glutamate, aspartate and alanine after administration of ammonium chloride to 48-hour fasted rats injected (im) 10 min. previously with dihydroxyacetone (2g./kg. body wt.).

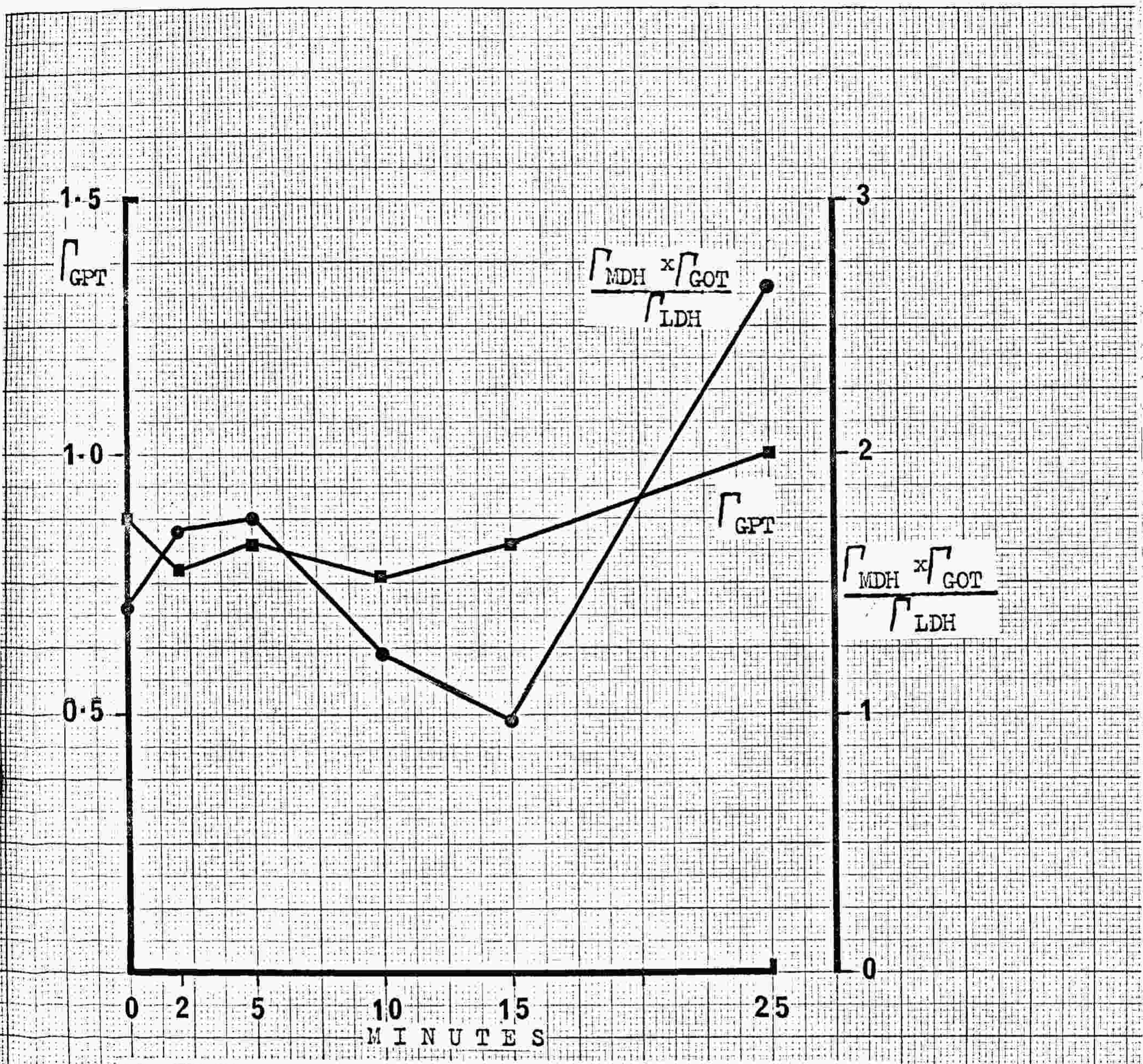


Fig.IV.22

The mass action ratio for alanine aminotransferase (Γ_{GPT}) and the indirect ratio for aspartate aminotransferase $\left(\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}\right)$ after administration of ammonium chloride to 48-hour fasted rats, injected (im) 10 min. previously with dihydroxyacetone (2g./kg. body wt.).

μmoles/g. at 10 min.) or even in fed rats (Figure IV.1, peak of 3.2 μmoles/g. at 5 min.). These results are good evidence for the hypothesis that the small extent of alanine synthesis in starved rats after administration of an ammonia load is due to the low hepatic pyruvate concentration in this situation. Since, in these experiments, the ammonia load was administered only 10 min. after dihydroxyacetone injection, long-term phenomena, such as enzyme induction, cannot be important. The smaller accumulation of aspartate in these experiments than in those with normal starved rats suggests that the high pyruvate concentration enabled alanine amino-transferase to compete more efficiently with aspartate aminotransferase for the available glutamate.

Apparent disequilibrium of glutamate dehydrogenase and hydroxybutyrate dehydrogenase 25 min. after administration of an ammonia load to starved rats.

In all the ammonia loading experiments so far reported here there was a tendency for oxoglutarate to 'overshoot', i.e. tissue contents of oxoglutarate always decreased rapidly after injection but on recovery reached levels at 25 min. which were greater than the initial level (Figures IV.3, IV.7, IV.15, IV.18). This effect was especially

striking in starved rats (Figure IV.15) where the 25 min. value was 2.5-fold greater than the initial level. It was therefore decided to investigate this phenomenon, in greater detail, in starved rats.

Starved (48 hour) rats were injected with the usual load of ammonium chloride and their livers freeze clamped 5, 25 and 50 min. afterwards. The following intermediates were measured: glutamate, oxoglutarate, ammonia, hydroxybutyrate, acetoacetate, isocitrate, citrate, malate, pyruvate, lactate, aspartate, NAD and NADH (Table IV.5). Ammonia was not measured in previous loading experiments because it was felt that a large variable error could be introduced immediately post-injection by contamination of the liver tissue with peritoneal fluid containing a high concentration of ammonia. Ammonia was measured in this experiment and the relatively small standard deviation of the 5 min. value suggests that there was no large error due to contamination. All the changes observed in the previous experiment with starved rats (Figures IV.13 to IV.16) were confirmed here and all the intermediates, including oxoglutarate, had virtually returned to their starting values by 50 min. Ammonia was 5-fold elevated at 5 min. post-injection but had returned to control levels by 25

Table I.5 Effect of NH_4Cl injection on some rat liver metabolites in fasted rats

Rats (male; 180-200 g.; 48-hour starved) were injected (i.p.) with NH_4Cl (2.5 mMoles/kg. body wt.), killed at the times indicated and their livers freeze-clamped. The control group (0 min.) were not injected. For other details see Chapter II. Results are in µMoles/g. fresh wt. ± S.D. There were 5 animals in the 0 min. and 50 min. groups and 4 animals in the 5 min. and 25 min. groups.

	0 min.	5 min.	25 min.	50 min.
Glutamate	2.88 ±0.251	3.02 ±0.589	1.74 ±0.793	2.47 ±0.299
Oxoglutarate	0.073 ±0.028	0.033 ±0.017	0.525±0.098	0.077±0.037
Ammonia	0.881 ±0.401	4.25 ±0.936	0.856±0.380	0.714±0.359
Hydroxybutyrate	1.62 ±0.414	1.15 ±0.776	1.45 ±0.284	1.34 ±0.250
Acetoacetate	0.870 ±0.154	1.23 ±0.601	0.893±0.140	0.793±0.092
Isocitrate	0.0094±0.0026	0.0054±0.0016	0.035±0.015	0.0098±0.015
Citrate	0.180 ±0.048	0.152 ±0.042	1.05 ±0.435	0.272 ±0.063
Malate	0.174 ±0.032	0.253 ±0.067	0.532±0.143	0.179±0.049
Pyruvate	0.023 ±0.004	0.036 ±0.020	0.028±0.002	0.024±0.007
Lactate	0.364± 0.143	0.512 ±0.529	0.537±0.151	0.328±0.115
Aspartate	0.894 ±0.216	2.83 ±1.80	0.731±0.095	0.842±0.181

min. Citrate and isocitrate behaved in a parallel way - both fell immediately on injection (5 min.), rose at 25 min. to levels 4-5 times higher than the control and returned to approximately normal values after 50 min. This elevation of citrate after ammonia injection is similar to that observed by Katanuma et al. (1966) in mice. These workers interpreted their finding as being due to a direct inhibition or inactivation of isocitrate dehydrogenase by ammonia leading to an accumulation of isocitrate and citrate.

The ratios $\frac{[NAD]}{[NADH]}$ mito, $\frac{[NADP]}{[NADPH]}$ cyto, and $\frac{\Gamma GOT \times \Gamma MDH}{\Gamma LDH}$ have been calculated from the data in Table IV.5 and are presented in Figure IV.23(a), (b), (c). The ratio $\frac{\text{total NAD}}{\text{total NADH}}$ is also plotted on Figure IV.23(c). There was fairly good agreement between the two values of the mitochondrial NAD/NADH ratio except at 25 min. where the value obtained from the glutamate dehydrogenase coupde was oxidised relative to the initial ratio though that obtained from the hydroxybutyrate dehydrogenase couple had returned to control values. There was also fairly good agreement between the two estimates of cytoplasmic $\frac{[NADP]}{[NADPH]}$ except at 25 min. where the ratio as calculated from isocitrate dehydrogenase was oxidised while that calculated from malic enzyme was reduced

Fig.IV.23(a)

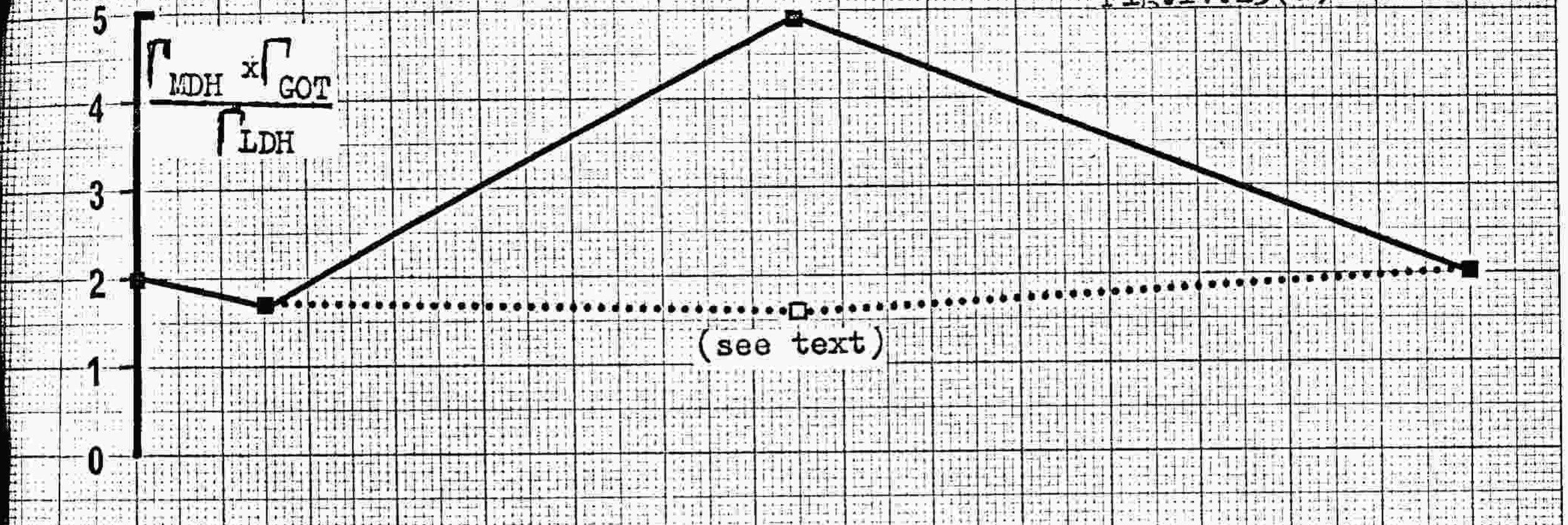


Fig.IV.23(b)

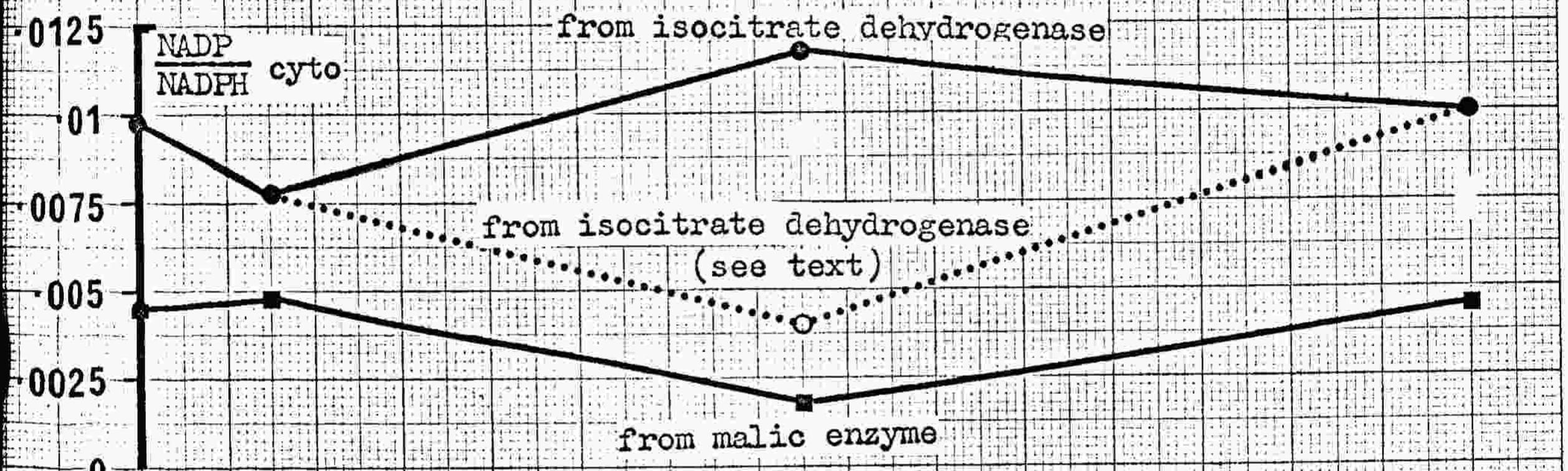
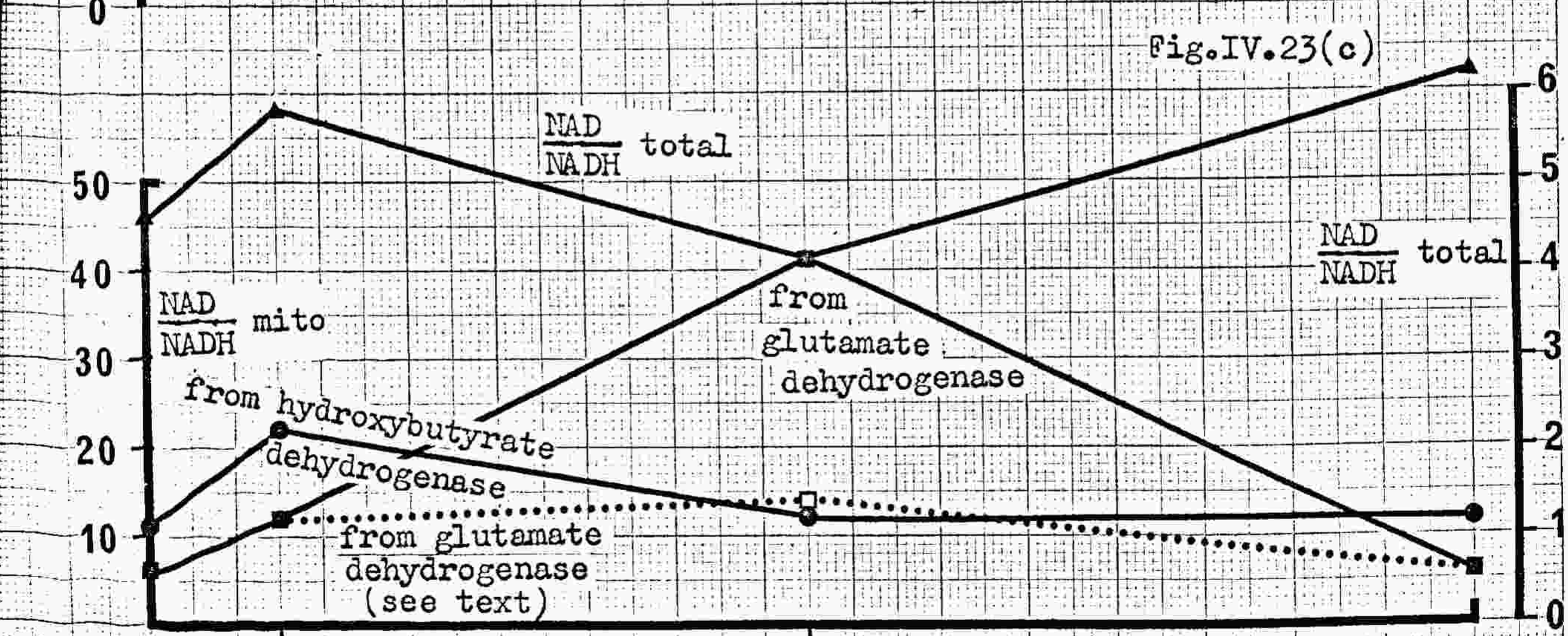


Fig.IV.23(c)



Figs.IV.23 (a),(b),(c).

The relationships in these figures are calculated from the data in Table.IV.5. For the significance of the dotted lines, see text.

relative to the initial ratios. The value of $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ was always close to 2.0 except at 25 min. where it reached a high value of 4.93, three times the theoretical value of 1.65. Thus in all these functions there was an apparent disequilibrium at 25 min. In addition, in Figure IV.23(c) the ratio $\frac{\text{total NAD}}{\text{total NADH}}$ parallels the $\frac{[NAD]}{[NADH]}$ mito calculated from the hydroxybutyrate dehydrogenase couple much closer than it does the ratio calculated from glutamate dehydrogenase. Thus, if the ratio calculated from total tissue contents of NAD and NADH is closely related to the $\frac{[NAD]}{[NADH]}$ mito (see Chapter III) the $\frac{[hydroxybutyrate]}{[acetoacetate]}$ at 25 min. is more likely to represent an accurate estimate of $\frac{[NAD]}{[NADH]}$ mito than is $\frac{[glutamate]}{[oxoglutarate] \times [ammonia]}$.

The only metabolite common to all three functions where apparent disequilibrium exists at 25 min. is oxoglutarate which has increased to a value about 3-times the initial liver content. It seems possible that oxoglutarate may be behaving in an abnormal way. If it is assumed that the real intracellular concentration of oxoglutarate at 25 min. is one-third of that actually measured and if the expressions involving oxoglutarate in Figures IV.23 (a),(b),(c) are recalculated on this basis then all three of the apparent disequilibria

disappear. This is illustrated in Figures IV.23 (a),(b), (c), where $\frac{[NAD]}{[NADH]}$ (glutamate dehydrogenase), $\frac{[NADP]}{[NADPH]}$ (isocitrate dehydrogenase) and $\frac{\lceil GOT \times \lceil MDH}{\lceil LDH}$ are all replotted (broken lines) on this basis. Thus the possibility must be considered that a large fraction of the hepatic oxoglutarate is not thermodynamically active at the site of glutamate dehydrogenase (mitochondrial matrix) or at the sites of cytoplasmic aspartate aminotransferase or cytoplasmic isocitrate dehydrogenase. An alternative explanation is that some of the enzymes concerned are inhibited or inactivated, either by the ammonia load per se or by change(s) in concentration(s) of effector(s) brough about by the ammonia load.

Effect of a second ammonia load at 25 min. on the apparent disequilibria at this time.

The two possibilities outlined above can be examined by the stratagem of administering a second ammonia load to these animals at 25 min. after the first one and studying the immediate (5 min.) reaction to it. If the relevant enzymes were inhibited or inactivated then the response to this second ammonia load might be expected to be different to the response to the original load. Alternatively, if a large portion of the oxoglutarate was sequestered, injection of a second ammonia load

should produce little change in its concentration. An important proviso is that ammonium ions alone cannot relieve the postulated enzyme inhibition or free the sequestered oxoglutarate.

Therefore the usual load of NH_4Cl was administered to starved (48-hour) rats which were then killed either 25 min. or 30 min. later. As there were no significant differences between the 25 or 30 min. livers they are grouped together as Group A (Table IV·6). Group B consisted of starved rats which were injected with the usual ammonia load, reinjected with the same load after 25 min. and killed five minutes later. An examination of the data in Table IV·6 and its comparison with the 5 min. data in Table IV·5 suggests that none of the three oxoglutarate-linked enzymes were inhibited at 25 min. The synthesis of aspartate demonstrated the efficient action of aspartate aminotransferase; the fall in isocitrate (parallel to the decrease in the ratio $\frac{[malate]}{[pyruvate]}$ from 24 to 9) testified to the functioning of isocitrate dehydrogenase and the decrease in the ratio $\frac{[hydroxybutyrate]}{[acetoacetate]}$ (from 2.36 to 0.66) illustrated the activity of glutamate dehydrogenase since this change could not be effected save by prior oxidation of $\frac{[NAD]}{[NADH]}$ mito as mediated by glutamate dehydrogenase.

