STUDIES ON THE METABOLISM OF GLUTAMINE

by

Khalid Iqbal

Thesis submitted for the degree of
Doctor of Philosophy
in the University of Edinburgh

Department of Biochemistry,
University of Edinburgh,
Edinburgh.

February 1969.

SUMMARY

Glutamine has been found by several workers to be present as the free amino acid in all animal tissues but little is known of its functions and the way it accumulates in the cells. The existence of the enzyme, glutamine synthetase which catalyses the synthesis of glutamine from glutamate and ammonia in the presence of ATP and a divalent cation, has been well established in brain, liver and kidney, but it is much less certain whether it occurs in skeletal and cardiac muscle.

Hormones play an important role in the regulation of the activity of several enzymes in various tissues. Although the growth hormone is known to stimulate protein synthesis, very few studies have been made on its effect in the regulation of glutamine synthesis, although glutamine may be thought of as a reservoir and transport form of amino-N, in tissues, and is essential for nucleic acid synthesis.

The present studies were carried out as part of an investigation into the origin and function of glutamine in skeletal and cardiac muscles and to explore the effect of growth hormone, if any, on its synthesis in these tissues. Since glutamine synthetase activity is already known to exist in kidney, this tissue was also studied in parallel with skeletal and cardiac muscle, so as to be able both to check the efficiency of the methods employed and to see whether the enzyme if it existed in the skeletal and cardiac muscles would be similar to the kidney enzyme.

Glutamine synthetase can be assayed by making use of the fact that hydroxylamine will serve as second substrate in place of ammonia. The product of the reaction is γ-glutamyl-hydroxamic acid, which can be quantitatively determined through the colour of the complex which it forms with ferric ions.

In order to make sure that the colour produced was due to the enzyme activity and to obtain a preparation giving maximum activity, it was necessary to study extraction methods before determining optical density. This was achieved by employing a dialysed high-speed supernatant, the activity of which was found to be about 3 - 4 times greater than that found in the homogenate.

On average 183·2 ± 65·1 units glutamine synthetase activity/g. wet tissue was found in kidney by the hydroxamate method using dialysed high-speed supernatants; 19·1 ± 10·3 units/g. were found in skeletal muscle and 2·5 ± 0·8 in heart. In the case of cardiac muscle, no activity could be detected in some of the extracts by the hydroxamate method, and the activity could also not be measured by the NADH oxidation method (see below). These results have been compared with those of others and the possible reasons for the differences in results have been discussed.

Inorganic phosphate was incidently found to be an inhibitor of the enzyme, both in kidney and muscle extracts. In order to study the kinetics of the phosphate inhibition, a method for freeing the extracts from most of the interfering ATP-ase, and a more sensitive assay method, in which ADP does not accumulate,

were developed. The enzyme was separated from ATP-ase by precipitation with 1·5 M and 1·8 M ammonium sulphate for muscle and kidney respectively. The assay method, measures glutamine synthetase activity by measuring the rate of ADP production. This is coupled to oxidation of NADH by pyruvate and lactic dehydrogenase. Adenosine diphosphate is re-phosphorylated with phosphoenol-pyruvate and pyruvate kinase; adenylate kinase was also added to remove traces of adenosine monophosphate which interfered with the assay.

Inorganic phosphate was found to be a competitive inhibitor of ATP for the muscle enzyme and a noncompetitive inhibitor for the kidney enzyme. Possible reaction mechanisms which would account for these findings have been discussed.

Several differences in the properties of the enzymes in muscle and kidney extracts were found (including this difference in inhibition by phosphate) and it is suggested that the enzyme in muscle is an isoenzyme of that found in kidney.

Bovine growth hormone both <u>in vivo</u> as well as <u>in vitro</u> was found to have no significant effect on the synthesis of glutamine in any of the tissues studied. The type of inhibition in the two tissue enzymes was also not affected.

CONTENTS

ACKNOWLEDGEMENTS

I must say thank you so much both to my supervisor, Dr. J.H. Ottaway for his advice and encouragement in carrying out this work, and to Prof. R.B. Fisher for providing the laboratory facilities.

I am also grateful to Dr. W.N.M. Ramsay for his helpful discussions and advice.

My thanks are also due to the members of Dr. Ottaway's research group for their cordial co-operation during my stay in this Department.

SOME OF THE ABBREVIATIONS USED IN THIS THESIS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATP-ase	adenosine triphosphatase
BGH	bovine growth hormone
CP	creatine phosphate
CPK	creatine phosphokinase
CTP	cytidine triphosphate
EDTA	diamino-ethane-tetracetic acid
AGTA	ethylene glycol-bis (β-aminoethyl ether) N,N'-tetra acetic acid
GHA	γ-glutamylhydroxamic acid
G. synth.	glutamine synthetase
GTP	guanidine triphosphate
ITP	inosine triphosphate
LDH	lactic dehydrogenase
MK	myokinase
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
PBrP	Pentabromophenol
PcP	pentachlorphenol
PEP	phosphoenolpyruvate
Pi	inorganic phosphate
Pk	pyruvate kinase
SDC	sodium deoxycholate
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
UTP	uridine triphosphate

C H A P T E R I

INTRODUCTION

INTRODUCTION

Amongst the free amino acids, glutamate and glutamine are found in the highest concentrations in many animal tissues; the values reported by Herbert, Coulson and Hernandez (1966) are shown in Table I. Our present knowledge about the origin and functions of these amino acids in tissues is rather limited. The major biosynthetic pathway leading to the synthesis of glutamine has long been known to be from glutamate and ammonia, the reaction catalysed by the enzyme, glutamine synthetase as shown in equation 1:

1. $HOOC.CH(NH_2).CH_2.CH_2COOH + ATP + NH_3 \xrightleftharpoons{Mg^{++}} HOOC.CH(NH_2).CH_2.CH_2.CONH_2 + ADP + Pi.$

Table I.