Table IV.6

Effect of a second injection of NH_4Cl on some rat liver metabolites in fasted rats

Rats (male; 180-200 g.; 48-hour starved) were injected (i.p.) with NH_4Cl (2.5 mMoles/kg. body wt.) and either killed 25 min. or 30 min. later (group A), or reinjected with NH_4Cl (2.5 mMoles/kg. body wt.) at 25 min. and killed 5 min. later (group B). For other details see Chapter II. Results are in μMoles/g. fresh wt. ± S.D. There were 8 animals in group A and 4 in group B.

	Group A	Group B
Glutamate	1.74 ±0.391	3.82 ±0.889
Oxoglutarate	0.291±0.151	0.053 ±0.014
Ammonia	0.646±0.202	3.32 ±0.595
Hydroxybutyrate	1.51 ±0.538	0.812 ±0.488
Acetoacetate	0.645±0.216	0.920 ±0.449
Isocitrate	0.028±0.0083	0.0076±0.0025
Citrate	0.890±0.292	0.235 ±0.036
Malate	0.666±0.305	0.320±0.082
Pyruvate	0.028±0.005	0.034 ±0.003
Lactate	0.651±0.177	0.490 ±0.162
Aspartate	0.564±0.165	1.88 ±0.971

These responses are similar to those obtained (see Table IV.5) after a single ammonia load to starved rats. A most important result from this experiment was the removal of over 80% of the liver oxoglutarate. Since this implies that most of the oxoglutarate is rapidly available to glutamate dehydrogenase it makes the explanation of the 25 min. disequilibria by postulating a sequestration of oxoglutarate extremely tenuous.

The parameters which exhibited apparent disequilibrium at 25 min. have been calculated from the data in Table IV.6 and are presented in Figures IV.24(a),(b),(c). It can be seen that this second injection of ammonia restored near-equilibrium to all three functions. The expression $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ decreased from 3.28 to 1.2 (near the equilibrium value of 1.65); the cytoplasmic $\frac{[NADP]}{[NADPH]}$ ratio as calculated from the reactants of isocitrate dehydrogenase became more reduced while that calculated from malic enzyme became more oxidised so that the 25 min. disagreement between the two estimates has largely disappeared; also the disagreement between the two estimates of $\frac{[NAD]}{[NADH]}$ mito has decreased.

A major difference between the present experiment and the immediate response of the normal starved rat to an ammonia load is evident in the behaviour of the ratio

Fig.IV.24(a)

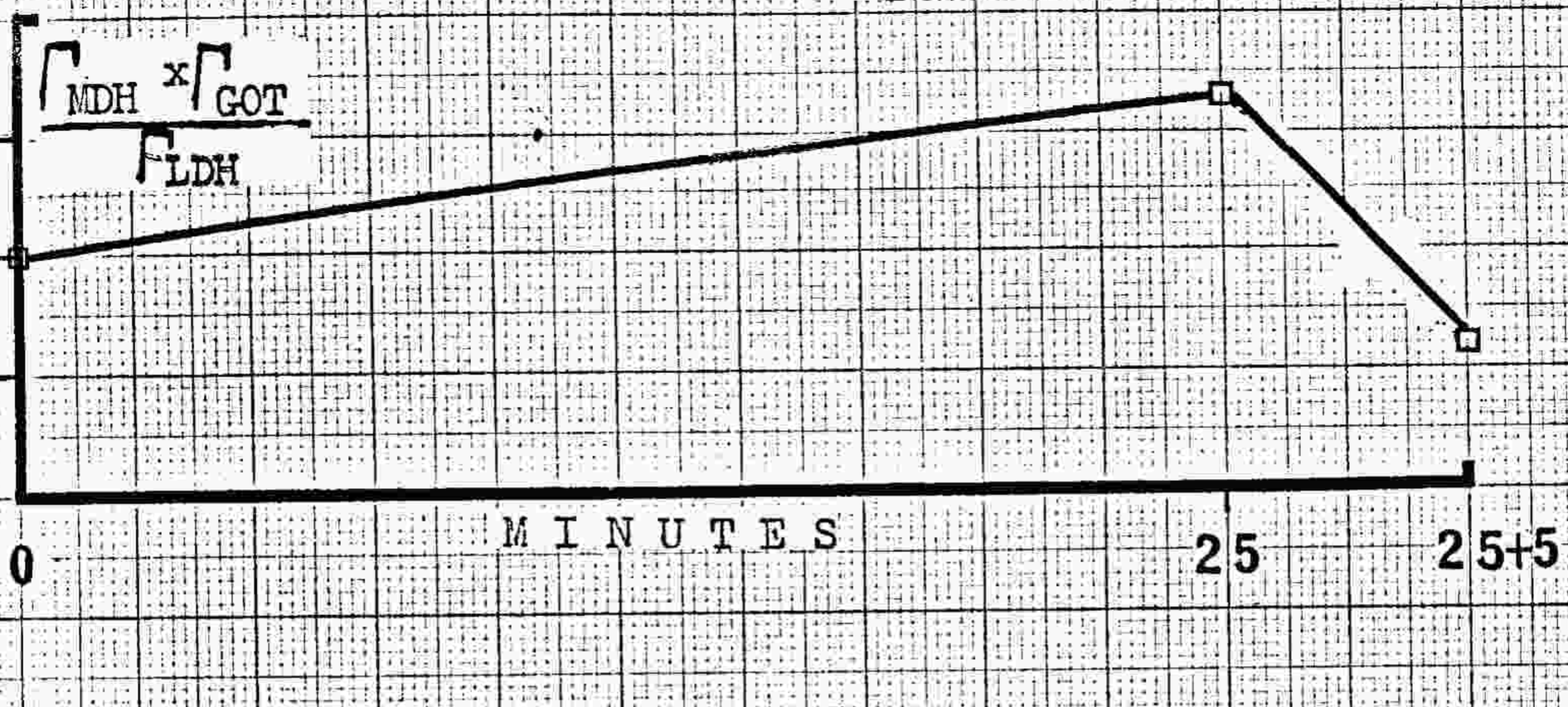


Fig.IV.24(b)

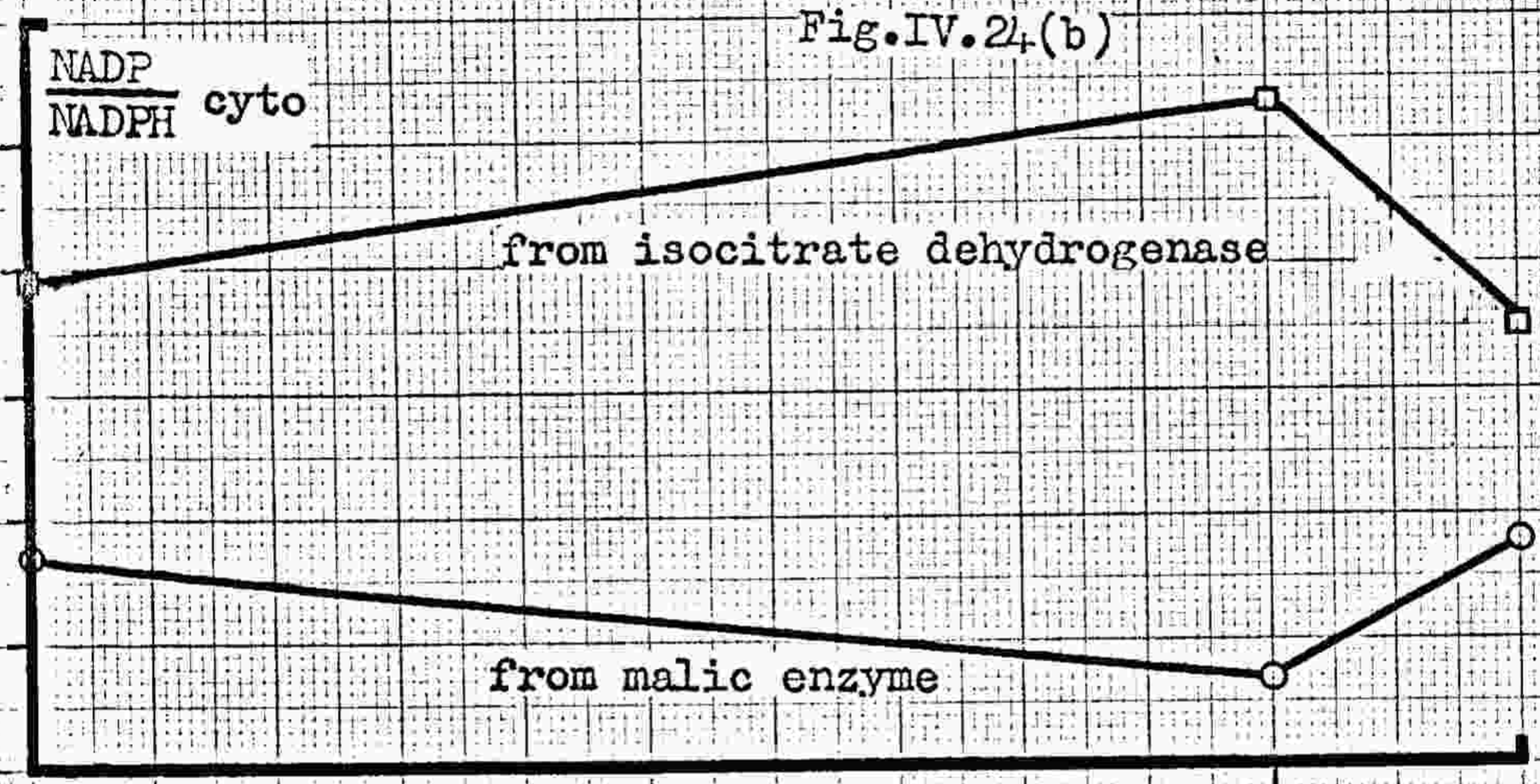
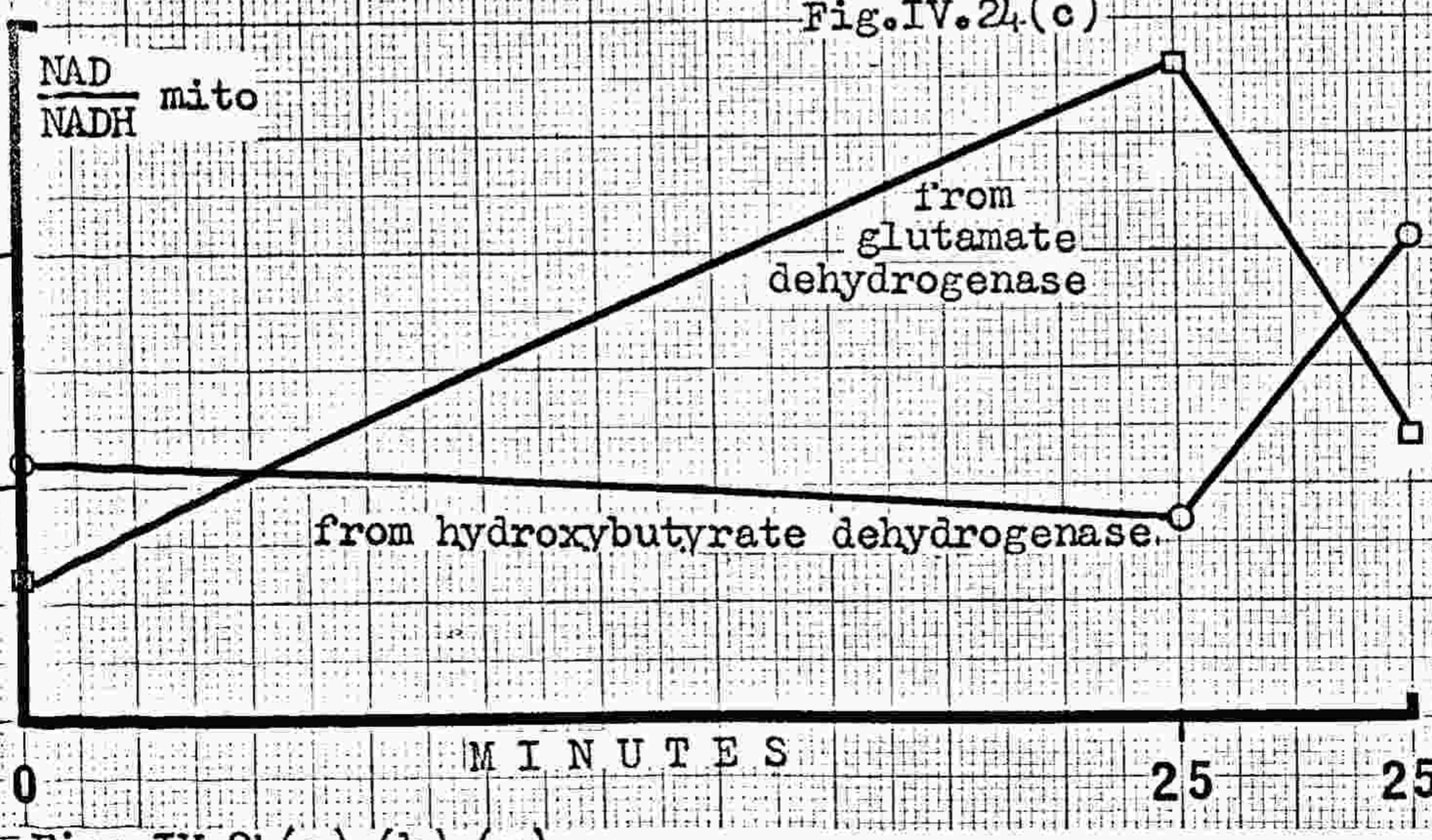


Fig.IV.24(c)



Figs.IV.24(a),(b),(c).

The relationships in these figures are calculated from the data in Table IV.6., except for the zero time data which was taken from Table IV.5.

$\frac{[\text{glutamate}]}{[\text{oxoglutarate}] \times [\text{ammonia}]}$ and a consideration of its alterations together with alterations in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ demonstrates that it is the glutamate dehydrogenase reactants which give an incorrect estimate of the mitochondrial redox state at 25 min. Since the reactants of the glutamate and hydroxybutyrate dehydrogenases share the same pool of pyridine nucleotides it follows that the influx of a large quantity of ammonia should indeed cause a decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ but in a defined manner. Firstly, via glutamate dehydrogenase, the $\frac{[\text{NAD}]}{[\text{NADH}]}$ ratio should become more oxidised - manifesting itself as a decreased $\frac{[\text{glutamate}]}{[\text{oxoglutarate}] \times [\text{ammonia}]}$ ratio. Secondly the oxidised $\frac{[\text{NAD}]}{[\text{NADH}]}$ ratio should, via hydroxybutyrate dehydrogenase, cause a decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$. This ratio did indeed decrease (2.36 to 0.88) but the ratio $\frac{[\text{glutamate}]}{[\text{oxoglutarate}] \times [\text{ammonia}]}$ increases (9.26 to 21.7). This increase indicates a reduction of the $\frac{[\text{NAD}]}{[\text{NADH}]}$ couple but since ammonia is an oxidant with respect to the mitochondrial redox state (via glutamate dehydrogenase) such a reduction is quite inexplicable. Also since the decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ can only be brought about subsequent to a decrease in the ratio $\frac{[\text{glutamate}]}{[\text{oxoglutarate}] \times [\text{ammonia}]}$ the observed increase in this

latter ratio indicates that the measured ratio (at 25 min.) does not represent that effective at the glutamate dehydrogenase site. Thus the glutamate dehydrogenase couple does not give an accurate estimate of $\frac{[NAD]}{[NADH]}$ mito to 25 min. after an ammonia load.

Lack of alanine or aspartate synthesis in heart and brain after an ammonia load.

The synthesis of alanine and aspartate in the liver following administration of an ammonia load showed that the sequential action of glutamate dehydrogenase and alanine (or aspartate) aminotransferase can serve to remove excess tissue ammonia. It was therefore of interest to see whether this mechanism was active in other tissues.

Ammonia metabolism in the heart has not been widely studied. Nevertheless the heart contains appreciable activity of the necessary enzymes (Williamson et al., 1967b; Awapara & Searle, 1952) and therefore it could respond to an ammonia load in the same way as liver. To test this possibility fed rats were injected with the usual NH_4Cl dose and killed after 5 min. and their hearts rapidly freeze-clamped. The tissue was treated as in Chapter II. None of the metabolites

measured (Table IV-7) altered significantly. There was no synthesis of alanine or aspartate and the mass action ratio for alanine aminotransferase was far from the equilibrium value.

Brain might be expected to remove ammonia by alanine formation. Hathaway et al. (1965) demonstrated a slow formation of alanine in brain (about 0.5 μmoles/g./30 min.) after the injection of convulsant such as dieldrin, picrotoxin or telodrin. A smaller increase was apparent after glutamine injection. Therefore fed rats were injected (i.p.) with either the usual dose (2.5 mMoles/kg.) of NH_4Cl or with 10 mMoles/kg. body wt. This latter dose is lethal - convulsions occurred at about 7-8 min. and death within 5 min. of these. Both groups of rats were decapitated 5 min. after the injection - their heads being allowed to drop into liquid nitrogen. The frozen heads were then treated as described in Chapter II. In neither group of rats (Table V-8) was there any appreciable synthesis of alanine and aspartate. In fact the group which received the lethal dose (Group C) showed a 40% decrease in the tissue aspartate content. The alanine aminotransferase mass action ratio was far from the equilibrium value but care must be taken in interpreting

Table IV.7

Effect of NH_4Cl injection on some metabolites in rat heart

Rats (male; 180-200 g.) were injected (i.p.) with 0.9% NaCl (control group) or with NH_4Cl (2.5 mMoles/kg. body wt.) were killed 5 min. later and their hearts freeze-clamped. For other details see Chapter II. Results are expressed as µMoles/g. fresh wt. and represent the means of three rats.

	Control group	NH_4Cl injected group
Glutamate	6.57	6.23
Aspartate	2.72	2.53
Alanine	0.741	0.737
Lactate	1.90	2.18
Pyruvate	0.041	0.055
Citrate	0.299	0.352
Oxoglutarate	0.019	0.020
Glutamine	3.14	3.27
$\frac{\text{Lactate}}{\text{Pyruvate}}$	46.3	39.6
$\Gamma_{G.T}$	0.052	0.046
$\frac{\Gamma_{mMDH} \times \Gamma_{GOT}}{\Gamma_{sMDH}}$	1.22	0.841

Table IV.8

Effect of NH_4Cl injection on some metabolites in rat brain

Rats (male; 180-200 g.) were injected (i.p) with NH_4Cl (2.5 mMoles/kg. body wt. for group B; 10 mMoles/kg. body wt. for group C), and 5 min. later were decapitated and their heads plunged into liquid nitrogen. Group A were not injected. For other details see Chapter II. Results are expressed as μMoles/g. fresh wt. and represent the means of three rats.

	Group A	Group B	Group C
Glutamate	8.46	9.85	7.70
Aspartate	2.88	2.63	1.80
Alanine	0.507	0.584	0.559
Lactate	4.00	4.39	4.87
Pyruvate	0.149	0.135	0.208
Malate	0.327	0.298	0.344
Oxoglutarate	0.050	0.042	0.059
$\frac{\text{Lactate}}{\text{Pyruvate}}$	26.9	32.5	23.4
Γ_{GPT}	0.020	0.018	0.021
$\frac{\Gamma_{mMDH} \times \Gamma_{GOT}}{\Gamma_{sMDH}}$	1.40	1.22	0.938

this because of the known compartmentation of glutamate in brain (Berl et al., 1962). Thus neither brain nor heart behave analogously to the liver in its ability to remove excess ammonia by synthesising alanine or aspartate.

<u>Effect of injection of pyruvate and oxaloacetate on the metabolism of the liver.</u>

Some of the conclusions derived from the previous experiments - that the aminotransferases are normally near equilibrium within the liver cell - are amenable to test by injection of the corresponding α-keto acids. This should cause a rapid net synthesis of the respective amino acids by a simple mass action effect. Sodium pyruvate (2.5 mMoles/kg.) was injected (i.p.) to rats which were then killed 5 min. later and their livers freeze-clamped. There was (Table IV.9) a considerable net synthesis of alanine. None of the other metabolites measured changed appreciably except lactate (which doubled) and of course pyruvate itself. The mass action ratio for alanine aminotransferase was far from the equilibrium value (1.48). It is thought that this may be because of an artifactually high pyruvate content caused by contamination of the

Table IV.9

Effect of pyruvate injection on some metabolites in rat liver

Rats (male; 180-200 g.) were injected (i.p) with sodium pyruvate (2.5 mMoles/kg. body wt.) or with 0.9% NaCl. They were killed 5 min. later and their livers freeze-clamped. For other details see Chapter II. Results are expressed as μMoles/g. fresh wt. and represent the means of three rats.