Free amino acids (glutamine and glutamate) contents of rat's tissues.

The values given are as m moles/kg. wet tissue.

Tissue	Glutamine	Glutamate
Brain	2·59	7·89
Heart	5·99	4·45
Kidney	0·32	4·09
Liver	2·68	1·64
Muscle	2·34	1·10
Plasma	0·55	0·15

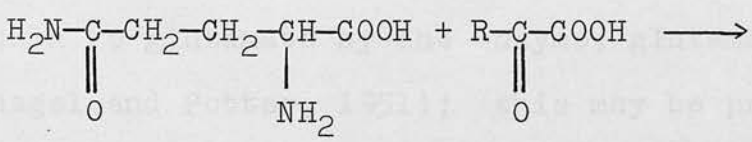
It is the activity of this particular enzyme in animal tissues which has been the subject of investigation for this thesis.

The enzyme has been studied by several workers but mainly in brain (Elliott, 1951; Meister and his collaborators, Meister, 1956, 1962, 1965; Berl, 1966; Meister, 1968), liver (Speck, 1949a, 1949b; Wu, 1961; de Duve, Baudhuin and Wattiaux, 1962), retina (Krebs, 1935; Piddington and Moscona, 1965) and also kidney (Reiner and Hudson, 1953; Richterich and Goldstein, 1958). Only one detailed report on the synthesis of glutamine in rat skeletal muscle and cardiac muscle showing very low levels of enzyme activity (4·4 and 4·6 μ moles of γ-glutamylhydroxamic acid (GHA) synthesised per g. wet tissue per hour) has appeared (Trush, 1963). This worker assayed the enzyme activity by measuring optical density of a cherry red complex of Fe^{3+} ions with γ-glutamylhydroxamic acid (GHA). The latter is formed when ammonia in equation 1 is substituted by hydroxylamine (a method devised by Lipmann and Tuttle, 1945) (see equation 2 given in the later pages).

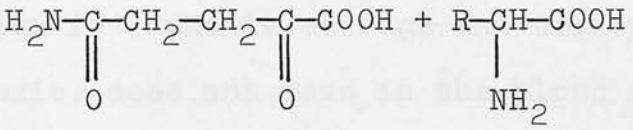
The present investigation therefore aimed to confirm if possible that glutamine synthetase is present in rat skeletal and cardiac muscle; to ascertain whether the enzyme if present is similar to that known to exist in other tissues, i.e. brain, kidney and liver (the enzyme from these sources being similar, kidney was investigated for the purposes of this thesis), and to investigate whether pituitary growth hormone either <u>in vivo</u> or <u>in vitro</u> affects the rate of the enzyme reaction in muscle tissues in particular, for this hormone (as discussed in detail later in the text) is known to increase the intracellular accumulation of

glutamine in certain animal tissues (Peekham and Knobil, 1962) which may be a result of an increase in glutamine synthetase activity. The inhibition of glutamine synthetase by inorganic phosphate (Pi), which was incidentally observed in the course of these studies, was also investigated.

The importance of glutamine synthetase is obvious both from the significant biochemical functions of glutamine, its physiological role and the wide distribution of the enzyme that catalyses its synthesis both in plants, micro-organisms and in animals. L(+) Glutamine, a neutral amino acid participates in a number of biosynthetic pathways, for instance those leading to the synthesis of purines, glucosamine, histidine, p-aminobenzoic acid, asparagine, anthranilic acid, phenyl-acetylglutamine, diphosphopyridine nucleotide and guanosine-5-phosphate (Meister, 1956, 1962). The amide group of glutamine is chemically extremely labile; phosphate, arsenate and bicarbonate catalyse the nonenzymic deamidation of glutamine and pyrrolidone carboxylic acid is formed. Glutamine in liver and kidney transaminates with a large number of α-keto acids, with subsequent hydrolysis of the α-keto acid-ω-amide catalysed by a specific transaminase-amidase [see equations (a) and (b)] forming the corresponding amino acid, α-ketoglutarate and ammonia (Meister, 1956):

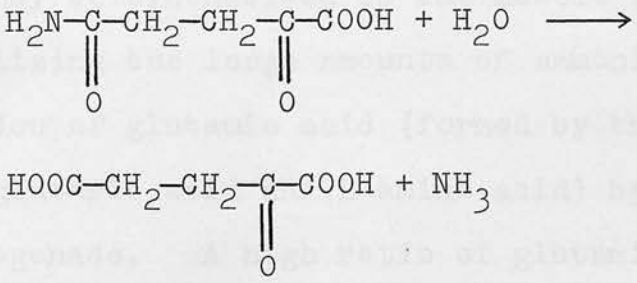
(a)


Glutamine α-ketoacid



α-ketoglutaramic acid α-Amino acid

(b)



α-ketoglutaric acid.

Glutamine which may serve as an ammonia store is enzymically deamidated in mammalian brain, liver, retina, kidney (Krebs, 1935) and muscle (Trush, 1963; Ottaway, 1969) by the enzyme called glutaminase. Deamidation of glutamine also occurs by reversal of glutamine synthesis or in association with ω-transfer reactions (discussed below in detail).

The physiological significance of glutamine is mainly due to its role in the detoxification and possibly also in the storage and transport of ammonia. Increased levels of brain and blood ammonia cause coma and convulsions (McDermott and Adams, 1954; Seegmiller, Schwartz, and Davidson, 1954) which could be due to inhibition of the citric acid cycle by lowering of the intracellular concentration of α-oxoglutarate, following its reduction to glutamate by the enzyme, glutamate dehydrogenase (Recknagel and Potter, 1951); this may be prevented by utilization of ammonia for glutamine synthesis. Conversion of ammonia to glutamine amide nitrogen may in fact represent an important

mechanism for ammonia storage and transport. Ammonium ion being very toxic, does not pass in the blood as such from the peripheral tissues to the liver but is probably transported as glutamine which may be synthesised in the muscle (by glutamine synthetase) by utilizing the large amounts of ammonia produced due to the oxidation of glutamic acid (formed by the transamination of α-ketoglutaric acid and L-amino acid) by the enzyme, glutamate dehydrogenase. A high ratio of glutamine to glutamate in body fluids suggest that the amide may also function in the transport of glutamic acid for it enters the tissues relatively easily as compared to glutamic acid (Schwerin, Bessman and Waelsch, 1950; Tigerman and MacVicar, 1951).

Although dietary glutamic acid and glutamine are not needed by mammals for growth or maintenance of nitrogen equilibrium, certain mammalian cells like a mouse fibroblast cell and a human uterine carcinoma, when grown in chemically defined media, do specifically require L-glutamine for growth and survival (Eagle, 1955). This may be due to its reactivity in certain transamination reactions, transpeptidation and in providing nitrogen for purine synthesis. This suggests that the cells in which glutamine is not synthesised depend on the tissues which do synthesise it, through the blood stream. Glutamine supplied from blood stream or medium may therefore be a limiting factor for protein synthesis in those cells which lack glutamine synthetase. It is, therefore, interesting that many cells have a very high glutamine concentration which may be because of their

inability to synthesise it (by glutamine synthetase), and the glutamine transported by blood stream is stored in such cells for the metabolic activities.

It is desirable at this point to describe in detail both the properties of glutamine synthetase and the mechanism which has been proposed for the synthesis (Krishnaswamy, Pamiljans and Meister, 1962; Meister, Krishnaswamy and Pamiljans, 1962; Meister, 1962, 1965, 1968).

PROPERTIES OF GLUTAMINE SYNTHETASE

The synthesis of glutamine has been found to be reversible by several workers (Levintow and Meister, 1954; Boyer, Mills and Fromm, 1959); the equilibrium constant at pH 7·0 and 37°C is $1{\cdot}2 \times 10^{-3}$. In reaction 2, (equation on page 7) the equilibrium constant for the hydroxamate formation lies farther to the right than the amide synthesis (Ehrenfeld, Marble and Meister, 1963; Meister, 1965) for the hydroxamate formation has been reported to proceed virtually to completion.

Glutamine synthesis is an endothermic reaction (Krebs, 1935); ATP and Mg^{++} were found to accelerate the synthesis of glutamine (Bujard, 1947; Leuthardt, 1947). Speck (1947) and Elliott (1948) established that the energy requirement for glutamine synthetase activity in cell free systems could be derived from the hydrolysis of ATP.

Glutamine synthetase, as mentioned earlier, also catalyses the formation of γ-glutamylhydroxamic acid when ammonia in the

equation 1 is substituted by hydroxylamine as shown below in equation 2. This latter reaction (equation 2) has been extensively made use of as a method for the assay of glutamine synthetase activity (see Chapter II for detailed discussion). Both the synthesis of glutamine and the hydroxamate formation are associated with cleavage of stoichiometric amounts of ATP (Speck, 1949b).

2. $HOOC.CH(NH_2).CH_2.CH_2.COOH + ATP + NH_2OH \xrightleftharpoons{Mg^{++}}$

$HOOC.CH(NH_2).CH_2.CH_2.CONHOH + ADP + Pi.$

The enzyme which catalyses the synthesis of glutamine and γ-glutamylhydroxamic acid also catalyses a transfer reaction as shown in equation 3 (Meister, 1956, 1962, 1965, 1968).

3. $HOOC.CH(NH_2).CH_2.CH_2.CONH_2 + NH_2OH \xrightleftharpoons[Pi \text{ or } Asi]{ATP \text{ or } ADP}$

$HOOC.CH(NH_2).CH_2.CH_2.CONHOH + NH_3$

Like reactions 1 and 2, divalent cation, i.e. Mg^{++}, Mn^{++} or Co^{++} is required for this transfer reaction. Catalytic quantities of adenosine diphosphate (ADP) or ATP and inorganic phosphate or arsenate are also required for this reaction. This γ-glutamyl transfer reaction is doubtless catalysed by the same enzyme, glutamine synthetase, but for the purpose of the present investigation only reactions 1 and 2 have been studied and are referred in this text as glutamine synthetase activity.

The glutamine synthetase activity shows considerable specificity both with regard to its substrates and its requirement

for divalent cation as an activator. D-Glutamate can substitute for L-glutamate both in hydroxamate formation and the synthesis of glutamine, but the rate of the latter reaction is much slower with D-glutamate than with its enantiomorph (Levintow and Meister, 1954; Khedouri and Meister, 1965). L-Glutamate in the enzyme system can also be substituted by several rather less active dicarboxylic amino acids (Levintow and Meister, 1953; Lichtenstein, Ross and Cohen, 1953; Levintow, Meister, Kuff and Hogeboom, 1955; Kagan, Manning and Meister, 1965; Kagan and Meister, 1966; Wellner, Zoukis and Meister, 1966).

Ammonia in the glutamine synthetase system can be substituted by hydroxylamine, hydrazine, methylamine or glycine ethyl ester. The corresponding γ-glutamyl derivative is formed in each case (Speck, 1949b; Elliott, 1951; Levintow and Meister, 1954).

Guanidine triphosphate (GTP), inosine triphosphate (ITP), cytidine triphosphate (CTP) and uridine triphosphate (UTP) are much less active than adenosine triphosphate (ATP) in glutamine synthesis (Levintow, Meister, Kuff and Hogeboom, 1955; Monder, 1965).