	Saline injected	Pyruvate injected
Lactate	0.895	1.82
Pyruvate	0.097	1.26
Glutamate	2.33	2.20
Oxoglutarate	0.150	0.167
Hydroxybutyrate	0.155	0.151
Acetoacetate	0.063	0.063
Alanine	1.69	2.77
Aspartate	1.06	1.25
Malate	0.348	0.368
Lactate / Pyruvate	9.24	1.44
Gln	1.12	0.167

liver, during the freeze-clamping operation, by pyruvate still in the abdominal cavity. This is supported by the fact that the $\frac{[\text{lactate}]}{[\text{pyruvate}]}$ ratio decreases to a value 15.6% that of the control (GPT decreased to a value 14.9% that of the control) while neither the tissue content of malate or oxaloacetate (as shown by the ratio $\frac{[\text{Oxoglutarate}]\ [\text{Aspartate}]}{[\text{Glutamate}]}$) changed appreciably, thus indicating no change in the cytoplasmic $\frac{[\text{NAD}]}{[\text{NADH}]}$ ratio. An artifactually high liver pyruvate content would explain both these apparent anomalies.

It was decided to use starved rats for the injection of oxaloacetate because preliminary experiments which employed fed rats suggested a decrease in the $\frac{[\text{Hydroxybutyrate}]}{[\text{Acetoacetate}]}$ ratio. This was difficult to establish in fed rats because the preparations of hydroxybutyrate dehydrogenase used to estimate ketone bodies contained traces of malate dehydrogenase. This is not normally a problem because of the low levels of oxaloacetate (10^{-6}M) usually encountered in tissues but it became a serious problem after the administration of oxaloacetate. So as to minimise this interference starved rats were used and oxaloacetate was removed before the determination of acetoacetate by the addition of malate dehydrogenase to the cuvette. The intravenous (sephanous

vein) route of injection was used while the rats were under light ether anaesthesia. This route was employed so that the oxaloacetate reached the liver rapidly thus minimizing the extent of spontaneous decarboxylation to pyruvate. When such rats were killed 5 min. after injection of oxaloacetate there was a large (300%) increase in aspartate (Table IV.10). There were also large increases in pyruvate (presumably because of spontaneous decarboxylation of oxaloacetate) and lactate as well as in oxoglutarate. The ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ decreased significantly.

This decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ may result from the penetration of oxaloacetate through the mitochondrial membrane and reaction with intramitochondrial NADH, thus oxidising the mitochondrial redox state. Alternatively it could be due to the increased oxoglutarate concentration (presumably caused by the transamination of oxaloacetate with glutamate) oxidising the mitochondrial redox state via glutamate dehydrogenase.

Discussion

The removal of ammonia in the liver by the formation of alanine and aspartate.

The results of the experiments in this chapter are

Table IV.10

Effect of oxaloacetate injection on some metabolites in rat liver

Rats (male; 180-200 g.) were injected I.V. (sephanous vein, while under ether anaesthesia) with either 0.9% saline or sodium oxaloacetate (2.5mMoles/kg. body wt.). They were killed 5 min. later and their livers freeze clamped. For other details see Chapter II. Results are expressed in μMoles/g. fresh wt. ± S.D. and are the means of 4 rats. The concentration of pyruvate and oxaloacetate in blood was measured and a correction was made for its contribution to the liver results by the method of Bohorst (1959)

	Saline injected	Oxaloacetate injected	P
Glutamate	1.79 ±0.240	1.74 ±0.180	
Oxoglutarate	0.039±0.042	0.206±0.096	
Lactate	0.403±0.058	1.10 ±0.598	
Pyruvate	0.023±0.012	0.105±0.052	
Hydroxybutyrate	2.12 ±0.320	1.25 ±0.883	
Acetoacetate	0.638±0.334	0.575±0.289	
Oxaloacetate	$<$0.015	0.194±0.079	
Aspartate	0.448±0.081	1.46 ±0.089	
Malate	0.463±0.138	1.01 ±0.067	
$\frac{\text{Hydroxybutyrate}}{\text{Acetoacetate}}$	3.40 ±0.572	2.12 ±0.572	P<0.02

in agreement with the concept of glutamate dehydrogenase, hydroxybutyrate dehydrogenase, alanine aminotransferase and aspartate aminotransferase all being mutually near to equilibrium in the liver. The increases in alanine and aspartate after injection of ammonia suggest that in some cases the formation of these amino acids can effectively remove ammonia when the urea cycle is overloaded. This removal is, of course, temporary and when the 'ammonia-crisis' is over the reversal of the aminotransferases and glutamate dehydrogenase releases ammonia and the appropriate keto acid. This ammonia may then be removed by the urea cycle and the keto acids oxidised. Since all the reactions occur very close to equilibrium there are no appreciable changes in free energy involved. Thus the sequential action of glutamate dehydrogenase and alanine (or aspartate) aminotransferase constituted a simple, reversible, non-energy requiring means for temporarily removing excess ammonia in the liver. The extent to which it can operate depends, of course, on the provision of relatively large quantities of pyruvate and oxaloacetate. Pyruvate may easily be provided by glycolysis. There is also no problem in the supply of oxaloacetate. Ross et al. (1967) obtained a rate of

glucose formation from pyruvate in the isolated perfused rat liver of 1.02 μmoles/g. liver/minute. Since this implies a conversion of pyruvate to oxaloacetate of 2.04 μmoles/g./min. via pyruvate carboxylase it suggests that the provision of the much smaller quantities of oxaloacetate needed for aspartate synthesis after ammonia injection could easily be accomplished by pyruvate carboxylase.

Figure IV.25 shows the changes in the sum of (Glutamate + Aspartate + Alanine) after administration of an ammonia load. This data is compiled from the experiments already described. It can be seen that in normal fed rats there is an increase in the sum of these amino acids of 4.1 μmoles/g. liver. Since the livers of these rats weighed on average 8.0 g./200 g. rat, the amount of ammonia removed by liver glutamate dehydrogenase is 32.8 μmoles or 6.5% of the administered load. In rats preinjected with arginine the net increase was only 1.05 μmoles/g. representing 1.7% removal by liver glutamate dehydrogenase. In the fasting rat the net increase is 5.3 μmoles/g. liver. Since the weight of the liver in these animals was 6.5 g./200 g. rat this represents a removal of 6.9% of the administered ammonia. When fasting rats had been preinjected with dihydroxyacetone

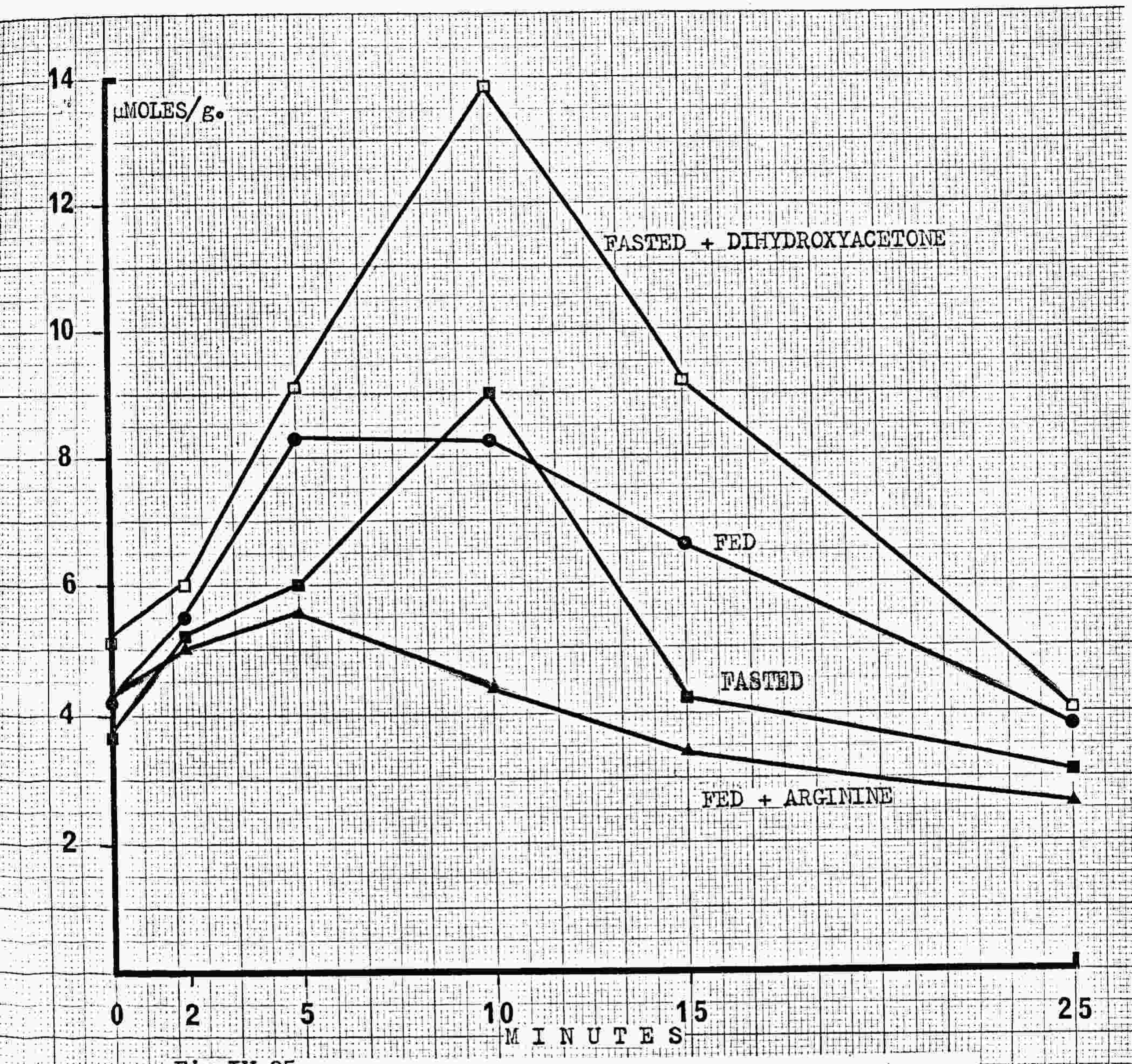


Fig.IV.25

Synthesis of (Glutamate+alanine+aspartate) in rat liver after administration of ammonium chloride. Data taken from Figs.IV.1, IV.11, IV.13 and IV.21.

the net increase was 8.7 µmoles/g. liver or 11.3%.

<u>The failure of glutamine in the liver to increase after an ammonia load.</u>

Synthesis of glutamine in the liver has been suggested as a mechanism for the control of the liver ammonia level (Kamin & Handler, 1967). Duda & Handler (1958) injected (i.v.) N^{15}-labelled ammonium lactate to starved rats and found that by far the greatest amount of label (in the compounds they analysed in the liver) was in the amide group of glutamine. Much less was found in urea and glutamate and only trivial amounts in alanine and aspartate. Very different results were obtained in the experiments reported in this chapter. Alanine and aspartate were always elevated while glutamine increased only slightly in fed rats and actually showed a decrease in starved rats. These anomalies may be partly explained by the fact that Duda & Handler used relatively small doses of ammonia (about 170 µmoles/kg. body wt.) and furthermore found that on increasing the ammonia load a much smaller percentage of the injected dose was incorporated into glutamine. Thus it appears that at small ammonia doses glutamine synthetase predominates over glutamate dehydrogenase in

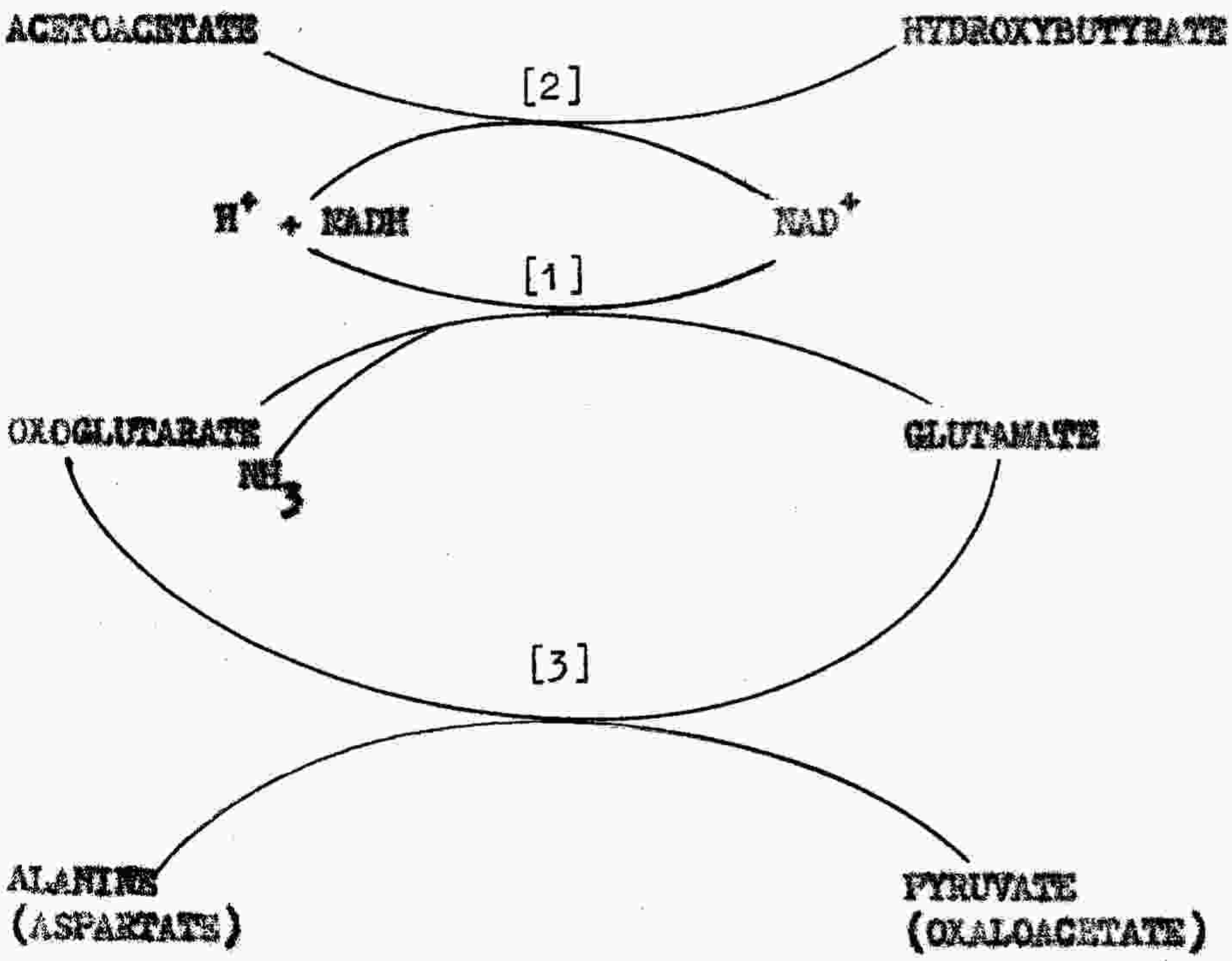
the removal of ammonia but on administration of larger quantities the dehydrogenase becomes quantitatively much more important. The mechanism for this is not known but may be related to the observation that high ammonia concentrations tend to inhibit rat liver glutamine synthetase (Speck, 1949).

<u>The changes in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ after an ammonia load</u>.

In all the experiments reported here there was an immediate decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ after an ammonia load (Figures IV.4, IV.9, IV.16, IV.20). Since it is the contention in this thesis and in other work emanating from this laboratory (Williamson et al., 1967b, Veech, 1968) that glutamate dehydrogenase and hydroxybutyrate dehydrogenase are in equilibrium with the same pool of intramitochondrial pyridinenucleotides, the decreased $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratio is taken to reflect an oxidation of the mitochondrial NAD-NADH system by ammonia brought about via glutamate dehydrogenase. However, work in other laboratories is not completely in agreement with this scheme so that some of the principal objections are discussed below.

Chance (1967) has suggested that the change in

hydroxybutyrate/acetoacetate after NH_4Cl might be due to "an alkalinization of the intracellular contents of the liver" rather than due to an effect of ammonia per se. A consideration of the data obtained together with the scheme (below) contradicts this suggestion.



ENZYMES

[1] Glutamate dehydrogenase.

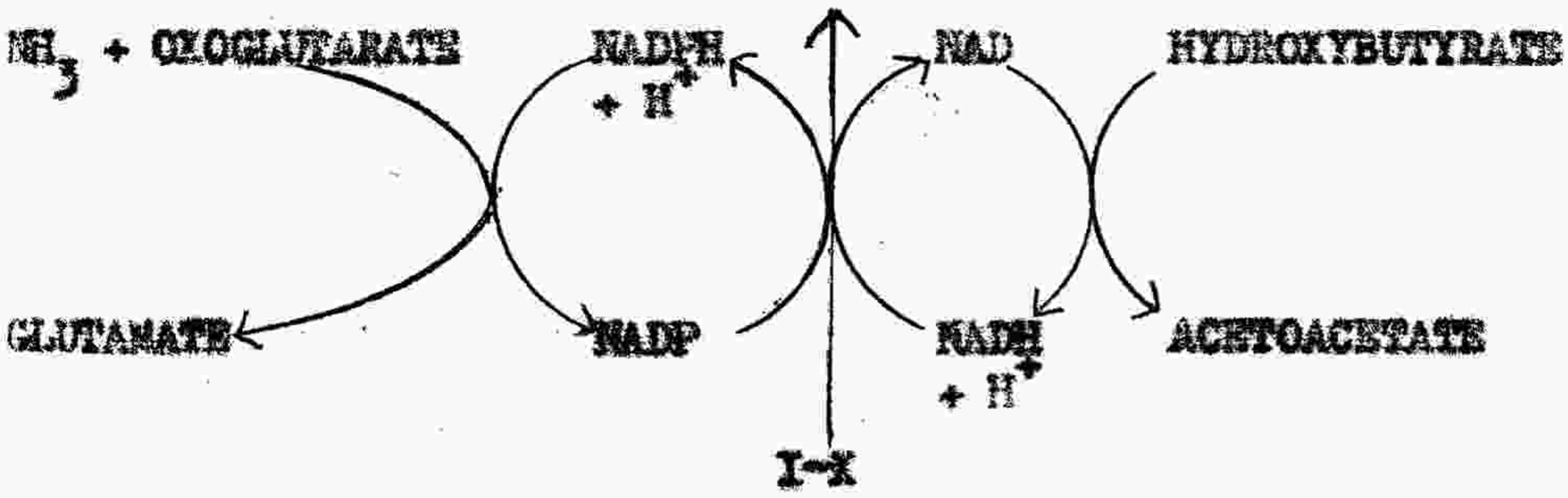
[2] Hydroxybutyrate dehydrogenase.

[3] Aminotransferase.

This scheme illustrates the postulated relationships between the reactants of glutamate dehydrogenase, hydroxybutyrate dehydrogenase and alanine (or aspartate) aminotransferase - the enzymes being mutually at equilibrium. If the primary disturbance of these equilibria were a fall in H^+ then one would certainly expect a decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ as suggested by Chance. One would also expect a fall in the tissue contents of glutamate, alanine and aspartate - in fact a large increase in the sum (glutamate + aspartate + alanine) is recorded. On the other hand, if, as proposed here, the primary disturbance of these equilibria is the increased ammonia concentration an increase in the sum (glutamate + aspartate + alanine) and a decrease in the ratio $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ would occur.

Klingenberg & Slenczka (1959) first suggested that glutamate dehydrogenase in isolated mitochondria reacted preferentially with NADP and only very slowly (if at all) with NAD. If this were true for the <u>in vivo</u> situation then a different interpretation would have to be given for the change in the $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratio after ammonia injection. Charles et al. (1967) suggested that the energy-linked transhydrogenase catalyses a

rapid equilibration of the hydroxybutyrate and the glutamate dehydrogenase couples. This could occur as in the following scheme.



Since the changes in the tissue content of ammonia, glutamate, oxoglutarate, hydroxybutyrate and acetoacetate on ammonia injection and during ischaemia were very close to what one would predict on the basis of a simple sharing of the same pool of NAD-NADH by both dehydrogenases, the alternative scheme (above) must be compatible with these observations. This does not appear to be the case.

Assuming that glutamate dehydrogenase is at equilibrium <u>in vivo</u> with the nucleotides NADP and NADPH (as it appears to be in isolated mitochondria: Papa et al., 1967) then the ratio $\frac{[NADP]}{[NADPH]}$ (calculated from

the levels of glutamate, oxoglutarate and ammonia in fed rats - Chapter III) in the mitochondrion becomes 20.1. This, in turn, must mean that the energy-linked transhydrogenase cannot be at equilibrium since, according to Lee & Ernster (1964) the equilibrium constant for this transhydrogenase is

$$\frac{[NAD]\,[NADPH]}{[NADH]\,[NADP]} = 480$$ see footnote (1)

and this would give a ratio for NAD/NADH of 9,648. The actual ratio as estimated from the hydroxybutyrate dehydrogenase system is 10.2. Thus the transhydrogenase

---

(1) This, of course, is not a very meaningful number. According to Lee & Ernster (1966) the reaction catalysed by the transhydrogenase is:

$$NADP + NADH + I\sim X \rightleftharpoons I + X + NADPH + NAD,$$

so that the correct equilibrium expression is $\frac{[NADPH]\,[NAD]\,[X]\,[I]}{[NADP]\,[NADH]\,[X\sim I]}$. This creates an immediate impasse because I, X and I~X (if they exist) are not at the moment amenable to measurement. Lee & Ernster measured the nucleotides for the 'half-equilibrium-constant' (quoted above in text) in mitochondria oxidising succinate and one can only assume that under <u>in vivo</u> conditions the ratio $\frac{[X]\,[I]}{[X\sim I]}$ is not too different from what it was in these mitochondria.

must be far out of equilibrium in the direction of NADH and NADP. If, therefore, the energy-linked transhydrogenase is not even powerful enough to achieve equilibrium under steady-state conditions in the fed rat it is extremely difficult to understand how it can react sufficiently rapidly to an ammonia load and rapidly oxidise sufficient NADH to cause the observed change in the ratio hydroxybutyrate/acetoacetate.