Amongst various cations, only Mn^{++}, Co^{++} and Fe^{++} ions have been shown to replace Mg^{++} in the activation of glutamine synthetase; Fe^{++} is much less active as compared to the other cations (Greenberg and Lichtenstein, 1959; Monder and Jacobson, 1964; Monder, 1965).

The pH optimum for glutamine synthesis activity by preparations obtained from pigeon liver (Speck, 1949a, 1949b) and sheep

brain (Elliott, 1951) has been found to be in the range of 7·0 to 7·4. Later Greenberg and Lichtenstein (1959), and Monder (1965) from their studies on sheep brain glutamine synthetase has concluded that the optimum pH depends on the nature and concentration of divalent cations individually, their relative concentration where more than one cation is present, and the ratio of ATP to metal ion. These workers found that the pH optimum was about 7·2 with ATP:Mg^{++} ratios from 1:2 to 1:20, and the broadening of the optimal pH range was observed with a ratio of 1:40; an increase in cation concentration shifted the optimum pH to acidic values.

The purified brain glutamine synthetase is completely inactive in the absence of Mg^{++} or Mn^{++} (Meister, 1962). Speck (1949b), and Baerle, Goldstein and Dearborn (1957) reported that Mg^{++} was essential in the synthesis of glutamine. A 0·02 M Mg^{++} concentration was shown to give the optimum enzyme activity (Elliott, 1951). As discussed below in the mechanism of action of the enzyme, Mg^{++} and ATP are required for the binding of glutamate to the enzyme. A Km value of $2{\cdot}5 \times 10^{-3}$ for glutamate, $1{\cdot}8 \times 10^{-4}$ for NH_4^+, $1{\cdot}5 \times 10^{-4}$ for hydroxylamine and $2{\cdot}5 \times 10^{-3}$ for ATP has been reported by several workers (Speck, 1949b; Elliott, 1951; Meister, 1962; Pamiljans, Krishnaswamy, Dumville and Meister, 1962) for glutamine synthetase.

MECHANISM OF GLUTAMINE SYNTHESIS

Most of the work on the mechanism of glutamine synthesis in animal tissues is due to Meister and his collaborators; Meister (1962, 1965, 1968) has on several occasions reviewed the contributions of various workers in this field as discussed below.

The first step in the synthesis of glutamine is the relatively optically non specific activation of glutamate (i.e. L- and D-forms), which is followed by a more specific reaction of the activated glutamate intermediate (activated L-glutamate is more active than the corresponding form of D-glutamate) with ammonia. This was demonstrated by ultra centrifugation and filt-ration techniques and also by a pulse-labeling enzymatic procedure using highly purified enzyme (Krishnaswamy, Pamiljans and Meister, 1962; Pamiljans et al., 1962). [Hydroxylamine is not affected by this specificity of the activated glutamate intermediate for hydroxylamine is known to react even non enzymically with acylphosphates (Levintow and Meister, 1953)]. It was later on established that nucleotide and metal ions were a requirement for the binding of glutamate to the enzyme and that binding is associated with cleavage of ATP to ADP and inorganic orthophosphate (Pi), which are also bound and are a part of the activated enzyme-glutamate complex; this activated form of glutamate reacts with ammonia to yield glutamine, or in the absence of ammonia, it may undergo cyclization to form pyrrolidone carboxylate (the rate of this reaction is considerably much slower than the synthesis of glutamine or the hydroxamate) (Meister, 1965, 1968). Divalent cation and

ADP or ATP are also required for the binding of glutamine to the enzyme in arsenolysis (equation 4) and γ-glutamyl transfer reactions (equation 3) catalysed by the enzyme.

4. $HOOC.CH(NH_2).CH_2.CH_2.CONH_2 + H_2O \xrightarrow{\text{Arsenate}}$

$HOOC.CH(NH_2).CH_2.CH_2.COOH + NH_3$

Wellner and Meister (1966) have recently reported that nucleotides are tightly bound to the enzyme; direct evidence for binding of ATP and ADP to glutamine synthetase was found by gel filteration experiments with columns of sephadex. These workers also observed that in glutamyl transfer reactions the binding of ATP to the enzyme is not associated with its cleavage to ADP, and the bound ATP is available for glutamine synthesis.

All the reactions catalyzed by purified preparation of enzyme studied by various workers (Krishnaswamy, Pamiljans and Meister, 1962; Meister, 1962; Meister, Krishnaswamy and Pamiljans, 1962) can be diagrammatically interpreted as shown in Fig. I, a modified form of that given by Krishnaswamy, Pamiljans and Meister (1962); Meister, Krishnaswamy and Pamiljans (1962); Meister (1962, 1965, 1968), so as to make it convenient to discuss my own findings as reported in later pages. Meister has postulated that the activated enzyme glutamate complex involves adenosine diphosphate (ADP) and γ-glutamyl phosphate (III, Fig. I); this intermediate reacts with nucleophilic agents to yield another intermediate IV or VI, and undergo cyclization. Representation of the activated glutamate as γ-glutamyl phosphate is supported both by the reports of several workers (Boyer, Koeppe and Luchsinger, 1956; Kowalsky, Wyttenbach,

Langer and Koshland, 1956) that the transfer of an oxygen atom from glutamate to inorganic phosphate, and that chemically synthesised β-aminoglutaryl phosphate, an expected intermediate in the enzymic synthesis of β-glutamine from β-glutamate, is utilized by glutamine synthetase for the synthesis of ATP which is analogous to the experiments with tripeptide (glutathione) synthetase and succinyl thiokinase (Meister, 1968). The hypothesis that enzyme bound glutamyl phosphate is formed in the reactions catalysed by glutamine synthetase has been further supported by the finding that the enzyme catalyses the synthesis of ATP from ADP and β-aminoglutaryl phosphate (Meister, 1965).

INHIBITION OF GLUTAMINE SYNTHETASE

This section of the properties of glutamine synthetase was intentionally left for discussion after describing the mechanism of enzyme action, so as to give the reader a clearer picture of the way the inhibitions are caused.

Adenosine diphosphate (ADP) has long been known to cause inhibition of glutamine synthetase being competitive to ATP (Speck, 1949b; Elliott, 1951). Elliott (1951) reported 50% inhibition of the crude preparation of sheep brain enzyme at an ADP:ATP ratio of 0·3. Meister (1962, 1965, 1968) has suggested that ADP and ATP are bound at the same site and the formation of enzyme-ADP or enzyme-ADP-glutamate complexes (see Fig. I) causes this inhibition. According to Cleland (1963), this is standard product inhibition.

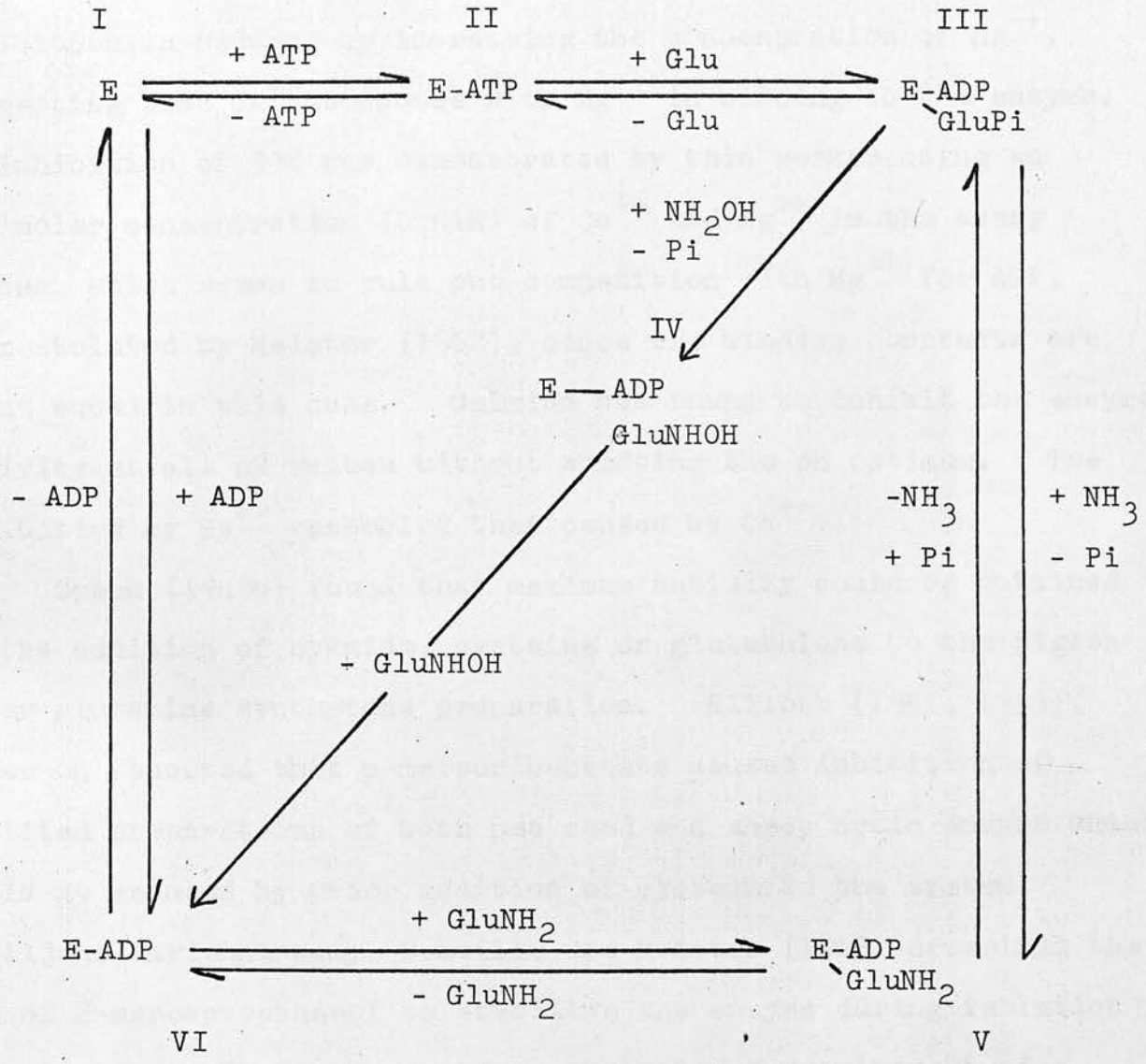


Fig. 1.

Mechanism of action of glutamine synthesis and hydroxamate formation, according to Krishnaswamy, Pamiljans and Meister (1962), Meister, Pamiljans and Krishnaswamy (1962) and Meister (1962,1965, 1968).

Glutamine synthesis: I ⟶ II ⟶ III ⟶ V ⟶ VI ⟶ I

Hydroxamate formation: I ⟶ II ⟶ III ⟶ IV ⟶ VI ⟶ I

E = enzyme: Glu = glutamate; GluPi = glutamylphosphate;
Pi = inorganic phosphate; Glu-NH_2 = glutamine;
Glu-NHOH = glutamylhydroxamate.