Of course the above argument does not hold if glutamate dehydrogenase or hydroxybutyrate dehydrogenase (or both) do not catalyse an equilibrium. It is not possible to prove that either one is in equilibrium without assuming that they share the same pool of NAD-NADH and proving that they are both in equilibrium. There is no independent <u>proof</u> that either enzyme must catalyse an equilibrium but there is much circumstantial evidence strongly suggesting that this is so. Especially important is the very high activity of these enzymes in rat liver. In the case of glutamate dehydrogenase the great decrease in the content of oxoglutarate on administration of ammonia is precisely what one would predict from mass-action considerations if an equilibrium existed. In the case of hydroxybutyrate dehydrogenase, the 'dead-end' status of hydroxybutyrate

as a metabolite and the low rate of ketogenesis in fed rats makes it very likely that equilibrium is closely approached *in vivo*.

That the reactants of both enzymes behave as if they were mutually at equilibrium even after five minutes of ischaemia (Chapter III) is especially incompatible with the idea that the energy-linked transhydrogenase mediates between them. If the 'apparent' equilibrium were brought about through the transhydrogenase then one would expect a disequilibrium to appear during ischaemia since the lack of oxygen would cause an acute shortage of I~X to drive the transhydrogenase. Although there is some ATP in the liver at this time (see Table IV.4, Chapter III) its concentration is greatly decreased and because of the decrease of the phosphorylation potential (ATP/ADP.Pi) that may reasonably be supposed to occur in this situation it would be quite impossible for I~X to be synthesised by a reversal of oxidative phosphorylation during ischaemia. Lee & Ernster (1964) have shown that as soon as a supply of energy (i.e. the oxidation of succinate) ceases, the transhydrogenase equilibrium proceeds rapidly towards the non-energy-linked transhydrogenase position (NADPH.NAD/NADP.NADH = 0.79). That the reactants of

glutamate dehydrogenase and hydroxybutyrate dehydrogenase still appear to maintain mutual equilibrium even after five minutes of ischaemia is good evidence that energy-linked processes are not involved in its maintenance.

<u>The behaviour of oxoglutarate 25 min. after an ammonia load</u>.

The behaviour of oxoglutarate 25 min. after an ammonia load is extremely puzzling. The experiments recorded in Tables IV.5 and IV.6 and in Figures IV.23 and IV.24 show that 25 min. after the administration of an ammonia load to starved rats (at a time when all the ammonia had been removed) agreement between the following sets of expressions was poor:

(a) between glutamate dehydrogenase and hydroxybutyrate dehydrogenase;

(b) between malic enzyme and isocitrate dehydrogenase;

(c) between $\frac{\Gamma_{GOT} \times \Gamma_{MDH}}{\Gamma_{LDH}}$ and its theoretical equilibrium value.

Oxoglutarate is the only metabolite common to all of the above three sets of expressions and its content in liver is 5-fold elevated at 25 min. Furthermore, when its concentration is arbitrarily assumed to be one-third of the measured liver content the disagreements listed

above disappear. This makes the hypothesis that most of the measured oxoglutarate is not available at the enzyme sites an attractive one. However the removal of over 80% of this oxoglutarate within 5 min. of a second ammonia load shows that most of the oxoglutarate is available for reaction with glutamate dehydrogenase thus making sequestration unlikely. Moreover the changes in the metabolite pattern immediately after this second ammonia load suggests that none of the relevant enzymes were inhibited at this time.

Further work will be required to explain these interesting findings. No satisfactory hypothesis is available at this time.

The experiments reported in this Chapter were carried out in collaboration with Dr. D.A. Heus.

CHAPTER V

AMMONIA METABOLISM AND THE KIDNEY

In the acidotic condition, renal breakdown of glutamine provides the major part of the ammonia excreted in exchange for conserved cations (Pitts, 1964; Lotspeich, 1967). Kidney cortex from acidotic rats, in addition to the enhanced capacity for glutamine hydrolysis (D.A. Hems, unpublished experiments) has an increased capacity for gluconeogenesis from glutamine, glutamate and oxoglutarate (Goodman et al., 1966). Recent measurements of the renal arterio-venous difference of glucose showed an increased output by the kidney of the acidotic dog (Steiner et al., 1968). Thus it appears that an appreciable proportion of the 5-carbon skeleton of glutamine may be converted to glucose by the kidney cortex during acidosis.

A detailed investigation of the renal content of metabolites possibly concerned in these phenomena has not been carried out. Alleyne (1967), however, has measured some metabolites in rapidly frozen rat kidney

and has reported significant decreases, in acidosis, in the content of citrate, oxoglutarate and malate. There was no change in the content of aspartate and pyruvate but phosphoenolpyruvate increased significantly. Alleyne interpreted this elevation of phosphoenolpyruvate as indicating that the enhancement of gluconeogenesis in acidosis was the result of increased activity of the enzyme, phosphoenolpyruvate carboxykinase.

The paucity of data on the content of intermediates in kidney probably reflects the difficulties inherent in the interpretation of such values. Liver is a fairly homogeneous tissue, yet the problems of sub-cellular compartmentation may render data on the tissue content of metabolites uninterpretable. Since the kidney consists of an anaerobic, glucose-utilising medulla and an aerobic cortex capable of producing glucose, it might be suggested that total metabolite measurements are not very useful. However, it is a premise, in this Chapter, that such measurements are probably a good index of the metabolite levels of the cortex. This assumption is based on two considerations:

(a) the medulla comprises only about 15% of the kidney weight;

(b) in some cases it was possible, after freeze-clamping,

to distinguish cortex from medulla in the frozen tissue. In these cases, the cortex was carefully chipped away and extracted separately. Measurement of the content of many intermediates in this type of preparation (largely free of medulla) revealed no appreciable difference from whole kidney extracts.

This chapter, therefore, presents measurements, in whole kidney, of amino acids, reactants of dehydrogenases, adenine nucleotides, glycolytic intermediates and certain other metabolites during a variety of metabolic conditions. These measurements are assumed to represent the cortical contents of the relevant metabolites and their functional inter-relations are discussed. A principal aim of this work was to establish which enzyme reactions may be at equilibrium in cortical cells and then to exclude these enzymes from the large number of potential rate-limiting reactions within the cell. Measurements of dehydrogenase reactants were of interest in connection with (i) the state of oxidation of the rapidly frozen tissue, (ii) the possible perturbation of equilibria by acidosis, since H^+ is a reactant in most dehydrogenase reactions, and (iii) comparison with the state of these reactions in the liver. Measurements of the glycolytic intermediates

were carried out in acidotic and in starved rats with a view to delineating the steps that are responsible for the enhanced gluconeogenic capacity in these states. Interest in the hypoxic kidney arose from an investigation of aspects of renal anaerobic glycolysis (Krebs, Hems & Gasa, to be published) and from the fact that an analysis of theaerobic-anaerobic transition in the whole organ has not been carried out, although this situation has been investigated to some extent in kidney slices (Ju, 1964; Inerwood & Newsholme, 1967). The kidney content of some amino acids, citrate cycle intermediates and dehydrogenase reactants are shown in Table V.1. In these experiments the 7-10 day acidotic rats were made acidotic by replacing the drinking water with 1.5% NH_4Cl. In the 12-hour acidotic rats, a cannula was introduced into the right atrium via the right jugular vein. The rats were then placed in restraining cages and 0.25N HCl (4 mls./100 g. body wt.) was infused over 4-5 hours. That these treatments caused acidosis was established by measuring the bicarbonate concentration in mixed venous blood. In normal rats a value of 24.8 mM was obtained; this was decreased to 20 mM after the NH_4Cl method and to 8-10 mM after the HCl infusion (D.A. Hems, unpublished experiments). Ischaemia was brought about by

Table V.1 The content of some amino acids, citrate cycle intermediates and dehydrogenase reactants in whole rat kidney.

Nembutal anaesthesia was employed except in the series marked with an asterisk (*) where the rats were killed by cervical dislocation. For further details see text and Chapter II. Results are in μmoles/g. fresh wt. ± S.D. The number of observations are given in parentheses.

Condition of rat	Glutamine	Glutamate	Oxoglutarate	Ammonia	Alanine	Aspartate	Citrate	Malate
Normal, fed.	1.72±0.421 (6)	3.01±0.675 (9)	0.290±0.132 (6)	0.878±0.231 (6)	0.863±0.174 (6)	0.387±.085 (6)	0.387±0.085 (7)	0.374±0.116 (6)
Starved (48 hours)	1,09±0.130 (8)	2.70±0.372 (12)	0.230±0.069 (11)	1.71± 0.394 (8)	0.819±0.059 (3)	0.746±0.068 (6)	-	0.269±0.048 (8)
Acidotic (7-10 days)	1.17±0.246 (5)	2.49±0.56 (6)	0.113±0.038 (6)	2.82±0.85 (5)	0. 674±0.048 (4)	0.926±0.019 (5)	0.175±0.028 (8)	0.136±0.04 (5)
Acidotic (12 hours)	0.861±0.225 (3)	2.32±0.361 (6)	0.050±0.037 (6)	4.60±0.953 (3)	-	0.773±0.048 (3)	-	0.174±0.103 (6)
Normal, fed ischaemic (2 min)	1.03±0.330 (5)	5.17±0.352 (8)	0.035±0.022 (5)	1.54±0.269 (5)	-	0.944±0.063 (5)	0.446±0.180 (9)	0.471±0.055 (5)
Starved (48 hours), ischaemic (2min)	0.750±0.100 (5)	3.50±0.265 (5)	0.044±0.018 (7)	3.25±1.32 (7)	0.548±0.075 (3)	0.742±0.054 (5)	0.472±0.160 (9)	0.302±0.042 (7)
Starved *(48 hours)	0.840±0.280	2.58±0.339	0.323±0.085	1.01±0.290	-	0.740±0.269	0.374±0.974	0.215±0.049

Table V.1 continued overleaf.

Table V.1 continued.

Condition of rat	Lactate	Pyruvate	Hydroxy-butyrate	Aceto-acetate	Lactate/Pyruvate	Hydroxy-butyrate/Aceto-acetate	Renal Venous Ammonia (mM)	Calculated intracellular ammonia (mM) (see text)
Normal, fed.	1.17±0.15 (6)	0.080±0.037 (6)	0.088±0.037 (6)	0.032±0.013 (6)	13.1	2.7	0.116±0.034 (6)	0.287
Starved (48 hours)	0.831±0.302 (10)	0.056±0.014 (9)	1.18±0.315 (11)	0.169±0.064 (11)	14.8	7.0	0.093±0.053 (7)	0.230
Acidotic (7-10 days)	1.58±0.540 (5)	0.104±0.010 (6)	0.080±0.011 (5)	0.025±0.006 (5)	15.2	3.2	0.464±0.123 (4)	0.938
Acidotic (12 hours)	1.33±0.10 (4)	0.058±0.035 (5)	-	-	22.9		-	-
Normal, fed ischaemic (2 min)	4.05±0.70 (5)	0.029±0.012 (6)	0.135±0.057 (5)	<0.02	139	>6.8	-	0.503
Starved (48 hr) ischaemic (2 min)	3.74±0.974 (7)	0.021±0.008 (7)	1.05±0.447 (7)	0.06±0.05 (7)	156	17.5	-	0.437
*Starved (48 hr)	0.530±0.211	0.038±0.010	0.740±0.195	0.217±0.063	13.9	3.4	-	-

severing the renal artery and renal vein and allowing the kidney to remain *in situ* for the required length of time.

Calculation of intracellular ammonia concentration

Anaesthesia was employed in this group of rats to allow sampling of renal venous blood for ammonia determination. The measurements of ammonia in frozen kidney do not represent the intracellular concentration because its concentration in urine is high. Ammonia is in diffusion equilibrium in the kidney (Balagura & Pitts, 1962; Stone et al., 1967); consequently its intracellular concentration may be calculated from the equation

$$\frac{\text{ammonia intracell}}{\text{ammonia extracell}} = \frac{1 + 10^{pK-pH\,intracell}}{1 + 10^{pK-pH\,extracell}} \quad *$$

For the purpose of these calculations the intracellular pH was assumed to be 7.0 in all cases. The pH of renal venous blood was taken as 7.40 except in acidosis when a value of 7.30 was used. (These values are based on measurements in this laboratory by D.A. Hems.) The concentration of ammonia in renal venous blood represents

---

* For a fuller description of this, see the Discussion to Chapter VI.

pK refers to the reaction $NH_4^+ \rightleftharpoons NH_3 + H^+$

[Ammonia] extracell. The concentration after ischaemia was calculated by assuming that there is no appreciable change in the pH of all the fluid phases of the kidney and that the increased tissue content of ammonia is still in diffusion equilibrium. Intracellular ammonia after ischaemia is then given by the expression

$$\frac{\text{Total Kidney content of ammonia (ischaemic)}}{\text{Total Kidney content of ammonia (in vivo)}} \times \text{calculated intracellular ammonia (in vivo)}$$

These assumptions are not amenable to experimental test at present.

Equilibrium between glutamate dehydrogenase and hydroxybutyrate dehydrogenase in the kidney.

If the calculated intracellular ammonia concentrations are approximately correct, it should be possible to test whether the glutamate and hydroxybutyrate dehydrogenases are mutually at equilibrium in kidney as they are in liver. The high activities of these enzymes in kidney (Williamson et al., 1967b) suggest that they may attain equilibrium. When the ratios of mitochondrial $\left[\frac{NAD}{NADH}\right]$ were calculated (as in Chapter I) from the reactants of these two enzymes the following values were obtained:

	$\frac{[NAD]}{[NADH]}$ from glutamate dehydrogenase	$\frac{[NAD]}{[NADH]}$ from hydroxybutyrate dehydrogenase
Fed	7.1	7.5
Starved	5.3	2.9
Acidotic (7-10 days)	11.0	6.3
Fed, ischaemic (2 min.)	0.98	2.9
Starved, ischaemic (2 min.)	1.4	1.2

There was generally good agreement between the two estimates of the $\frac{[NAD]}{[NADH]}$ ratio. The agreement when the kidney became anaerobic (and the content of many of the reactants changed greatly) was especially convincing.

The value of $\frac{[NAD]}{[NADH]}$ obtained with fed rats was very similar to that found in the liver of such rats by Williamson et al. (1967b). The ratio $\frac{[hydroxybutyrate]}{[acetoacetate]}$ (7.0) in starved rats was much higher than the liver value and than previous measurements of this ratio in kidney carried out in this laboratory. These latter measurements were made on rats that had been killed by cervical dislocation so that it seemed possible that Nembutal, used in the present experiment, could be the cause of the discrepancy. Therefore a separate group of starved rats were killed by cervical dislocation and the ketone bodies measured in their kidneys (Table V.1).

The $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratio was 3.4, half that found in the Nembutal-anaesthetised group, indicating that Nembutal *per se* may have caused a reduction of the pyridine nucleotides of renal mitochondria.

Lactate/Pyruvate ratio of the kidney.

The lactate/pyruvate ratio in all the *in vivo* kidneys, except the 12-hour acidotic rats, was between 13 and 15. This is similar to, but a little higher than, normal fed liver values. The high value (22.9) in 12-hour acidotic rats could be caused by a slight intracellular acidosis.

Amino acid metabolism in the kidney.

That the contents of certain gluconeogenic amino acids decreased in liver in gluconeogenic conditions (starvation, diabetes) has been shown by Williamson et al. (1967a). In the present study (Table V.1) there was a similar effect in the kidney in both starved and acidotic rats. The kidney content of glutamine, glutamate, alanine and aspartate decreased in these states. This indicates that in these gluconeogenic situations the kidney utilises, to some extent, tissue amino acids as glucose precursors.

The mass action ratio for alanine aminotransferase was calculated and compared with the equilibrium constant (1.48). As oxaloacetate was not measured in these experiments, the composite relationship $\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}$ (at equilibrium = 1.65) was used as an index of aspartate aminotransferase equilibrium. The following values were obtained:

	Γ_{GPT}	$\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}$
Fed	0.934	3.75
Starved	0.820	3.63
Acidotic (7-10 days)	0.294	4.70
Acidotic (12 hours)	-	2.59
Fed, ischaemic (2 min.)	-	2.10
Starved, ischaemic (2 min.)	0.287	4.82

The mass action ratios for alanine aminotransferase were close to the equilibrium constant in fed and starved rats but in acidotic rats agreement was poor. After 2 min. ischaemia the value in starved kidney had also gone out of equilibrium. Thus the enzyme may normally be sufficiently active to catalyse an equilibrium <u>in vivo</u> but not active enough to cope with the rapid changes during ischaemia.

The composite relationship $\frac{\Gamma_{MDH} \times \Gamma_{GOT}}{\Gamma_{LDH}}$ was in all cases larger than the theoretical value (1.65) but never more than 3-fold larger. This suggests that the three enzymes involved in this expression (lactate dehydrogenase, cytoplasmic malate dehydrogenase and cytoplasmic aspartate aminotransferase) are all close to equilibrium in the kidney.

Adenine nucleotides in the kidney.

The content of adenine nucleotides in rat kidney was measured in a variety of conditions (Table V.2). The $\frac{ATP}{ADP}$ ratio in fed rats was 1.07 which is much less than the value (2.72) obtained by other workers in this laboratory (Nishiitsutsuji-Uwo et al., 1967) using identical analytical techniques. As these workers employed Nembutal as an anaesthetic it was considered that this might be the cause of the discrepancy. Therefore the adenine nucleotides were measured in a group of rats anaesthetised with Nembutal. The $\frac{ATP}{ADP}$ ratio in these animals (Table V.2) was 1.77; thus Nembutal may, in part, account for the discrepancy. An even larger effect was found in starved rats where the $\frac{ATP}{ADP}$ ratio of 1.09 in cervically dislocated rats increased to 2.19 after Nembutal. These effects of Nembutal were

Table V.2. The content of adenine nucleotides in whole rat kidney

The rats were killed by cervical dislocation except in the goups marked with an asterisk(*) where Nembutal anaesthesia was employed. Results are in μmoles/g. fresh wt. ± S.D.

	No. of observations	AMP	ADP	ATP	Total adenine nucleotides	$\frac{ATP}{ADP}$	$\frac{(ATP)\times(AMP)}{(ADP)^2}$
Normal, Fed	(7)	1.43± 0.22	1.54± 0.13	0.421± 0.08	3.19	1.07	0.335
*Normal, Fed	(5)	1.71± 0.26	0.964± 0.18	0.247± 0.134	2.92	1.77	0.454
Starved (48 hrs.)	(6)	1.38± 0.13	1.27± 0.21	0.435± 0.07	3.08	1.09	0.370
*Starved (48 hrs.)	(3)	1.9?± 0.29	0.91± 0.24	0.214± 0.05	3.11	2.19	0.514
Acidotic (7-10 days)	(7)	1.34± 0.07	1.22± 0.21	0.459± 0.122	3.02	1.10	0.413
Acidotic (12 hours)	(3)	1.64± 0.3	1.07± 0.19	0.464± 0.212	3.17	1.53	0.465
Normal, Fed ischaemic (30 sec.)	(3)	0.453± 0.015	1.30± 0.010	1.38± 0.010	3.11	0.333	0.353
Normal, Fed ischaemic (1 min.)	(4)	0.446± 0.107	1.12± 0.08	1.56± 0.06	3.13±	0.400	0.557
Normal, Fed ischaemic (2 min.)	(4)	0.367± 0.03	0.787± 0.045	1.20± 0.086	2.35	0.466	0.711

also apparent in AMP contents, which decreased almost 2-fold in the anaesthetised rats. It is unlikely that anoxia in the kidneys of the cervically dislocated rats is responsible for this discrepancy as all the other signs of anoxia were absent. These kidneys had a lower $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratio than those from Nembutal-treated rats as well as a lower tissue content of lactate and similar $\frac{[\text{lactate}]}{[\text{pyruvate}]}$ ratios.

The content of total adenine nucleotides was extremely constant in all conditions except ischaemia. In the ischaemic kidney there was no loss after 1 min. but after 2 min. only 73% remained. This loss could be due to either dephosphorylation or deamination of AMP. The ATP content was particularly susceptible to anoxia - after only 30 sec. ischaemia it had fallen from 1.43 to 0.435 μmoles/g. fresh wt.

The adenylate kinase mass action ratio in all the <u>in vivo</u> conditions was between 0.3 and 0.7. This is close to the equilibrium values quoted by Gevers (1966) so that, if adenylate kinase is located in kidney as it is in liver (outer mitochondrial membrane), it probably controls the relative amounts of the nucleotides in the cytoplasm.

Glycolytic intermediates in the kidney

The kidney contents of most glycolytic intermediates were measured in a variety of conditions (Table V.3). The adenine nucleotides (Table V.2) must also be considered in conjunction with the glycolytic metabolites. For the purposes of this study, starvation and acidosis are regarded as gluconeogenic (or, potentially gluconeogenic) situations.

The effects of starvation and chronic acidosis (7-10 days) on these intermediates were quite similar, while the pattern in acute (12 hr.) acidosis was very different. In the former:

(a) all the intermediates from fructose diphosphate to pyruvate increased and these increases were always more pronounced in the chronic acidotic rats;

(b) the kidney contents of glucose-6-phosphate and fructose-6-phosphate decreased in the chronic acidotic rats while they had either increased (glucose-6-phosphate) or remained unchanged (fructose-6-phosphate) on starvation.