Elliott (1951) showed Ca^{++} to be a strong inhibitor of glutamine synthesis by the purified sheep brain enzyme; this inhibition is reduced by increasing the concentration of Mg^{++}, suggesting that Ca^{++} competes with Mg^{++} in binding to the enzyme. An inhibition of 93% was demonstrated by this worker using an equimolar concentration (0·01M) of Ca^{++} and Mg^{++} in the assay system, which seems to rule out competition with Mg^{++} for ATP, as postulated by Meister (1962), since the binding constants are about equal in this case. Calcium was found to inhibit the enzyme activity at all pH values without shifting the pH optimum. The inhibition by Ba^{++} resembled that caused by Ca^{++}.

Speck (1949b) found that maximum activity could be obtained by the addition of cyanide, cysteine or glutathione to the pigeon liver glutamine synthetase preparation. Elliott (1951, 1953), later on reported that p-mercuribenzoate caused inhibition of purified preparations of both pea seed and sheep brain enzyme which could be reduced by prior addition of cysteine to the system. Pamiljans, Krishnaswamy, Dumville and Meister (1962) described the use of 2-mercaptoethanol to stabilize the enzyme during isolation and storage. These observations suggested a requirement of a sulphydryl group for maximum enzyme activity. The dithiol-mediated inhibition by arsenite has suggested the presence of only one sulphydryl group of glutamine synthetase (Wu, 1965). Wu (1964c) has reported that glutamine synthetase contain a metal ion and a dithiol component. The presence of a metal ion in glutamine synthetase (Wu, 1964c) and the fact that excess of Mg^{++} inhibits

the enzyme activity (Speck, 1949b; Trush, 1963; Wu, 1964c) shows that the metal ion directly binds to the enzyme and not as Mg ATP as postulated by Meister (1962), for the enzyme inhibition by excess of the metal ion may well be due to its binding to ATP and hence making ATP unavailable to the enzyme; the enzyme inhibition due to excess of ATP reported by Trush (1963) in crude extracts may be due to accumulation of ADP due to the high levels of ATP-ase in it.

Methionine sulfone, methionine sulfoxide and methionine sulfoximine inhibit the glutamine synthetase activity by competing with glutamate in binding to the enzyme (Elliott and Gale, 1948; Speck, 1949; Meister, 1968). Both crystal violet and fluoride are also known to be the inhibitors of glutamine synthetase (Speck, 1949; Elliott, 1951).

I feel it important to mention here that many of these inhibitors of glutamine synthetase (as discussed above) are either products of the enzyme reaction (like ADP) or naturally occurring in tissues (e.g. Ca^{++}). The estimation in crude extracts may therefore be misleading and the answers may depend on the method of measurement e.g. whether ADP accumulates or not. The mechanism of enzyme action (as already discussed) put forward by Meister (1962, 1965, 1968) for instance is based on the enzyme measurements by the hydroxamate method in which ADP and Pi accumulate in the system and hence may be looked upon with some suspicion.

GROWTH HORMONE AND NITROGEN METABOLISM

It is probably not out of the way here to describe in a little detail the various facts which induced the author to study, as mentioned earlier, the effect of growth hormone on the rate of the enzyme reaction and to investigate also whether growth hormone treatment causes enzyme activity to appear in muscle, if not present in the control animals.

A considerable amount of evidence now exists that growth hormone exerts its effect on protein metabolism primarily on the processes of protein synthesis (Friedberg and Greenberg, 1948; Russell, 1951, 1955). Ulrich, Tarner and Li (1954) for instance have reported a decrease in albumin synthesis after hypophysectomy of a rat and an increase on treatment with growth hormone.

The role of growth hormone in the regulation of various enzymes involved in protein metabolism has been investigated by several workers in recent years (Reid and Stevens, 1958; Panda, Goel, Mansoor and Talwar, 1962; Liberti, Colla, Pilsum and Ungar, 1966; Lakatna, Pilsum and Ungar, 1966); this has been reviewed by Knox, Auerbach and Lin, (1956); and Knobil and Hotchkiss (1964). The growth hormone treated hypophysectomized rats have been shown to have a much greater kidney transamidinase activity as compared to the hypophysectomized animals (Ungar and Pilsum, 1966).

Both amino acid transport and protein synthesis are stimulated by growth hormone (Kipnis and Reiss, 1960). Kostyo (1964) has recently shown that the action of growth hormone on protein bio-

synthesis in muscle is not mediated solely through its action on membrane amino acid transport. But on the other hand the effect of the hormone on transport may be dependent to some extent on the stimulation of protein synthesis (Kostyo, 1968). According to Korner (1965), growth hormone makes energy for protein biosynthesis available by stimulating the synthesis of particular messenger RNA molecules in adipose tissue, which in turn causes the synthesis of enzymes needed for fat metabolism.

Growth hormone *in vitro* has been reported to accelerate the incorporation of amino acids including glutamine and asparagine (Knobil and Hotchkiss, 1964), glycine (Manchester and Young, 1959) and leucine (Kostyo and Knobil, 1965; Hjalmarson, 1968) into the protein of hypophysectomized rat diaphragm, but the hormone has no effect on diaphragm from normal rat (Manchester and Young, 1959; Kostyo and Knobil, 1965). According to Korner (1959) growth hormone treatment increases the ability of normal rat liver microsomes to incorporate amino acid into protein.

An increase in the accumulation of amino acids by certain tissues including muscle due to growth hormone treatment of hypophysectomized rats was demonstrated by Riggs and Walker (1960). Peekham and Knobil (1962) reported that the addition of bovine growth hormone to intact diaphragms from hypophysectomized rats significantly increased the intracellular accumulation of glutamine, asparagine, glycine, alanine, threonine, proline, histidine and tryptophan, as measured by isotopic techniques but not that of the dicarboxylic acids.