The changes in these metabolites in acute acidosis were almost diametrically opposite to those in chronic acidosis. Glucose-6-phosphate and fructose-6-phosphate were elevated,

Table V.3 The content of some glycolytic intermediates in whole rat kidney

Condition of rat	Lactate	Pyruvate	Phospho-enol pyruvate	2-Phospho-glyceric acid	3-Phospho-glyceric acid	α-Glycero phosphate	Total triose phosphates	Fructose diphosphate	Fructose-6-phosphate	Glucose-6-phosphate	Glucose	Inorganic phosphate
Normal, Fed	0.476± 0.222 (13)	0.027± 0.01 (13)	0.035± 0.01 (12)	0.014± 0.005 (12)	0.085± 0.030 (12)	0.114± 0.029 (13)	0.016± 0.006 (12)	0.010± 0.004 (13)	0.013± 0.006 (10)	0.039± 0.012 (12)	2.72± 0.37 (6)	4.05± 0.513 (9)
Starved (48 hr.)	0.424± 0.13 (14)	0.028± 0.01 (14)	0.051± 0.015 (11)	0.016± 0.010 (9)	0.114± 0.032 (12)	0.177± 0.045 (14)	0.017± 0.004 (8)	0.012± 0.005 (9)	0.013± 0.003 (7)	0.059± 0.005 (9)	1.69± 0.48 (5)	4.25± 0.466 (10)
Acidotic (7-10 days)	0.483± 0.120 (13)	0.029± 0.007 (13)	0.063± 0.019 (13)	0.021± 0.008 (13)	0.154± 0.042 (13)	0.130± 0.022 (13)	0.023± 0.009 (9)	0.014± 0.005 (10)	0.009± 0.003 (7)	0.034± 0.014 (12)	2.57± 0.196 (7)	3.83± 0.524 (11)
Acidotic (12 hours)	1.32± 0.102 (4)	0.058± 0.024 (4)	0.038± 0.015 (6)	0.007± 0.007 (5)	0.075± 0.029 (6)	0.139± 0.098 (6)	0.014± 0.012 (3)	0.003± 0.003 (3)	0.021± 0.010 (3)	0.08± 0.018 (4)	-	4.82± 0.501 (5)
Fed, Ischaemic (30 sec.)	2.07± 0.128 (3)	0.021± 0.006 (3)	0.014± 0.003 (3)	< 0.005	0.045± 0.012 (3)	0.356± 0.019 (3)	0.032± 0.003 (4)	0.017± 0.005 (3)	0.006 0.003 (3)	0.021± 0.004 (3)	-	6.08± 0.392 (3)
Fed, Ischaemic (1 min.)	2.10± 0.691 (4)	0.012± 0.005 (4)	0.012± 0.005 (4)	< 0.005	0.040± 0.009 (4)	0.308± 0.071 (4)	0.032± 0.006 (11)	0.023± 0.004 (4)	0.006± 0.005 (3)	0.019± 0.005 (3)	-	7.18± 0.080 (4)
Fed, Ischaemic (2 min.)	5.04± 0.670 (12)	0.010± 0.007 (12)	0.013± 0.006 (12)	<0.005	0.024± 0.016 (12)	0.396± 0.059 (12)	0.038± 0.016 (11)	0.043± 0.009 (11)	<0.005	<0.005	1.53± 0.156 (4)	8.72± 0.637 (12)

Rats were killed by cervical dislocation. Results are in μmoles/g. fresh wt. ± S.D. No of observations is in parentheses

the intermediates from fructose diphosphate to 2 phosphoglycerate decreased, phosphoenol pyruvate remained virtually unchanged and pyruvate was doubled.

In ischaemia, of course, the rate of glycolysis greatly increases and many workers have used this situation to evaluate the mechanisms which may limit glycolysis *in vivo*. In the kidney (Table V.3), glucose-6-phosphate and fructose-6-phosphate decreased during ischaemia, fructose diphosphate and triose phosphates increased and the metabolites from 3-phosphoglycerate to pyruvate all decreased. The decreased kidney contents of 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate are very different to the experiments of Lowry et al. (1964) on ischaemic mouse brain, and Williamson (1966) on anoxic perfused heart, where those intermediates were always increased.

Discussion

Decreased oxoglutarate, malate and citrate in acidosis

Alleyne (1968) observed a decrease in oxoglutarate, malate and citrate in acidotic kidneys. No explanation was proposed for these effects. All these decreases, however, may derive simply from the elevated intracellular ammonia concentration if the network of equilibria that

are known to operate in liver are also functional in kidney cortex. This may be elaborated as follows:

(a) If glutamate dehydrogenase is at equilibrium and the $\frac{[NAD]}{[NADH]}$ ratio in the mitochondrion does not change, then an elevated ammonia concentration will produce/lowered oxoglutarate concentration. This is observed.

(b) If isocitrate dehydrogenase is at equilibrium in the cytoplasm and if the cytoplasmic $\frac{[NADP]}{[NADPH]}$ ratio does not alter, then a decreased oxogluterate concentration will cause a decreased isocitrate concentration. If aconitase is at equilibrium then the decreased isocitrate will lower the citrate concentration. This is observed.

(c) If aspartate aminotransferase is at equilibrium then the fall in oxogluterate will produce an equivalent decrease in oxaloacetate since the contents of the other two reactants, glutamate and aspartate decrease to the same extent so that the ratio $\frac{[glutamate]}{[aspartate]}$ remains steady. This decreased oxaloacetate will decrease the content of malate provided that malate dehydrogenase is at equilibrium in the cytoplasm and the $\frac{[NAD]}{[NADH]}$ ratio in this compartment does not alter greatly. This decrease in malate is

also observed in the kidney of the acidotic rat.

Glycolytic intermediates in starvation and acidosis.

It is extremely difficult to interpret the changes in the glycolytic intermediates (relative to the normal fed rat) in these potentially gluconeogenic situations because no information is available on the rate of glucose production (if indeed there is glucose production) by rat kidneys in vivo. There is evidence that the kidney of the dog increases its output of glucose in acidosis (Steiner et al., 1968) but there is no comparable data either for the acidotic or fasted rat. Thus the evaluation of changes in these intermediates must be extremely tentative.

If the acutely acidotic rat kidney does produce more glucose, then the decreased fructose diphosphate and increased fructose-6-phosphate levels suggest that the mediation of this postulated effect may be via an activation of fructose diphosphatase. Similar cross-over points have been shown by Newsholme & Underwood (1966) when gluconeogenesis is stimulated in kidney cortex slices.

The increase in phosphoenolpyruvate found in kidneys from chronically acidotic rats was similar to that

recorded by Alleyne (1968). The contents of 2-phosphoglycerate, 3-phosphoglycerate, total triose phosphates and fructose diphosphate were also elevated. As suggested by Alleyne, an increased activity of phosphoenolpyruvate carboxykinase may well be responsible for these results. The cytoplasmic oxaloacetate concentration probably decreased, as evidenced by decreases in the expressions $\frac{[Malate][Pyruvate]}{[Lactate]}$ (from 0.028 to 0.009) and $\frac{[Aspartate][Oxoglutarate]}{[Glutamate]}$ (from 0.107 to 0.042). The cytoplasmic concentrations of GTP and ITP could not be measured but, if ATP is used as an index, their concentrations did not change appreciably on acidosis. Thus, if the substrates for phosphoenolpyruvate carboxykinase have either not changed (GTP, ITP) or have decreased (oxaloacetate), an increased flux through this enzyme in acidosis (i.e. conversion of glutamine to glucose) necessitates either its activation or induction. A necessary prerequisite for this mechanism is that the phosphoenolpyruvate carboxykinase reaction is normally far from equilibrium.

Glycolytic intermediates in ischaemia.

The tissue contents of glycolytic intermediates have been particularly well studied during aerobic/anaerobic transition by Lowry et al. (1964) in mouse brain and by

Williamson (1966) in perfused rat heart. The same general picture was obtained in both these systems: the intermediates from glucose to fructose-6-phosphate decreased in anoxia, fructose diphosphate was greatly elevated and the intermediates from the triose phosphates to phosphoenolpyruvate were also elevated; pyruvate was slightly elevated in some experiments, slightly decreased in others. These results were interpreted as an activation of phosphofructokinase brought about primarily by the decrease in ATP and the concomitant increase in the levels of inorganic phosphate and AMP. Underwood & Newsholme (1967) have also demonstrated a fall in glucose-6-phosphate and a rise in fructose diphosphate on addition of cyanide to kidney cortex slices. As the properties of rat kidney cortex phosphofructokinase are similar to those from other sources (i.e. an inhibition by ATP , potentiated by citrate and relieved by AMP and fructose diphosphate and an activation by ADP and inorganic phosphate, Underwood & Newsholme, 1967) an activation of this enzyme in kidney cortex could also increase the rate of glycolysis.

The mass action ratio for the phosphoglucoisomerase reaction remained very constant during ischaemia (0.333 <u>in vivo</u>, 0.361 after 30 sec., and 0.315 after 1 min.).

The apparent equilibrium constant for this reaction is 0.47 (Hess, 1963), so that phosphoglucoisomerase appears to be in equilibrium.

The 'crossover' between fructose diphosphate and fructose-6-phosphate in the ischaemic kidney is a similar phenomenon to that found by previous workers in other systems. The apparent equilibrium constant for this reaction is 1,200 (Hess, 1963). The calculated mass action ratios are 0.720 in vivo, 6.38 after 30 sec. ischaemia, 9.58 after 1 min. and 18.4 after 2 min. Thus phosphofructokinase is normally far from equilibrium but in ischaemia it comes closer to equilibrium. That this occurs during greatly increased flux and with decreased concentrations of substrates (both ATP and fructose-6-phosphate decrease) necessitates an activation of this enzyme.

It seems probable that the enzyme steps from the triose phosphates to 3-phosphoglycerate are all close to equilibrium. Veech (1968) derived the following equation to describe the mutual equilibrium of glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase and 3-phosphoglycerate kinase:

$$* \frac{[\text{3-phosphoglycerate}]}{[\text{Glyceraldehyde-3-phosphate}]} \times \frac{[\text{ATP}]}{[\text{ADP}] \times [P_i{}^{2-}]} \times \frac{[\text{Lactate}]}{[\text{Pyruvate}]}$$

$$= \frac{K_{GADH} \times K_{3PGkinase}}{K_{LDH}} = \frac{59}{1.11 \times 10^{-4}} \text{ M} \quad \text{at pH 7.0}$$

If, in addition, it is assumed that triosephosphate isomerase is at equilibrium, then $\left[\frac{\text{total triose phosphates}}{23}\right]$ may be substituted for glyceraldehyde-3-phosphate. ($K_{eq} = \frac{[\text{Dihydroxyacetonephosphate}]}{[\text{Glyceraldehyde-3-phosphate}]} = 22$, Bergmeyer et al., 1965). The equation then becomes:

$$\frac{[\text{3-phosphoglycerate}]}{[\text{total triose phosphates}]} \times \frac{[\text{ATP}]}{[\text{ADP}] \times [P_i{}^{2-}]} \times \frac{[\text{Lactate}]}{[\text{Pyruvate}]}$$

$$= \frac{59 \times 10^4}{1.11 \times 23} \text{ M} = 23{,}110 \text{ M}.$$

The values for this expression obtained in the kidney were 64,000 in vivo, 19,800 after 30 sec. ischaemia, 21,700 after 1 min. and 26,700 after 2 min. These are sufficiently close to the theoretical equilibrium value to justify the conclusion that the segment of glycolysis from dihydroxyacetone phosphate to 3-phosphoglycerate is quite close to equilibrium even during the increased flux caused by ischaemia.

---

* The $HPO_4{}^{2-}$ values are calculated from the inorganic phosphate measurements, assuming a pH of 7.0, and are expressed as Molar.

The ischaemic kidney content of 2-phosphoglycerate was too low to measure accurately so that no statement can be made about the proximity to equilibrium of phosphoglycerate mutase. The <u>in vivo</u> mass action ratio of 0.165 compared to the apparent equilibrium constant of 0.17 (Hess, 1963) indicated that it is normally very close to equilibrium.

Pyruvate kinase was very far from equilibrium in all conditions; its mass action ratio was 0.823 <u>in vivo</u>, 0.500 after 30 sec. of ischaemia, 0.400 after 1 min. and 0.360 after 2 min. According to Hess (1963) the apparent equilibrium constant is 15,000. The substrates for this enzyme did not increase during ischaemia - after 30 sec. ADP showed no change in content (1.34 to 1.30), while phosphoenolpyruvate decreased (0.035 to 0.014). An increased flux through a non-equilibrium enzyme without increases in substrate concentrations necessitates an activation of the enzyme.

The mechanism for the activation of pyruvate kinase is not known but the following observations may be of use in formulating a hypothesis:

(a) Tanaka et al. (1965) have shown that there are two isoenzymes of pyruvate kinase in tissues, a muscle-type (M-type) found in muscle, brain, heart, liver,

kidney etc., and a liver type (L-type) found only in liver and kidney. Only the L-type varied in activity with dietary and hormonal conditions.

(b) Passeron et al. (1967) showed that the L-type of pyruvate kinase is strongly inhibited by low concentrations of Cu^{2+} but that low fructose diphosphate concentrations (0.066mM) prevented this inhibition. This concentration of fructose diphosphate, in the absence of Cu^{2+}, also enhanced pyruvate kinase activity, especially after pre-incubation.

(c) ATP inhibits liver pyruvate kinase (Seubert & Huth, 1965).

The following hypothesis may then be tentatively proposed to explain the increased glycolysis during ischaemia in the kidney.

(a) During ischaemia, ATP formation does not keep pace with its utilization so that there is an increase in inorganic phosphate and ADP. The ADP increase may be small and momentary and, through the mediation of adenylate kinase, it causes a large percentage increase in AMP. The combination of decreased ATP (an inhibitor) and increased inorganic phosphate and AMP (an activator and a deinhibitor,

respectively) increases the activity of phospho-fructokinase.

(b) As the concentration of ATP decreases its inhibitory action on pyruvate kinase is lessened; the elevated fructose diphosphate (a result of the phosphofructo-kinase activation) also increases the activity of the enzyme. Thus pyruvate kinase is enabled to catalyse an increased conversion of phosphoenol-pyruvate to lactate without increased substrate concentrations.

(c) As the amount of glucose removed in 2 min. ischaemia (Table V.3) agrees well with the amount of lactate accumulating it is likely that glucose is the substrate for this anaerobic glycolysis. As the concentrations of ATP and glucose will be progressively decreasing, this suggests that an activation of hexokinase must also be necessary if this enzyme is out of equilibrium. However, the overall tissue content of glucose is probably a poor indicator of the concentration at the enzyme site so that whether, or not, hexokinase is at equilibrium in the kidney cannot be decided.

In this hypothesis, the elevated fructose diphosphate serves both to activate phosphofructokinase and pyruvate

kinase. A similar scheme has been proposed by Hess (1966) for glycolysis in yeast based on the great activation of yeast pyruvate kinase by fructose diphosphate and the crossover points obtained at pyruvate kinase during oscillations in glycolysis. Hess uses the term 'positive feed forward' to describe the activation of pyruvate kinase by fructose diphosphate. That activators of pyruvate kinase in anoxia were not observed in brain and heart may be related to the absence of the fructose diphosphate sensitive (L-type) isoenzyme in these tissues. It might, therefore, be anticipated that such an activation would occur in the ischaemic liver which possesses this isoenzyme. Preliminary experiments strongly suggest that this is the case.

CHAPTER VI

THE CONCENTRATIONS OF AMMONIA IN BLOOD AND TISSUES

It is well established that there are large concentration gradients between plasma and tissues for the amino acids (Holden, 1962). Schimassek & Gerok (1965) have recently demonstrated that these concentration gradients are, in part, maintained by the liver and can also be demonstrated in the isolated perfused liver. No information is available as to whether there are also concentration gradients for ammonia. Since ammonia in solution exists both as ammonium ions (NH_4^+) and ammonia gas (NH_3) and since gases are highly diffusible it might be predicted that no gradient could exist. This chapter will be concerned with:

(a) demonstrating that such a gradient exists <u>in vivo</u>;

(b) studying some of its properties in the perfused liver, and

(c) a discussion of the possible mechanisms by which it is maintained.

Evidence for an ammonia gradient

While it has long been appreciated that the ammonia content of whole blood is extremely low (about 10 µM, see Reif, 1960 and Bromberg et al., 1960) its content in tissues has not been easy to measure with accuracy. That appreciable quantities are found in many tissues is shown in Table VI.1. It can be seen that there is at least a 10-fold gradient across all the tissues examined.

The ammonia content of portal venous blood is known to be considerably higher than in blood from the systemic circulation. This is primarily due to the action of bacteria in the colon on dietary amino acids. Administration of Neomycin greatly decreases the ammonia concentration of portal venous blood (Silen et al., 1955). Most of this ammonia is removed by the liver and converted to urea and this uptake by the liver occurs continuously against a concentration gradient. To illustrate this point the concentrations of ammonia in the blood vessels serving the liver were measured (Table VI.2) in the rat. Since the relative blood flow through the liver is approximately 75% from the portal vein and 25% from the hepatic artery, the hepatic extraction of ammonia may be calculated as being more

Table VI.1

Ammonia content of arterial blood and some freeze-clamped tissues from rat and dog

The blood and tissues were treated as described in Chapter II. Ether anaesthesia was used to obtain the rat thigh muscle; the dogs were anaesthetised with Nembutal. The no. of observations is in parentheses'

Rat

Arterial whole blood	0.02 µmoles/ml.	(9)
Liver	0.712 µmoles/g.	(5)
Abdominal muscle	0.867 " "	(7)
Kidney	0.878 " "	(6)
Brain	0.340 " "	(3)
Thigh muscle	0.255 " "	(2)
Spleen	0.204 " "	(2)
Heart	0.199 " "	(2)

Dog

Arterial whole blood	0.052 µmoles/ml.	(2)
Muscle	0.761 µmoles/g.	(2)
Brain	0.918 " "	(2)
Pancreas	1.94 " "	(2)

Table VI.2

Ammonia content in the liver and its blood vessels

All the data refer to fed rats. The number of observations is shown in parenthesis.

Liver	0.712 µmoles/g. fresh wt.	(5)
	= 0.975 µmoles/ml. tissue water.	
Arterial Blood	0.020 µmoles/ml.	(9)
Portal Venous Blood	0.261 " "	(9)
Hepatic Venous Blood	0.031 " "	(10)

Ratio $\frac{\text{Liver ammonia}}{\text{Hepatic venous ammonia}}$ = 31.4

than 80%. The gradient between the liver and the venous outflow is 23-fold. In fact, this gradient is higher still since the concentration of ammonia in tissue water is greater than the tissue content (0.71 μmoles/g.). As the water content of the liver is 73%, the intracellular concentration is likely to be about 0.97 mM so that the gradient is 31.4-fold. Seligson & Hirahara (1957) have shown that in human venous blood the erythrocytes contain 2.8 times more ammonia than the plasma. If this also holds for rat blood it would result in a plasma concentration in hepatic venous blood of 0.0094 μmoles/ml. and would mean that the effective liver/plasma gradient is more than 100-fold.

The agreement between the mitochondrial $\frac{[NAD]}{[NADH]}$ ratios as calculated from the reactant of glutamate dehydrogenase and hydroxybutyrate dehydrogenase (Chapter III and Williamson et al., 1967b) demonstrates that these enzymes are close to equilibrium. Such agreement would not be achieved if any of the measured metabolites (hydroxybutyrate, acetoacetate, glutamate, oxoglutarate and ammonia) were bound to any appreciable extent within the cell. Thus the observed gradient cannot be explained by invoking the existence of a large fraction of the intracellular ammonia in a bound form.

The ammonia gradient in the perfused liver

The study of the liver-blood ammonia gradient *in vivo* is difficult because conditions cannot be altered easily. In view of the success in demonstrating amino acid gradients in the isolated perfused liver (Schimassek & Gerok, 1965) it was decided to see whether this system could be used as a model for studying the ammonia gradient. The technique employed is described in Chapter II. Oleate (2mM) was included as a substrate as, without fatty acids in the perfusion medium, the redox state of the mitochondrial pyridine nucleotides becomes oxidised relative to the *in vivo* state (R. Hems, personal communication). Livers from fed rats were perfused for 38 min. and at this time 75 μmoles of NH_4Cl were added to the medium, bringing the ammonia concentration up to about 0.5mM. Samples were taken at intervals from the medium and at the end of the experiment (100 min.) the liver was freeze-clamped. The $\frac{[lactate]}{[pyruvate]}$ and $\frac{[hydroxybutyrate]}{[acetoacetate]}$ ratios were used as criteria of the physiological state of the perfused organ as unsatisfactory perfusions tend to cause anoxia in the liver which would increase these ratios (see Chapter III).

There was an initial lactate concentration in the medium of 0.806mM (Table VI.3) which increased to 1.33mM

Table VI.3 Removal of added ammonia by the perfused liver

Livers (from fed rats) were perfused with a medium containing 2mM oleate. After 38 min., 75 μmoles of NH_4Cl were added. Medium samples were taken at the times indicated. The average weight of the livers was 8.05 g. For further details see text. Gas phase: 95% O_2/5% CO_2. Results are in μmoles/ml. ± S.D. and are the means of four perfusions.