According to Wu (1964a, 1964b) a change in protein synthesis, RNA synthesis and DNA synthesis affects the activity of glutamine synthetase. Kirk and Moscona have recently demonstrated that protein synthesis is required for the increase in retinal glutamine synthetase activity (Kirk and Moscona, 1963; Kirk, 1965; Moscona and Kirk, 1965); these authors showed this in cultures of retinal tissue by using inhibitors of protein and RNA synthesis. This was further established by Wu (1964a) who showed that administration of puromycin and of p-fluorophenyl-alanine which inhibit protein synthesis in vivo also inhibited the glutamine synthetase activity of rat liver.

On the basis of these findings by various workers, it was thought to be very interesting to learn if the muscle enzyme activity is also affected by growth hormone treatment.

C H A P T E R II

MATERIALS AND METHODS

(1) MATERIALS

(2) METHODS

(A) Preparation of tissue extracts

(B) Glutamine synthetase assay

(i) Hydroxamate method

(ii) NADH Oxidation method

(C) Phosphate determination

(D) Determination of ADP

MATERIALS AND METHODS

(1) MATERIALS

Only glass distilled water was used for making up all solutions.

Growth Hormone

Bovine pituitary growth hormone (BGH) was a gift from the Endocrinology Study Section of National Institutes of Health, U.S.A. The lot, N.I.H.-GH-B11, used in the present investigations was stated to have a potency of 0·81 USP units per mg.

Lactic dehydrogenase (LDH) was a crystalline suspension in 2·2 M $(NH_4)_2SO_4$ solution; pH approximately 6, prepared from pig heart. It was analytical grade having a specific activity of approximately 360 U/mg.

Myokinase (MK) was a suspension of 3·2 M $(NH_4)_2SO_4$ solution with a pH of approximately 6 and was of analytical grade having a specific activity of approximately 360 U/mg. It was prepared from rabbit muscle.

Pyruvate kinase (Pk). Pyruvate kinase was an analytical grade product and was crystalline suspension in 2·1 M $(NH_4)_2SO_4$ (pH approximately 6) prepared from rabbit muscle. Its specificity was approximately 150 U/mg.

Creatine phosphokinase (CPK) was of analytical grade (lyophilized dry powder, free from salt) and was prepared from rabbit muscle; its specific activity was approximately 18 U/mg. protein.

All these four enzymes were purchased from C.F. Boehringer & Soehne, GmbH Mannheim, W. Germany. They were kept in a

refrigerator at about 2°C except CPK which was kept dessicated in a deep freeze (ca - 20°C).

ADP, ATP, CP, NADH & PEP

Adenosine-5-diphosphoric acid (ADP), trisodium salt; adenosine-5-triphosphoric acid (ATP), a crystalline disodium salt; creatine phosphoric acid (CP), disodium crystalline salt; reduced nicotinamide adenine dinucleotide (NADH), disodium salt; and phosphoenol pyruvate (PEP), crystalline monopotassium salt were all purchased from C.F. Boehringer & Soehne, GmbH Mannheim, W. Germany. These chemicals were stored in a deep freeze (ca - 20°C) in a dessicator.

γ-Glutamylhydroxamic acid (GHA)

γ-Glutamylhydroxamate was obtained from Sigma Chemical Co., U.S.A. It was stored in a deep freeze at about -20°C in a dessicator over P_2O_5 and it was found to be anhydrous by C, H and O analysis (Dr. Minnis of this Department was kind enough to carry out this analysis).

Ammonium sulphate $[(NH_4)_2SO_4]$

Ammonium sulphate, especially low in heavy metals for enzyme work, was a product of The British Drug Houses (BDG) Ltd., England.

Ferric nitrate $[Fe(NO_3)_3.9H_2O]$

Ferric nitrate, laboratory chemical grade, was purchased from May & Baker Ltd., England.

2'-Mercaptoethanol

Mercaptoethanol was an Eastman Chemical product of Kodak Ltd., U.S.A. It was stored in a refrigerator (ca 2°C).

Pentabromophenol (PBrP)(C_6Br_5OH) was a 'technical' grade product of Aldrich Chemical Company, Inc. Milwaukee, Wis., U.S.A. It was recrystallised from ethanol before use.

Pentachlorophenol (PCP)(C_6Cl_5OH) was a 'fine chemicals' grade product of Hopkin & Williams Ltd., England.

Sodium deoxycholate (SDC) was a product of British Drug Houses (BDH) Ltd., England.

Sodium dodecylsulphate (SDS) was purchased from Koch Light Labs., Ltd., England.

di-Sodium hydrogen orthophosphate was obtained from Dr. Ottaway. It was BDH 'Analar' grade recrystallised from EDTA solution. It contained 2 molecules of water of crystallization.

Ethylene glycol-bis (β-aminoethyl ether) N,N'-tetra acetic acid (EGTA), and L-glutamic acid, mono sodium salt, were purchased from Sigma Chemical Co., U.S.A.

Diamino-ethane-tetraacetic acid (EDTA), imidozole, and sodium azide were of 'laboratory reagent' grade and were purchased from The British Drug Houses Ltd., England.

All the rest of the chemicals used in the present investigation were 'Analar' grade from British Drug Houses Ltd., England.

Animals used

Female albino rats, of a local strain, weighing about 225 g were employed for the present investigation, since they grow very slowly and were hence suitable for studying the effects of growth hormone as shown later in the text. They were fed ad lib on a rat-cake diet with free access to water. An animal was killed, after giving it an anaesthetic (ether), by removing its heart, which makes it very convenient to wash the blood from the tissue.