	0'	10'	40'	43'	46'	49'	55'	70'	85'	100'
Ammonia	0.103± 0.020	0.060± 0.017	0.418± 0.020	0.332± 0.000	0.282± 0.032	0.204± 0.060	0.104± 0.024	0.072± 0.024	0.069± 0.020	0.069 0.010
Urea	0.198± 0.090	0.994± 0.140	0.957± 0.104				1.18± 0.153	1.33± 0.216	1.51± 0.305	1.61± 0.269
Lactate	0.806± 0.287	1.33± 0.439	1.21± 0.304				1.19± 0.407	0.935± 0.739	0.732± 0.673	0.601± 0.412
Pyruvate	0.010± 0.004	0.017± 0.001	0.020± 0.010				0.026± 0.018	0.029± 0.009	0.030± 0.027	0.047± 0.033
Lactate / Pyruvate		78.2	60.5				45.8	32.2	24.4	12.8
Hydroxybutyrate	0.024± 0.022	0.430± 0.143	1.46± 0.122				1.78± 0.195	1.96± 0.299	2.10± 0.474	2.22± 0.600
Acetoacetate	0.027± 0.024	0.072± 0.010	0.373± 0.151				0.674± 0.402	0.829± 0.447	1.02± 0.541	1.39± 0.894
Hydroxybutyrate / Acetoacetate		5.97	3.91				2.64	2.36	2.06	1.60

after 10 min. (due to the washing out of the lactate which accumulated during the anoxic phase of the operation) and thereafter decreased to a concentration of 0.601mM at 100 min. Pyruvate slowly increased in concentration from 0.018 mM before perfusion to 0.047 mM at 100 min. The $\frac{[\text{lactate}]}{[\text{pyruvate}]}$ ratio was quite high (78.2) after 10 min. perfusion but returned to a normal <u>in vivo</u> value (12.8) at 100 min. There was a production of ketone bodies from oleate at a fairly linear rate of about 0.75 µmoles/g. liver/min. over the first 55 min. which then dropped to about half this rate. The $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratio was high (5.97) at 10 min. but decreased gradually to a value of 1.50 after 100 min. All of these observations may be taken as evidence for a healthy functioning perfused liver.

There was a large increase in the urea concentration of the perfusion medium after 10 min. due to the washing out of urea from the liver. During the next 30 min. the rate of urea production from endogenous precursors was 0.221 µmoles/g./min., which increased to 0.266 µmoles/g./min. after the addition of ammonia to the medium. Towards the end of the perfusion the rate decreased to about 0.16 µmoles/g./min. Barak & Beckenbauer (1966) recorded an endogenous rate of urea production of 0.25 µmoles/g./min.

in perfused livers from fed rats, while Hems et al. (1966) found a rate of 0.1 μmoles/g./min. in livers from starved rats.

The initial ammonia concentration in the medium was reduced from 0.103 mM to 0.060 mM within 10 min. When, at 38 min., 75 umoles of NH_4Cl was added to the medium this was rapidly removed until a steady value of about 0.07 mM was reached. This is approximately double the concentration found in hepatic venous blood. The rate of ammonia removal between 40 min. and 43 min. was 0.51 μmoles/g./min. which is well below the maximum rate of urea synthesis (Hems et al., 1966 observed a production of 0.8 μmoles urea/g./min. from 10 mM NH_4Cl) so that the removal of ammonia in the present experiments may be determined merely by the flow rate through the liver. This rapid removal of ammonia to a low concentration is extremely similar to that reported by Glogner (1966). Hems et al. (1966), however, found that the perfused liver could never reduce the ammonia concentration of the medium to less than 0.5mM.

The liver was freeze-clamped immediately after the final medium sample was taken (100 min.) and various metabolites measured in it (Table VI.4). The $\frac{[\text{lactate}]}{[\text{pyruvate}]}$ and $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ ratios were quite similar to those

Table VI.4

Content of various metabolites in livers from fed rats after 100 min. perfusion

For details see text. Results are in μmoles/g. fresh wt. ± SD. and represent the mean of four perfusions.

Lactate	0.750 ± 0.456
Pyruvate	0.064 ± 0.010
$\frac{\text{Lactate}}{\text{Pyruvate}}$	11.7
Hydroxybutyrate	1.24 ± 0.520
Acetoacetate	0.923 ± 0.475
$\frac{\text{Hydroxybutyrate}}{\text{Acetoacetate}}$	1.34
Glutamate	2.13 ± 0.302
Oxoglutarate	0.239 ± 0.103
Ammonia	0.612 ± 0.082
ATP	2.00 ± 0.424
ADP	0.796 ± 0.186
AMP	0.324 ± 0.076
$\frac{\text{Hydroxybutyrate x oxoglutarate x ammonia}}{\text{Acetoacetate x Glutamate}}$	9.2×10^{-2} M
$\frac{\text{Liver ammonia}}{\text{Medium ammonia}}$	9.25 ± 2.81

found for the medium at this time. The contents of the adenine nucleotides were comparable to those reported by Hems et al. (1966) in the perfused livers of starved rats; the ratio of $\frac{ATP}{ADP}$ was 2.51.

The concentration of ammonia in the liver was 0.612 μmoles/g. so that there was a 9-fold liver/perfusion medium gradient at the end of the perfusion. The value for the expression $\frac{[\text{hydroxybutyrate}] \times [\text{oxoglutarate}] \times [\text{ammonia}]}{[\text{acetoacetate}] \times [\text{glutamate}]}$ was 9.2×10^{-2}mM. This is very close to the theoretical value (7.9×10^{-2}mM) for equilibrium between the two mitochondrial dehydrogenases so that as already mentioned intracellular binding of ammonia cannot be invoked to explain the gradient in the perfused liver.

Effect of anaerobiosis on the ammonia gradient

If the observed ammonia gradient is a functional one then its maintenance must require the expenditure of energy. In anaerobiosis the gradient should, therefore, be diminished or even abolished. This can be conveniently studied in the perfusion system where anaerobiosis is easily attained. Perfusion was therefore carried out as before (2mM oleate was included in the medium so as to be comparable with the aerobic perfusions), except that:

(a) the gas used was 95% N_2 / 5% CO_2

(b) sodium cyanide (2mM final concentration) was added to the medium just before perfusion and an equal quantity was added every 30 min. to replace that which may be blown off in the artificial lung.

Perfusion was continued for 70 min. The concentrations of some metabolites in the medium are shown in Table VI.5. The high ratios of $\frac{[\text{lactate}]}{[\text{pyruvate}]}$ and $\frac{[\text{hydroxybutyrate}]}{[\text{acetoacetate}]}$ indicate the reduced redox states of the intracellular pyridine nucleotide pool. There was a large increase in the concentration of lactate in the medium as a result of anaerobic glycolysis. There was also a small but steady increase in the concentration of ketone bodies in the medium. In the hour between 10 min. and 70 min. this represented a synthesis of 0.768 μmoles/g. liver. The reason for this is not known but Krebs & Gascoyne (1968) have also reported an anaerobic synthesis of ketone bodies in liver homogenates.

There was an initial 'wash-out' of tissue urea in the first 10 min. of perfusion, similar to that observed in the aerobic perfusions, but once this was complete (sometime between 10 min. and 40 min.) there was no longer any production of urea. The ammonia concentration

Table VI.2 Anaerobic perfusion of the liver

Livers (from fed rats) were perfused with a medium containing 2m% oleate and 2m. NaCN. Medium samples were taken at the times indicated. The average weight of the livers was 8.9 g. Results are in μmoles/ml. ± S.D. and are the means of four perfusions. Gas phase: 95% N_2/5% CO_2. For other details see text.

	0'	10'	40'	70'
Ammonia	0.124±0.036	0.163±0.028	0.242±0.031	0.348±0.044
Urea	0.114±0.010	0.464±0.067	0.643±0.135	0.631±0.124
Lactate	1.14 ±0.248	3.82 ±0.783	5.98 ±1.22	7.06 ±1.45
Pyruvate	0.020±0.015	0.034±0.011	0.033±0.006	0.023±0.003
Lactate / Pyruvate	-	112	181	307
Hydroxybutyrate	0.004±0.004	0.036±0.009	0.096±0.017	0.087±0.064
Acetoacetate	0.005±0.003	0.009±0.006	0.009±0.005	0.007±0.006
Hydroxybutyrate / Acetoacetate	-	4.0	6.2	12.4

Table VI.6

Content of various metaboiltes in livers from fed rats after 70 min. anaerobic perfusion

For details see text. Results are in μmoles/g. fresh wt. ± SD. The no. of perfusions are given in parenthesis.

Lactate	4.58	± 0.974	(4)
Pyruvate	0.019	± 0.020	(4)
$\frac{\text{Lactate}}{\text{Pyruvate}}$	241		
Hydroxybutyrate	0.105	± 0.058	(4)
Acetoacetate	0.010	± 0.012	(4)
$\frac{\text{Hydroxybutyrate}}{\text{Acetoacetate}}$	10.5		
Glutamate	1.02	± 0.058	(4)
Oxoglutarate	0.015	± 0.020	(4)
Ammonia	1.17	± 0.344	(4)
ATP	0.064		(2)
ADP	0.209		(2)
AMP	1.11		(2)

$$\frac{\text{Hydroxybutyrate} \times \text{oxoglutarate} \times \text{ammonia}}{\text{Acetoacetate} \times \text{glutamate}} \qquad 18 \times 10^{-2}\ \text{mM}$$

$\frac{\text{Liver ammonia}}{\text{Medium ammonia}}$	3.34	± 0.598	(4)

in the medium steadily increased at a rate equal to about 3.0 μmoles/g. liver/hour.

Immediately after the final (70 min.) sample of perfusion medium was taken the livers were freeze-clamped and the tissue content of certain metabolites was measured (Table VI.6). The levels of lactate, pyruvate, hydroxybutyrate and acetoacetate were very similar to their concentrations in the medium at this time. The levels of the adenine nucleotides were in accordance with the anaerobic state of the liver. The value for the expression $\frac{[\text{hydroxybutyrate}] \times [\text{oxoglutarate}] \times [\text{ammonia}]}{[\text{acetoacetate}] \times [\text{glutamate}]}$ was 2½-fold higher than the theoretical equilibrium value but, as the levels of oxoglutarate and acetoacetate were too low to measure accurately with the methods employed the calculated value for this expression may be in error.

The ammonia content of the liver was elevated to 1.17 μmoles/g. and the gradient $\frac{[\text{liver ammonia}]}{[\text{medium ammonia}]}$ was decreased to 3.34 (as opposed to 9.25 in the aerobic perfusion); this decrease in gradient was significant ($p < 0.01$). Thus it appears that energy from oxidative metabolism is necessary for the maintenance of the normal ammonia gradient in livers.

Discussion

Species of ammonia in aqueous solution

So far in this thesis 'ammonia' has been used as a generic term embracing its molecular species, but for the purposes of this discussion we must differentiate between the species. Ammonia exists in solution in two forms according to the equation:

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

The pK for this reaction is 9.02 (Jacquez et al., 1959). Thus the relative proportions of NH_3 and NH_4^+ in solution are pH-dependent. About one of every ten molecules at pH 8.0, one of every 100 molecules at pH 7.0 and one of every 1000 molecules at pH 6.0 exists as the free base, the remainder as ammonium ions. In this discussion the free base is designated as 'NH_3' and ammonium ions as 'NH_4^+'; the sum of these species is designated as 'ammonia'.

Possible mechanisms for the maintenance of the ammonia gradient

The problem to be discussed here is the maintenance of the ammonia gradient by the liver and the *in vivo* uptake of ammonia against this gradient. There appear

to be three mechanisms by which ammonia could become concentrated across the liver plasma membrane:

(a) free diffusion of NH_3 into the cell in response to a lower intracellular pH,

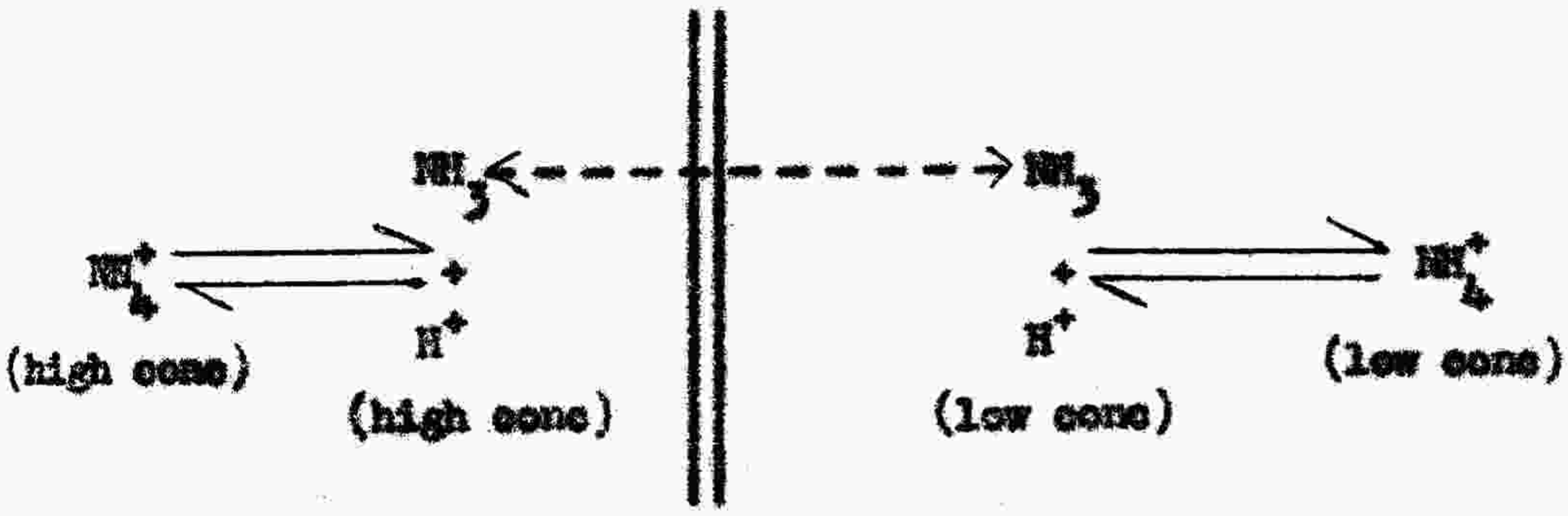
(b) distribution of NH_4^+ in response to a membrane potential, and

(c) active 'pumping' of NH_4^+ across the plasma membrane.

The first two mechanisms involve the passive movement of ammonia, although energy would have to be expended to maintain both the postulated pH gradient and the membrane potential. These mechanisms will now be discussed in greater detail.

Free Diffusion of NH_3

Jacobs (1940) has suggested that, because of its high lipid solubility and lack of charge, NH_3 readily diffuses across cell membranes, whereas NH_4^+ penetrates much more slowly because of its charge, its larger molecular diameter (due to hydration) and its low lipid solubility. Therefore NH_3 diffuses across cell membranes until its concentration becomes equal on both sides of the membrane; NH_4^+ then accumulates on the more acid side as in the following scheme:



If the membrane is absolutely impermeable to NH_4^+ then a 10-fold greater $[H^+]$ on one side would result in a 10-fold greater $[NH_4^+]$ on the same side. Since it is total ammonia that is experimentally measured (not NH_4^+), this relationship may be more conveniently expressed as follows (Milne et al., 1958):

$$\frac{[\text{total ammonia}]\ \text{intracell}}{[\text{total ammonia}]\ \text{extracell}} = \frac{1 + 10^{\text{pK-pH intracell}}}{1 + 10^{\text{pK-pH extracell}}}$$

While there is now little doubt that the renal excretion of ammonia is by the passive diffusion of NH_3 into a more acid solution (urine), (Pitts, 1964) the application of this mechanism to the liver gradient raises difficulties. From the above equation it can be calculated that (assuming a blood pH of 7.40) an intracellular pH of 5.89 is necessary to explain a 31.4-fold gradient between liver and blood. An even lower pH must be invoked if the membrane is slightly

permeable to NH_4^+.

There is no precise information on the pH of the liver cell. Indeed, since the cell is not a homogeneous aqueous phase but contains many different compartments, each liable to have different hydrogen ion activities, the concept of intracellular pH is not very meaningful. However, with a knowledge of the pH values and volumes of the individual compartments, it would be possible to calculate an 'overall' intracellular pH. For the purposes of ammonia distribution between liver and plasma it is the overall pH that is relevant. An estimate of this overall pH can be arrived at by measuring the total CO_2 in the freeze-clamped liver together with a knowledge of the pH and CO_2 concentration of blood. The rationale behind this experiment is that CO_2 equilibrates across the membrane and enters into equilibrium with HCO_3^- within the cell; therefore, with a knowledge of the liver content of HCO_3^- + CO_2 (= total CO_2) and the carbonic anhydrase equilibrium constant, the overall pH of the cell can be estimated. Krebs (1967) has estimated the overall pH of the liver on this basis and has reported a value of 7.22. It is possible, however, that this method may give an erroneously high estimate of the intracellular pH. It is assumed that the total measured CO_2 in freeze-

clamped liver represents only CO_2 and HCO_3^-, yet it is known that the amino groups of proteins can form carbamino compounds with CO_2. Carbamino formation means that the total measured liver CO_2 no longer represents just CO_2 and HCO_3^- and so the estimated liver HCO_3^- is higher than the actual HCO_3^-. The net result is an estimate of the intracellular pH which is too alkaline. Krebs (1967) assumed that the amount of carbamino-CO_2 is less than 10% of the total measured CO_2; this amount would not appreciably affect his estimate. In any case, as the pH falls the capacity of amino groups to form carbamino compounds decreases sharply, so that it is unlikely that the pH of the liver cell is less than 6.0 and that there is a great amount of carbamino-CO_2.

Thus, while there is no satisfactory direct estimate of the overall pH of the liver cell it is unlikely to be below 7.0 and certainly not 5.89, the pH necessary to justify passive diffusion of NH_3 across the liver plasma membrane as the mechanism for the ammonia gradient.

Distribution of NH_4^+ in response to a membrane potential

If it is accepted that the overall pH of the liver cell is not too dissimilar from the pH of blood then the

ratio $\frac{[NH_4^+]\text{intracellular}}{[NH_4^+]\text{extracellular}}$ will be quite similar to the ratio $\frac{[\text{total ammonia}]\text{ intracellular}}{[\text{total ammonia}]\text{ extracellular}}$ i.e. about 31.4. This gradient could be brought about by a potential across the membrane. Two conditions are necessary for this mechanism to be effective:

(i) NH_4^+ must be permeable through the membrane so that the potential can influence its distribution, and

(ii) NH_3 must be quite impermeable through the membrane because otherwise, since it would be at a higher concentration inside than out, it would diffuse back out of the cell thus counteracting the effect of the membrane potential.

At equilibrium (i.e. when the tendency of the cell to accumulate cations is just balanced by their tendency to diffuse out along a concentration gradient) the situation is described by the Nernst equation:

$$\text{potential difference (mV)} = -61 \log \frac{[NH_4^+]\text{intracellular}}{[NH_4^+]\text{extracellular}}$$

$$= -61 \log 31.4$$

$$= -91.3 \text{ mV}$$

Thus a membrane potential of -91 mV is required to explain the ammonia gradient; if NH_3 were slightly permeable the potential would be even higher. A search of the literature revealed no published data on the <u>in vivo</u>

potential of the liver cell membrane. Li & McIlwain (1957) measured the potentials of liver slices and obtained values of-36 mV for guinea pig slices and-34 mV for cat liver slices. These potentials, however, may be lower than the <u>in vivo</u> potentials because of the leakage of cations that may occur in incubated slices. Parsons & Van Rossum (1961) considered that the highest values recorded by Li & McIlwain (45 mV) may be close to the values likely to occur <u>in vivo</u>. It seems extremely unlikely that liver, a non-excitable tissue, has a membrane potential as high as-91 mV, so that an explanation of the ammonia gradient on this basis seems unlikely.

Active pumping of NH_4^+ across the membrane

It is conceivable that NH_4^+ could be actively pumped across the membrane. Again, a necessary precondition for this mechanism is that the diffusion of NH_3 out of the cell should be absent or very small. It is perhaps relevant that the Na^+, K^+-activated ATP-ase is also activated by NH_4^+ - NH_4^+ can replace the K^+ activation and is about equally as effective as K^+ (Skou, 1960; Kendi, 1964). That NH_4^+ can compete with K^+ for inward transport has been shown in erythrocytes by Post & Jolly

(1957). A similar effect has been suggested in cat cerebral cortex slices by Tower et al., (1961). As rat liver possesses an extremely high activity of the Na^+,K^+-activated ATPase (1,200 µmoles ATP hydrolysed/gm. protein/min. according to Albers, 1967) such a mechanism is, perhaps, a feasible explanation for the ammonia gradient observed in vivo across the rat liver.

CHAPTER VII

GENERAL DISCUSSION

As the principal problems arising from the experiments in this thesis were discussed at the end of the appropriate chapter, this discussion will be limited to some of the more general aspects of the work.

Use of anaesthetics for the measurement of tissue metabolites

In the present work anaesthesia was avoided whenever possible. Exceptions are the use of Nembutal to obtain samples of renal venous blood and ether to obtain samples of skeletal muscle. As a great number of investigators make use of anaesthetics prior to tissue sampling, an examination of their merits, relative to those of cervical dislocation, is necessary.

Cervical dislocation is only of use for rapidly obtaining tissues from the abdominal or thoracic cavities. Furthermore, in our hands, it is only feasible on animals of 250 g. body wt. or less. Its potential

disadvantages are,

(a) that the tissue may become slightly anoxic before it can be frozen, and

(b) that the handling immediately prior to death may cause alarm and stress to the animal.

It seems unlikely that anoxia is a serious problem for organs obtained from the abdominal cavity. Although the time between cervical dislocation and freezing of tissue may be as long as 10 sec., the time between the excision of the tissue and its freezing is usually 1-2 sec. As the heart is still beating and the blood pressure appears normal at the time of excision, it is this 1-2 sec. period that must be regarded as the potential anoxic time. Anoxia may, however, be a serious complication when heart is being sampled, as the time elapsing between the opening of the thorax and the freezing of the heart is normally about 5-7 sec. This reservation also applies to anaesthetised animals unless some form of artificial respiration is employed. While it is difficult to evaluate the extent of the stress caused prior to cervical dislocation of the animals, it may well be no greater than that caused by the injection of Nembutal or the administration of ether.

The use of anaesthetics allows the freezing of

tissues *in situ* (thus eliminating the possibility of anoxia) but there are reservations about their possible effects on metabolism. It is self-evident that any chemical that produces loss of consciousness must be interfering with metabolic processes in the brain; therefore, whenever possible, anaesthetics should be avoided in the measurement of metabolites in brain. That anaesthetics can affect extracerebral metabolism has been shown by Marquez-Julio & French (1967), who found substantial differences in the content of many metabolites in skeletal muscle from pentobarbitol or ether anaesthetised rats. Similar results were obtained by Dale (1965) in liver. Particularly striking was the highly significant difference between the hepatic pyruvate content of ether anaesthetised rats (0.220 μmoles/g.) and Nembutal anaesthetised rats (0.029 μmoles/g.). The demonstration by Aldridge & Parker (1960) of an inhibitory effect of many barbiturates on oxidative phosphorylation in liver mitochondria provides a further argument against the use of Nembutal.

In general, therefore, it is felt that for the estimation of metabolites in abdominal organs, cervical dislocation is the method of choice but that ether should be employed whenever an anaesthetic is required.

A possible physiological role for the glutamate dehydrogenase - aminotransferase mechanism in the removal of ammonia

There is little doubt that the urea cycle is not normally over-extended. The activities of the enzymes are quite high relative to the daily excretion of urea. Furthermore, it is well known that the normal functions of glutamate dehydrogenase and the aminotransferases lie in the area of amino acid metabolism, so that one may question their physiological importance in the removal of ammonia. Certainly in the normal course of events they are not of importance in the removal of ammonia, but there are some cases of hyperammonemia where they may have a role.

The more common causes of hyperammonemia are associated with cirrhosis, and it appears that the lesion responsible for hyperammonemia is not enzymatic but circulatory - the bypassing of the liver by a large fraction of ammonia-rich portal blood, due to portacaval shunts, leads to a high concentration of ammonia in the systemic circulation. According to Kirk (1936), who studied the effect of oral administration of ammonium salts to patients with cirrhosis and hepatitis, such

patients could metabolise ammonia perfectly well, provided that there was no shunt between the portal and peripheral venous systems. Thus the removal of ammonia by hepatic glutamate dehydrogenase and the aminotransferases can play no effective part in this type of hyperammonemia.

There are, however, a class of diseases in which this mechanism may be of importance in the removal of ammonia. These are the inborn metabolic defects which affect the urea cycle. Four distinct types have been reported: (a) hyperammonemia due to a deficiency of carbamylphosphate synthase (Russell et al., 1962); (b) hyperammonemia due to a deficiency of ornithine transcarbamylase (Freeman et al., 1964); (c) citrullinuria, probably due to a deficiency of argininosuccinic acid synthase (McMurray et al., 1963), and (d) argininosuccinic aciduria (Tomlinson & Westall, 1964). Argininosuccinic aciduria has been fairly well studied, considering its rarity. The activities of the urea cycle enzymes have been measured in an argininosuccinic aciduric child (Miller & McLean, 1967) and a huge reduction in the activity of argininosuccinase (3% of normal) has been shown. Nevertheless the activity present was sufficient to account for the production of about 30 g. urea per

day which is in the normal range for a child (16 year old boy) of this age. This helps to resolve one of the puzzling features of this disease - the maintenance of normal blood urea and the daily production of normal amount of urea. However, the argininosuccinase activity observed in the patient would only be adequate if the load presented to it was spread evenly throughout the day. This would not usually be the case and at times (after a meal) the capacity of the defective urea cycle would be overextended. At such times hyperammonemia is observed (Miller & McLean, 1967). This is precisely the situation in which the removal of ammonia by the formation of alanine and aspartate could be of use in the temporary storage of ammonia. When the immediate 'ammonia crisis' was over and the intrahepatic concentration of ammonia began to fall the reversal of the glutamate dehydrogenase and aminotransferase reactions (by mass action) could slowly supply ammonia to the urea cycle. Some evidence that this may actually occur is to be found in the work of Moser et al. (1967) who measured the plasma amino acids after an ammonia tolerance test on an argininosuccinic aciduric child. They found that the alanine concentration increased from 0.526 mM to 1.04 mM during the ammonia load. No other amino acid

increased by as much as this.

<u>Glutamate dehydrogenase in vivo</u>

There is considerable information available about the metabolism of glutamate by isolated mitochondria but much of it is not compatible with the concept that <u>in vivo</u> glutamate dehydrogenase is at equilibrium with mitochondrial NAD and NADH. The possibility that this enzyme reacts slowly with NAD <u>in vivo</u> has been discussed in Chapter IV. Klingenberg (1965) has suggested that glutamate dehydrogenase primarily operates in the direction of incorporation of ammonia into glutamate. This proposal is based on the slow deamination of added glutamate by mitochondria. Recent studies by de Haan & Tager (1968) have demonstrated that rat liver mitochondria are quite impermeable to oxoglutarate unless malate or malonate are present. They suggested that oxoglutarate-transport through the mitochondrial membrane is mediated by a malate-activated carrier. As glutamate oxidation is commonly studied in the presence of arsenite to inhibit further metabolism of oxoglutarate, high concentrations of oxoglutarate tend to accumulate in the mitochondrion and the reaction rapidly comes to equilibrium. That this is probably the cause of the slow oxidation of

glutamate was demonstrated by de Haan & Tager who showed that:

(a) in the presence of arsenite the amount of oxoglutarate in the mitochondria rose within 1 min. to about 40 n moles and then remained constant. There was a slow increase in the amount of oxoglutarate present in the incubation medium;

(b) in the presence of arsenite and malonate only about 10 n moles of oxoglutarate were found in the mitochondria but there was a large and rapid appearance of oxoglutarate in the incubation medium. In this experiment glutamate oxidation was stimulated 2½ times.

Thus it appears that glutamate dehydrogenase may be near equilibrium in isolated mitochondria and rapid oxidation of glutamate prevented by the attainment of equilibrium.

Klingenberg (1965) suggested that glutamate dehydrogenase and NADPH constitute a highly efficient system for the removal of ammonia in liver mitochondria, and this may be responsible for the low concentration of ammonia in blood. The experiments in this thesis suggest that such a view is no longer tenable. Glutamate dehydrogenase may indeed constitute an efficient ammonia

removal mechanism but by virtue of its <u>in vivo</u> equilibrium it maintains an appreciable (0.5-0.7 µmoles/g.) intracellular ammonia concentration. The low blood concentration of ammonia is brought about by the mechanism which maintains a gradient between cell and blood, not by glutamate dehydrogenase

APPENDIX

This appendix contains the data whuch was used to construct the figures in Chapters III and IV.

Table A.1 (corresponds to Figures III.1,2,3,4,5,6 and 7).

Metabolites in rat liver during ischaemia.

Rats (male; 180-200g) were killed by cervical dislocation and a portion of liver freeze-clamped immediately (in vivo). The blood vessels were then severed and the liver allowed to remain in situ for the times indicated and then freeze-clamped. Other details are in Chapter II.

Results are in μMoles/g. fresh wt. ± S.D. There were 5 rats in each group.

	in vivo	2 min.	5 min.
Ammonia	0.712 ± 0.271	0.769 ± 0.201	0.693 ± 0.257
Glutamate	2.35 ± 0.736	2.84 ± 0.574	2.65 ± 0.615
Oxoglutarate	0.165 ± 0.016	0.068 ± 0.002	0.041 ± 0.001
Hydroxybutyrate	0.105 ± 0.043	0.187 ± 0.028	0.201 ± 0.010
Acetoacetate	0.053 ± 0.020	0.028 ± 0.009	0.026 ± 0.015
Alanine	0.874 ± 0.647	1.53 ± 0.935	2.26 ± 0.607
Aspartate	1.19 ± 0.236	1.01 ± 0.311	0.927 ± 0.223
Malate	0.422 ± 0.180	0.549 ± 0.138	0.583 ± 0.082
Lactate	0.975 ± 0.432	3.08 ± 1.73	5.13 ± 2.19
Pyruvate	0.080 ± 0.031	0.052 ± 0.022	0.055 ± 0.014

Table A.2 (corresponds to Figures III. 8 and 9).

Metabolites in rat liver during ischaemia.

Experimental details as for Table A.1. Results are in μMoles/g. fresh wt. ± S.D. There were 4 rats in each group.

	in vivo	2 min.
Isocitrate	0.020 ± 0.010	0.010 ± 0.004
Oxoglutarate	0.115 ± 0.064	0.036 ± 0.014
Malate	0.435 ± 0.123	0.588 ± 0.140
Pyruvate	0.083 ± 0.026	0.060 ± 0.014

Table A.3 (corresponds to Figures III.10,11,12,and 13).

Metabolites in liver from L-cycloserine treated rats during ischaemia.

Rats (male; 180-200g) were injected (i.p.) with L-cycloserine (25 mg./kg. body wt.). After 60 min. they were killed by cervical dislocation and a portion of liver freeze-clamped immediately (in vivo). The other experimental details are as for Table A.1,

Results are in μMoles/g. fresh wt. ± S.D. There were 5 rats in each group.

	in vivo	2 min.	5 min.
Ammonia	0.635 ± 0.207	0.756 ± 0.227	0.770 ± 0.386
Glutamate	1.63 ± 0.428	1.91 ± 0.527	1.85 ± 0.404
Oxoglutarate	0.114 ± 0.061	0.044 ± 0.014	0.034 ± 0.017
Alanine	3.40 ± 0.977	3.46 ± 0.702	3.44 ± 0.923
Aspartate	0.736 ± 0.222	1.09 ± 0.242	1.30 ± 0.348
Malate	0.294 ± 0.153	0.271 ± 0.143	0.264 ± 0.116
Lactate	0.647 ± 0.506	3.11 ± 2.31	5.87 ± 2.58
Pyruvate	0.098 ± 0.083	0.071 ± 0.046	0.080 ± 0.028

Table A.4 (corresponds to Figures III.14 and 15).

Possible ammonia precursors in rat liver during ischaemia.

Experimental details as for Table A.1. There were 5 rats in each group.

	in vivo	2 min.	5 min.
Glutamine	4.68 ± 0.290	4.57 ± 0.500	4.26 ± 0.507
ATP	2.45 ± 0.358	1.44 ± 0.573	0.864 ± 0.302
ADP	1.17 ± 0.232	1.42 ± 0.158	1.39 ± 0.106
AMP	0.300 ± 0.108	0.779 ± 0.312	1.18 ± 0.505

Table A.5a. (corresponds to Figure III.16).

Metabolites in liver from starved rats during ischaemia.

Rats were male, 180-200g and fasted for 48 hours. Other experimental details as in Table A.1.

Results are in μMoles/g. fresh wt. Data from a typical experiment.

	in vivo	2 min.	5 min.
Glutamate	1.71	1.40	1.59
Alanine	0.378	0.784	1.77
Aspartate	0.519	0.676	0.579

Table A.5a. (corresponds to Figure III.17).

Metabolites in liver from starved, L-cycloserine treated rats during ischaemia.

Rats (male;180-200g; fasted for 48 hours) were injected (i.p.) with L-cycloserine (25 mg./kg. body wt.). After 60 min. they were killed by cervical dislocation and a portion of liver freeze-clamped immediately (in vivo). The other experimental details are as for Table A.1.

	in vivo	2 min.	5 min.
Glutamate	1.93	1.80	2.04
Alanine	3.71	3.95	3.93
Aspartate	0.294	0.761	1.02

Table A.6. (corresponds to Figures IV.1,2,3,4,5)

Effect of injection of ammonia on some metabolites in rat liver.

Rats (male; 180-200g) were injected (i.p.) with NH_4Cl (2.5 mMoles/kg. body wt.). They were killed by cervical dislocation at the times indicated and a portion of liver freeze-clamped immediately. The control rats (0 min.) were not injected. Other experimental details are in Chapter II.
Results are in μMoles/g. fresh wt. ± S.D. There were 4 animals in each group.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
Lactate	0.614± 0.244	1.31 ± 0.250	0.683 ± 0.097	0.537 ± 0.076	0.681 ± 0.107	0.697 ± 0.244
Pyruvate	0.073± 0.024	0.103± 0.042	0.081 ± 0.012	0.060 ± 0.011	0.087 ± 0.056	0.093 ± 0.040
Malate	0.332± 0.088	0.209± 0.082	0.178 ± 0.058	0.218 ± 0.079	0.327 ± 0.024	0.354 ± 0.061
Glutamate	2.05 ± 0.621	1.29 ± 0.137	1.95 ± 0.188	2.80 ± 1.25	3.16 ± 0.267	2.19 ± 0.709
Oxoglutarate	0.175± 0.040	0.058± 0.014	0.055 ± 0.020	0.062 ± 0.017	0.184 ± 0.123	0.228 ± 0.130
Hydroxybutyrate	0.232± 0.072	0.184± 0.100	0.179 ± 0.173	0.167 ± 0.077	0.208 ± 0.148	0.271 ± 0.228
Acetoacetate	0.090± 0.014	0.094± 0.020	0.143 ± 0.072	0.123 ± 0.039	0.112 ± 0.039	0.126 ± 0.062
Glutamine	5.03 ± 0.664	5.82 ± 0.660	5.59 ± 0.970	5.74 ± 1.02	5.64 ± 0.739	5.16 ± 1.26
Alanine	1.07 ± 0.365	2.50 ± 0.326	3.24 ± 1.00	2.75 ± 1.40	1.57 ± 0.636	0.685 ± 0.179
Aspartate	1.05 ± 0.198	1.69 ± 0.317	3.11 ± 0.551	2.70 ± 0.437	1.86 ± 0.824	0.899 ± 0.590
Urea	5.52 ± 0.836	6.80 ± 0.891	6.34 ± 0.312	6.20 ± 1.50	6.82 ± 1.22	6.04 ± 1.74

Table A.7. (corresponds to Figures IV.6,7,8,9 and 10).

Effect of injection of ammonia to L-cycloserine treated rats on some metabolites in their livers.

Rats (male; 180-200g) were injected (i.p.) with L-cycloserine (25 mg/kg. body wt.). 60 min. later they were injected (i.p.) with NH_4Cl (2.5 mMoles/kg.body wt.) and at the indicated intervals were then killed by cervical dislocation and their livers frozen. The control rats (0 min.) were not injected with NH_4Cl. Other experimental details are in Chapter II.
Results are in µMoles/g. fresh wt. ± S.D. There are 4 animals in each group.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
Lactate	0.632 ± 0.387	1.36 ± 0.540	0.753 ± 0.334	0.607 ± 0.221	0.417 ± 0.107	0.556 ± 0.213
Pyruvate	0.104 ± 0.088	0.125 ± 0.057	0.099 ± 0.063	0.082 ± 0.064	0.054 ± 0.045	0.057 ± 0.026
Malate	0.195 ± 0.068	0.121 ± 0.062	0.134 ± 0.070	0.236 ± 0.049	0.379 ± 0.049	0.413 ± 0.101
Glutamate	1.77 ± 0.329	1.38 ± 0.333	2.15 ± 0.283	2.89 ± 0.600	2.97 ± 0.834	1.34 ± 0.642
Oxoglutarate	0.196 ± 0.085	0.041 ± 0.018	0.051 ± 0.035	0.059 ± 0.027	0.092 ± 0.016	0.313 ± 0.190
Hydroxybutyrate	0.146 ± 0.076	0.168 ± 0.079	0.167 ± 0.022	0.244 ± 0.061	0.211 ± 0.053	0.198 ± 0.021
Acetoacetate	0.086 ± 0.004	0.159 ± 0.026	0.159 ± 0.069	0.199 ± 0.081	0.124 ± 0.034	0.105 ± 0.028
Alanine	2.56 ± 0.083	2.57 ± 0.361	2.39 ± 0.086	2.31 ± 0.169	2.94 ± 0.657	2.55 ± 0.769
Aspartate	0.575 ± 0.132	1.78 ± 0.282	2.21 ± 0.731	2.78 ± 0.802	2.19 ± 1.55	0.519 ± 0.239
Urea	4.36 ± 0.774	5.50 ± 1.56	5.67 ± 1.87	5.20 ± 1.80	5.69 ± 0.469	5.41 ± 1.44

Table A.8. (corresponds to Figure IV.11).

Effect of preinjection with L-arginine on the response of some amino acids in rat liver to an ammonia load.

Rats (male; 180-200g) were injected (i.p.) with L-arginine (3 mMoles/kg. body wt.). 60 min. later they were injected (i.p.) with NH_4Cl (2.5 mMoles/kg. body wt.). The other experimental details are as for Table A.7.
Results are in µMoles/g. fresh wt. ± S.D. There are 4 rats in each group.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
Glutamate	2.42 ± 1.20	1.48 ± 0.371	2.18 ± 0.792	2.23 ± 0.521	1.78 ± 0.573	1.38 ± 0.159
Alanine	0.728 ± 0.272	1.79 ± 0.692	1.57 ± 0.459	1.00 ± 0.176	0.723 ± 0.224	0.595 ± 0.331
Aspartate	1.12 ± 0.246	1.71 ± 0.635	1.80 ± 0.452	1.11 ± 0.270	0.873 ± 0.180	0.678 ± 0.175

Table A.9 (corresponds to Figures IV.13,14,15,16 and 17).

Effect of injection of ammonia to starved rats on some metabolites in their livers.

Rats (male; 180-200g; fasted for 48 hours) were injected (i.p.) with NH_4Cl (2.5 mMoles/kg. body wt.) They were killed by cervical dislocation at the times indicated and a portion of liver frozen immediately. The control rats (0 min.) were not injected. Other experimental details as in Chapter II.

Results are in μMoles/g. fresh wt. ± S.D. There were 3 rats in each group.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
Lactate	0.533 ± 0.417	1.05 ± 0.350	0.864 ± 0.697	0.744 ± 0.035	0.596 ± 0.196	0.504 ± 0.149
Pyruvate	0.035 ± 0.024	0.054 ± 0.020	0.044 ± 0.024	0.062 ± 0.030	0.043 ± 0.014	0.038 ± 0.020
Malate	0.285 ± 0.135	0.350 ± 0.165	0.399 ± 0.232	0.757 ± 0.282	0.609 ± 0.161	0.474 ± 0.163
Glutamate	2.67 ± 0.461	1.71 ± 0.397	2.50 ± 0.156	4.34 ± 1.33	3.15 ± 1.49	2.43 ± 1.43
Oxoglutarate	0.053 ± 0.017	0.033 ± 0.010	0.042 ± 0.035	0.050 ± 0.010	0.093 ± 0.073	0.134 ± 0.086
Hydroxybutyrate	1.65 ± 0.220	0.973 ± 0.346	1.34 ± 0.506	1.04 ± 0.476	1.68 ± 0.843	1.34 ± 0.185
Acetoacetate	0.347 ± 0.264	0.537 ± 0.221	0.622 ± 0.511	0.341 ± 0.133	0.355 ± 0.010	0.306 ± 0.109
Alanine	0.341 ± 0.210	1.37 ± 1.03	1.07 ± 0.811	1.62 ± 0.926	0.352 ± 0.235	0.201 ± 0.041
Aspartate	0.632 ± 0.359	2.10 ± 0.847	2.42 ± 1.79	3.02 ± 2.04	0.743 ± 0.503	0.465 ± 0.276
Glutamine	3.73 ± 1.15	3.79 ± 2.36	3.24 ± 2.16	1.93 ± 0.766	1.96 ± 0.801	2.23 ± 1.22

Table A.10 (corresponds to Figures IV.18,19,20,21 and 22).

Effect of injection of ammonia to starved, dihydroxyacetone treated rats on some metabolites in their livers.

Rats (male; 180-200g; fasted for 48 hours) were injected (i.m.) with dihydroxyacetone (2g/kg body wt.). 10 min. later they were injected (i.p.) with NH_4Cl (2.5 mMoles/kg body wt.) and at the indicated intervals they were killed by cervical dislocation and their livers freeze-clamped immediately. The control rats (0 min.) were not injected with NH_4Cl. Other experimental details are as in Chapter II.

Results are in μMoles/g fresh wt. and are the means of two experiments.

	0 min.	2 min.	5 min.	10 min.	15 min.	25 min.
Lactate	1.66	1.90	2.68	1.95	2.57	1.49
Pyruvate	0.136	0.126	0.168	0.155	0.281	0.130
Malate	0.342	0.343	0.321	0.414	0.484	0.549
Glutamate	2.63	1.77	2.35	3.70	3.14	2.13
Oxoglutarate	0.167	0.057	0.061	0.058	0.146	0.285
Hydroxybutyrate	0.630	0.628	0.417	0.462	0.467	0.493
Acetoacetate	0.266	0.282	0.258	0.287	0.227	0.202
Alanine	1.88	3.01	5.36	7.56	4.94	0.979
Aspartate	0.613	1.21	1.36	2.58	1.11	0.950

REFERENCES

Adam, H. (1963) in 'Methods of Enzymatic Analysis', p. 573. Ed. H.U. Bergmeyer. Academic Press: New York.

Albers, R.W. (1967) Ann. Rev. Biochem., 36, 727.

Aldridge, W.N. & Parker, V.H. (1960) Biochem. J., 76, 47.

Alleyne, G.A.O. (1968) Nature, 217, 847.

Awapara, J. & Seale, B. (1952) J. biol. Chem., 194, 497.

Bailey, N.T.J. (1959) 'Statistical Methods in Biology'. English Universities Press: London.

Baker, B.R., Lee, K.W., Skinner, W.A., Martinez, P.M. & Tong, E. (1966) J. Med. Pharm. Chem., 2, 633.

Balagura, S. & Fitts, R.F. (1962) Am. J. Physiol., 203, 11.

Barak, A.J. & Beckenbauer, H.C. (1966) Biochem. Pharm., 15, 1295.

Berenblum, I. & Chain, E.B. (1938) Biochem. J., 32, 295.

Bergmeyer, H.U. (1963) in 'Methods of Enzymatic Analysis', p. 37. Ed. H.U. Bergmeyer. Academic Press: New York.

Bergmeyer, H.U. & Bernt, E. (1963 a & b) in 'Methods of Enzymatic Analysis', p. 123 and p. 324. Ed. H.U. Bergmeyer. Academic Press: New York.

Bergmeyer, H.U., Gawehn, K., Klotzsch, H., Krebs, H.A. & Williamson, D.H. (1967) Biochem. J., 102, 423.

Bergmeyer, H.U. & Klotzsch, H. (1963) in 'Methods of Enzymatic Analysis', p. 131. Ed. H.U. Bergmeyer. Academic Press: New York.

Bergmeyer, H.U., Klotzsch, H., Moellering, H., Nelbock-Hochstetter, M. & Beaucamp, K. (1965) in 'Methods of Enzymatic Analysis', p. 998. Ed. H.U. Bergmeyer. Academic Press: New York.

Berl, S., Takagaki, G., Clarke, D.D. & Waelsch, H. (1962) J. biol. Chem., 237, 2562.

Berl, S., Takagaki, G., Clarke, D.D. & Waelsch, H. (1962) J. biol. Chem., 237, 2570.

Bernt, E. & Bergmeyer, H.U. (1963) in 'Methods of Enzymatic Analysis', p. 384. Ed. H.U. Bergmeyer. Academic Press: New York.

Bessman, S.P. (1956) in 'Inorganic Nitrogen Metabolism', p. 408. Eds. McElroy & Glass. The John Hopkins Press: Baltimore.

Bessman, S.P. & Bessman, A.N. (1955) J. clin. Invest., 32, 622.

Braunstein, A.E., Azarkh & Tin-Sen, S. (1961-62) Biokhimiya, 26, 760.

Brdiczka, D., Pette, D., Brunner, G. & Miller, F. (1968) Europ. J. Biochem., 5, 294.

Bromberg, P.A., Robin, E.D. & Forkner, C.E. (1960) J. clin. Invest., 39, 332.

Brown, R.H., Duda, G.D., Korkes, S. & Handler, P. (1957) Arch. Biochim. Biophys., 66, 301.

Brusilow, S.W. & Gordes, E.H. (1968) Am. J. Physiol., 214, 513.

Bublitz, C. & Kennedy, E.P. (1954) J. biol. Chem., 211, 951.

Bucher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) in 'Methods of Enzymatic Analysis', p. 253. Ed. H.U. Bergmeyer. Academic Press: New York.

Bucher, T. & Hohorst, H.J. (1963) in 'Methods of Enzymatic Analysis', p. 246. Ed. H.U. Bergmeyer. Academic Press: New York.

Caughey, W.S., Smiley, J.D. & Hellerman, L. (1957) J. biol. Chem., 224, 591.

Chance, B. (1967) Adv. Enz. Reg., 5, 435.

Chance, B., Schoener, B., Krejci, K., Russmann, H., Hesemann, M., Schnitger, H. & Bucher, T. Biochem. Z., 341, 325.

Chappell, J.B., & Crofts, A.R. (1965) Biochem. J., 95, 707.

Charles, R., Tager, J.M. & Slater, E.C. (1967) Biochem. Biophys. Acta, 131, 29.

Conway, E.J. (1962) 'Microdiffusion Analysis and Volumetric Error'. Crosby Lockwood: London.

Czok, R. & Eckert, L. (1963) in 'Methods of Enzymatic Analysis', p. 224. Ed. H.U. Bergmeyer. Academic Press: New York.

Dale, R.A. (1965) J. Physiol., 18, 701.

Duda, G.D. & Handler, P. (1958) J. biol. Chem., 232, 303.

Engel, P.C. & Dalziel, K. (1967) Biochem. J., 105, 691.

Folin, O. & Denis, W. (1912) J. biol. Chem., 11, 527.

Freeman, J.M., Nicholson, J.F., Masland, W.S., Rowland, L.P. & Carter, S. (1964) J. Pediat., 65, 1039.

Devers, M. (1966) D.Phil. Thesis, University of Oxford.

Glogner, P., Nieth, H., Wagner, H. & Gloede, K. (1966) Klin. Wschr., 44, 1040.

Goldberg, N.D., Passonneau, J.V. & Lowry, O.H. (1966) J. biol. Chem., 241, 3997.

Goodman, A.D., Fulsz, R.E. & Cahill, G.R. (1966) J. clin. Invest., 45, 612.

Greenstein, J.P., Winitz, M., Gullino, P., Birnbaum, S.M. & Otey, M.C. (1956) Arch. Biochem. Biophys., 64, 342.

Grisolia, S. & Cohen, P.P. (1953) J. biol. Chem., 204, 753.

Gruber, W. & Moellering, H. (1966) Biochem. Z., 346, 85.

de Haan, E.J. & Tager, J.M. (1968) Biochem. Biophys. Acta, 153, 98.

Hahn, M., Massen, O., Nencki, M. & Pawlow, J. (1893) Arch. expti. Pathol. u. Pharmakol., 32, 161.

Hathaway, D.E., Mallinson, A. & Akintorwa, D.A.A. (1965) Biochem. J., 94, 676.

Hems, R., Ross, B.D., Berry, M.N. & Krebs, H.A. (1966) Biochem. J., 101, 284.

Hems, R., Stubbs, M. & Krebs, H.A. (1968) Biochem. J., 107, 807.

Hess, B. (1963) in 'Control Mechanisms in Respiration and Fermentation', p. 333. Ed. B. Wright. Ronald Press Co.: New York.

Hess, B. (1966) Biochem. Biophys. Res. Commun., 24, 824.

Hohorst, H.J. (1963, a,b,c,d) in 'Methods of Enzymatic Analysis', pp. 328, 266, 215, 134. Ed. H.U. Bergmeyer. Academic Press: New York.

Hohorst, H.J., Kreutz, F.H. & Bucher, T. (1959) Biochem. Z. 332, 18.

Hohorst, H.J., Kreutz, F.H. & Reim, M. (1961) Biochem. Biophys. Res. Commun., 4, 159.

Hohorst, H.J. & Reim, M. (1963) in 'Methods of Enzymatic Analysis', p. 335. Ed. H.U. Bergmeyer. Academic Press: New York.

Holden, J.T. (1962) 'Amino Acid Pools'. Elsevier: Amsterdam.

Hopper, S. & Segal, H.L. (1962) J. biol. Chem., 237, 3189.

Jacobs, M.H. (1940) Cold Spring Hbr. Symp. Quant. Biol., 8, 30.

Jacquez, J.A., Poppell, J.W. & Jeltsch, R. (1959) J. Appl. Physiol., 14, 255.

Johnson, S. (1755) 'A dictionary of the English Language', London.

Kamin, H. & Handler, P. (1957) Ann. Rev. Biochem., 26, 419.

Katanuma, N., Okada, M., Katsumuma, T., Fujino, A. &

KIRK, E. Acta. Med. Scand., Suppl 78 (1936) 161

Matsuzawa, T. (1966) in 'Pyridoxal catalysis: enzymes and model systems'. Eds: E.E. Snell, A.E. Braunstein, E.S. Severin & Y.M. Torchinsky. Interscience: New York.

Katsuma, N., Okada, M. & Nishii, Y. (1966) Adv. Enz. Reg., 4, 317.

Kirsten, E., Gerez, C. & Kirsten, R. (1963) Biochem. Z., 337, 312.

Klingenberg, M. (1963 a & b) in 'Methods of Enzymatic Analysis', pp. 528, 531. Ed. H.U. Bergmeyer. Academic Press: New York.

Klingenberg, M. (1965) Biochem. Z., 343, 479.

Klingenberg, M. & Pfaff, E. (1966) in 'Regulation of Metabolic Processes in Mitochondria', p. 180. Eds. J.M. Tager, S. Papa, E. Quagliariello & E.C. Slater. Elsevier: Amsterdam.

Klingenberg, M. & Slenczka, W. (1959) Biochem. Z., 331, 486.

Knox, W.E. & Greengard, O. (1965) Adv. Enz. Reg., 3, 247.

Krebs, H.A. (1964) Proc. Roy. Soc. B., 159, 545.

Krebs, H.A. (1967) Adv. Enz. Reg., 5, 424.

Krebs, H.A. & Gascoyne, T. (1968) Biochem. J., 108, 513.

Krebs, H.A. & Henseleit, K. (1932) Hoppe-Seyl. Z., 210, 33.

Lamprecht, W. & Trautschold, I. (1963) in 'Methods of Enzymatic Analysis', p. 543. Ed. H.U. Bergmeyer. Academic Press: New York.

Lee, C.P. & Ernster, L. (1964) Biochim. Biophys. Acta., 81, 187.

McMurray, W.C., Rathburn, J.C., Mohyuddin, F. and Koegler, S.J.
Pediatrics 32, 347 (1963)

Lee, C.P. & Ernster, L. (1966) in 'Regulation of Metabolic Processes in Mitochondria', p. 218. Eds. J.M. Tager, S. Papa, E. Quagliariello & E.C. Slater. Elsevier: New York.

Lehninger, A.L. (1964) 'The Mitochondrion'. W.A. Benjamin: New York.

Li, C.L. & McIlwain, H. (1957) J. Physiol., 139, 178.

Lotspeich, W.D. (1967) Science, 155, 1066.

Lowry, O.H., Passonneau, J.V., Hasselberger, F.X. & Schulz, D.W. (1964) J. biol. Chem., 239, 18.

Marquez-Julio, A. & French, I.W. (1967) Canad. J. Biochem., 45, 1323.

Martin, J.B. & Doty, D.M. (1949) Analyt. Chem., 21, 965.

Mayor, F., Veloso, D. & Williamson, D.H. (1968) To be published.

Miller, A.L. & McLean, F. (1967) Clin. Sci., 32, 385.

Milne, M.D., Scribner, B.H. & Crawford, M.A. (1958) Am. J. Med., 24, 709.

Moser, H.W., Efron, M.L., Brown, H., Diamond, R. & Neumann, C.G. (1967). Am. J. Med., 42, 9.

Otto, K. (1965) Hoppe-Seyl. Z., 341, 99.

Newsholme, E.A. & Underwood, A.H., (1966) Biochem. J., 99, 24c.

Nishiitsutsuji-Uwo, J.M., Ross, B.D. & Krebs, H.A. (1967) Biochem. J., 103, 852.

Papa, S., Tager, J.M., Francoville, A., de Haan, E.J. & Quagliariello, E. (1967) Biochim. Biophys. Acta., 131, 14.

Parnas, I.K. & Heller, J. (1924) Biochem. Z., 151, 1.

Parsons, D.S. & Van Rossum G.D.V. (1961) Quart. J. Exptl. Physiol., 46, 353.

Passeron, S., de Asua, L.J. & Carminatti, H. (1967) Biochem. Biophys. Res. Commun., 27, 33.

Pfleiderer, G. (1963) in 'Methods of Enzymatic Analysis', p. 381. Ed. H.U. Bergmeyer. Academic Press: New York.

Pitts, R.F. (1964) Am. J. Med., 36, 720.

Post, R.L. & Jolly, P.C. (1957) Biochim. Biophys. Acta, 25, 118.

Ratner, S. (1954) Adv. Enz. Reg., 15, 319.

Recknagel, R.O. & Potter, V.R. (1951). J. biol. Chem., 241, 3904.

Reichelt, K.L., Kramme, E. & Treit, B. (1964), Scand. J. Clin. Lab. Invest., 16, 433.

Reif, A.E. (1960) Analyt. Biochem., 36, 727.

Rendi, R. & Uhr, M.L. (1964) Biochim. Biophys. Acta, 89, 520.

Rolleston, F.S. (1966) D.Phil. Thesis. Oxford.

Rosado, A., Flores, G., Mora, J. & Soberon, S. (1962) Am. J. Physiol., 203, 37.

Rosenthal, O., Gottlieb, B., Gorry, J.D. & Vars, H.M. (1956) J. biol. Chem., 223, 469.

Ross, B.D., Hems, R. & Krebs, H.A. (1967) Biochem. J., 102, 942.

Russel, A., Levin, B., Oberholzer, V.G. & Sinclair, L. (1962) Lancet, 2, 699.

Schmidt, E. & Schmidt, F.W. (1967) 'Guide to practical enzyme diagnosis'. Boehringer: Mannheim.

Schimassek, H. & Gerok, W. (1965) Biochem. Z., 343, 407.

Schimke, R.T. (1963) J. biol. Chem., 238, 1012.

Schmahl, F.W., Betz, E., Talke, H. & Hohorst, H.J. (1965) Biochem. Z., 342, 518.

Schoenheimer, R. (1949) 'The dynamic state of body constituents'. Harvard University Press: Cambridge, Mass.

Seligson, D. & Hirahara, K. (1957) J. Lab. Clin. Med., 49, 962.

Siebert, G. (1963) in 'Methods of Enzymatic Analysis', p. 316. Ed. H.U. Bergmeyer. Academic Press: New York.

Siebert, W. & Huth, W. (1965) Biochem. Z., 343, 176.

Silen, W., Harper, H.A., Mawdsley, D.L. & Welrich, W.L. (1955) Proc. Soc. Exper. Biol. Med., 88, 138.

Skon, J.C. (1960) Biochim. Biophys. Acta, 42, 6.

Speck, J.F. (1949) J. Biol. Chem., 179, 1387.

Start, C. & Newsholme, E.A. (1968) Biochem. J., 107, 411.

Steiner, A.L., Goodman, A.D. & Treble, D.H. (1968) Am. J. Physiol., 215, 211.

Stone, W.J., Balagura, S. & Pitts, R.F. (1967) J. clin. Invest., 46, 1603.

Tanaka, T., Harano, Y., Morimura, H. & Mori, R. (1965) Biochem. Biophys. Res. Commun., 21, 55.

Thoren, W. & Helmann, J. (1958) J. Neurochem., 2, 166.

Tomlinson, S. & Westhall, R.G. (1964) Clin. Sci., 26, 261.

Tower, D.B., Sherrett, J.R. & McKhann, G.M. (1961) in 'Regional Neurochemistry', p. 65. Eds: S.S. Kety & J. Elkes. Pergamon: Oxford.

Underwood, A.H. (1965) D.Phil. Thesis. University of Oxford.

Underwood, A.H. & Newsholme, E.A. (1967) Biochem. J., 104, 296.

Veech, R.L. (1968) D.Phil. Thesis. University of Oxford.

Warren, K.S. & Schenker, S. (1964) J. Lab. Clin. Med., 64, 442.

Williamson, D.H. (1967) D.Phil. Thesis. University of Oxford.

Williamson, D.H., Lopes-Vieira, O. & Walker, B. (1967a) Biochem. J., 104, 497.

Williamson, D.H., Lund, P. & Krebs, H.A. (1967b) Biochem. J., 103, 514.

Williamson, D.H., Mellanby, J. & Krebs, H.A. (1962) Biochem. J., 82, 90.

Williamson, J.R. (1966) J. biol. Chem., 241, 5026.

Wollenberger, A., Ristau, O & Schoffa, G. (1960) Pflug. Arch. ges. Physiol., 270, 399.

Wu, R. (1964) Biochem. Biophys. Res. Commun., 14, 79.

Yoshida, A. & Freese, E. (1964) Biochim. Biophys. Acta, 92, 33.

ABSTRACT

The work reported in this theais concerns the metabolism of ammonia in the liver and kidney of the rat. Glutamate dehydrogenase in the mitochondria of these tissues appears to be very close to equilibrium and thus ammonia is linked to a large number of other metabolites through the network of equilibrium enzymes in these tissues. These inter-relations and the factors influencing them in a number of different situations were studied.

Chapter I contains a historical introduction to the metabolism of ammonia and a discussion of the difficulties inherent in a study of its metabolism. Rapid freezing of tissues together with an enzymatic method for the determination of ammonia provide the best methods for the study of its *in vivo* metabolism. A very brief account of current knowledge concerning equilibrium enzymes is presented and the criteria necessary for establishing that the reaction of a given enzyme is close to equilibrium are discussed. As an arbitrary standard, it is proposed that the *in vivo* mass action ratio should differ from the apparent

equilibrium constant by no more than a factor of three before it may be considered to be 'close to equilibrium'.

Chapter II is concerned with the materials and experimental methods employed in the course of this work. Techniques for the rapid freezing of tissues, sampling of blood and perfusion of liver are described. Most of the analytical techniques were conventional enzymatic procedures involving the spectrophotometric measurement of oxidised or reduced pyridine nucleotides. A modification of the enzymatic determination of ammonia is described, whereby the troublesome non-specific oxidation of NADH can be eliminated.

Chapter III presents experimental results on the content of metabolites in ischaemic liver. This information is used to establish whether certain enzymes, known to be close to equilibrium <u>in vivo</u>, maintain near-equilibrium during increased flux. Adenylate kinase, alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, hydroxybutyrate dehydrogenase, isocitrate dehydrogenase (cytoplasmic) and malic enzyme were all found to be close to equilibrium during ischaemia.

Ammonia did not accumulate, although it is known to do so in other tissues in anoxia, but a steady increase in alanine was observed which could be prevented

by the prior administration of L-cycloserine (an inhibitor of alanine aminotransferase). This increase in alanine is thought to represent the removal of ammonia (arising during ischaemia from an unknown source) by the sequential action of glutamate dehydrogenase and alanine aminotransferase. These enzymes may represent a mechanism whereby the intracellular ammonia concentration is controlled.

Chapter IV examines these equilibrium enzymes in liver after perturbation by administration of an ammonia load. The behaviour of the reactants of these enzymes, in this situation, is generally in accordance with the postulated mutual equilibria except at 25 min. post-injection when disequilibrium is apparent in many systems. The possible reasons for these disequilibria are discussed.

The combined action of glutamate dehydrogenase and the alanine and aspartate aminotransferases mediate large increases in the liver content of alanine and aspartate, after the administration of ammonia. This alanine synthesis was studied in detail; it could be prevented by the prior administration of L-cycloserine, was decreased in starvation and was greatly increased by preinjection of dihydroxyacetone. As the liver content

of pyruvate was decreased in starved rats and increased after administration of dihydroxyacetone it was concluded that the maximal *in vivo* activity of alanine aminotransferase (in the direction of alanine synthesis) is governed by the intracellular pyruvate concentration.

Chapter V describes measurements of metabolites in freeze-clamped, whole kidney; these are taken as an approximation of their concentrations in the cortex. Evidence is obtained that glutamate dehydrogenase, hydroxybutyrate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and adenylate kinase are close to equilibrium.

The tissue contents of glycolytic intermediates were measured in fed, starved and acidotic rats to obtain information on the increased gluconeogenic capacity in starvation and acidosis, but insufficient evidence on the direction and rate of glycolytic/gluconeogenic flux *in vivo* under these conditions makes interpretation of the data difficult. These intermediates were also measured during ischaemia to establish which enzymes are activated to account for the increased glycolytic flux. The evidence suggests that phosphofructokinase and pyruvate kinase are activated during ischaemia.

Chapter VI demonstrates a considerable concentration gradient of ammonia between liver and blood and points out that the uptake of ammonia from portal venous blood is continuously occurring against this gradient. This gradient could be reproduced in the perfused liver and is greatly decreased during anaerobic perfusion. Three possible mechanisms for the maintenance of this gradient are discussed but sufficient data are not available to distinguish between them.

Chapter VII contains a discussion of some more general aspects of this work. The relative merits of cervical dislocation versus anaesthesia prior to freezing tissue for metabolite measurements are discussed. It is concluded that anaesthesia should be avoided whenever possible.

A physiological role for the glutamate dehydrogenase -aminotransferase system is proposed in the temporary removal of ammonia in diseases involving inborn errors of the urea cycle.

The status of glutamate dehydrogenase <u>in vivo</u> is discussed in the light of some recent <u>in vitro</u> studies which suggested that glutamate dehydrogenase reacts primarily to synthesise glutamate <u>in vivo</u> and not to catabolise it. It is concluded that the equilibrium

nature of this enzyme <u>in vivo</u> enables it to react, with great facility, in either direction.