For treatment with growth hormone (BGH), which was dissolved in 0·9% NaCl at a concentration of 2·5 mg./ml. by adjusting the pH to 9·0 - 9·5 with 0·1 M NaOH. A dose of almost 1 mg./day was injected subcutaneously into the experimental animals, which gave a gain in weight, as compared with controls, of about 2 g./day. A final dose was given 2-3 hours before killing the animal.

(2) METHODS

(A) PREPARATION OF TISSUE EXTRACTS

Step I.

Heart, both kidneys and about 2·5 g. of leg skeletal muscle (gluteus maximus) were quickly removed from the carcase and transferred to an ice-cold solution of 0·15 M NaCl + 0·005 M $NaHCO_3$. Each tissue was washed with an excess of this solution, blotted, weighed and left in a deep freeze (ca - 20°C) for a minimum period of 45 minutes. After this the tissue was chopped and finally homogenised, as recommended by Trush (1963), in 5 ml. of ice cold sodium chloride-sodium bicarbonate solution per g. of wet tissue. Heart and kidney were homogenised in a teflon glass

homogeniser, but the skeletal muscle being tougher was homogenised in an "Ultra-Turrax" (Janke and Kunkel, K.G., Stanfen i.Br.W. Germany). The homogenate was allowed to stand for about 2 hours in a refrigerator at 2°C for extraction of the enzyme, with frequent shaking.

Step II

This total homogenate as obtained above was centrifuged at a speed of 30,000 R.P.M. for 45 minutes in a Spinco Preparative Ultracentrifuge using the type 40 rotor at about 2°C. The residue was rejected and the supernatant was filtered through four layers of cheese cloth to remove any floating fat particles.

Step III

The supernatant was then dialysed against two changes each of 100 vols. of sodium chloride-sodium bicarbonate solution containing 1 mM EDTA and 5 mM 2'-mercaptoethanol for about 3 hours in a cold room at a temperature of about 5°C.

The enzyme preparation obtained was stored at about 2°C and used on the day of preparation.

Potassium salts (KCl and $KHCO_3$) were used instead of NaCl and $NaHCO_3$ in the enzyme preparation used for the assay by the NADH oxidation method (as described later in this chapter), since Na^+ inhibits pyruvate kinase (PK) (used in this method), while K^+ is an activator for this enzyme.

(B) GLUTAMINE SYNTHETASE ASSAY

(i) HYDROXAMATE METHOD

The most popular method for the assay of glutamine synthetase is the one due to Speck (1949) and Elliott (1951) which is based on the fact that γ-glutamylhydroxamic acid (GHA) is formed when ammonia in the reaction mixture is replaced by hydroxylamine [see Chapter I, equations (1) and (2)]. The γ-glutamylhydroxamic acid can then be estimated by means of its cherry-red complex with ferric ions (Lipmann and Tuttle, 1945). Moreover the equilibrium for the hydroxamate formation lies much farther to the right than for the synthesis of glutamine, since the standard free energy of hydrolysis of the hydroxamate has been reported by Ehrenfeld, Marble and Meister (1963) to be about 700 cal./mole.

This method is, however, not very sensitive; a solution of 1 μmole of GHA/ml. has an optical density of about 0·5, which is not very convenient for measuring the low levels of glutamine synthetase activity which are to be expected, for example, in muscle extracts. The sensitivity of the assay method was improved by replacing the ferric chloride in the reagent by colourless ferric salts, e.g. the nitrate or perchlorate (Ottaway and Khalid Iqbal, 1969). This approximately doubled the sensitivity of the estimation. The method was not developed at the beginning of the investigation. Therefore the ferric reagent (using ferric chloride) was employed in some of the experiments reported in Chapter III (i.e. from section A to section E).

Reagents

0·3 M imidazole-HCl buffer, pH 7·2

0·5 M Magnesium chloride

1·0 M Hydroxylamine.

A stock solution of 2 M hydroxylamine hydrochloride was neutralised with 2 N sodium hydroxide immediately before use and the volume was adjusted with water.

0·5 M sodium glutamate, pH 7·2

0·25 M 2'-Mercaptoethanol, pH 7·2

0·1 M Sodium ATP, pH 7·2

Ferric reagent (Lipmann and Tuttle, 1945), equal volumes of 8·5 N HCl; 15% $FeCl_3.6H_2O$ in 0·3 N HCl; and 36% Trichloroacetic acid.

Ferric reagent (Ottaway and Khalid Iqbal) equal volumes of 40% trichloroacetic acid (TCA) and 0·6 M $Fe(NO_3)_3.9H_2O$.

Procedure

Incubations were carried out at 37°C for 10 minutes (except where the enzyme activity was measured at different time intervals as mentioned in the text) in 15 ml. centrifuge tubes containing 150 µmoles (0·5 ml.) of imidazole-HCl buffer*, 300 µmoles (0·3 ml.) of hydroxylamine, 75 µmoles (0·3 ml.) of 2'-mercaptoethanol, 30 µmoles (0·3 ml.) of ATP together with enzyme + water to a final volume of 3·0 ml.

The amount of enzyme was adjusted so that not more than 3 µmoles of γ-glutamylhydroxamate (GHA) were formed, when the amount of hydroxamate produced was proportional to the enzyme added (Elliott, 1955).

* 60 µmoles of magnesium chloride (0.3 ml.), 150 µmoles of glutamate (0.3 ml.),

In all the assays carried out by this method except where specifically stated in the text, the tissue extract equivalent to 50 mg. wet tissue in the case of kidney or 250 mg. in the case of both cardiac and skeletal muscle per assay were employed.

After incubation, 2 ml. of the ferric reagent was added to each tube, mixed quickly, and after standing for about 10 minutes, the protein was removed by centrifuging for about 10 minutes in a bench centrifuge. The hydroxamate in the supernatant was estimated by measuring the absorbance at a wavelength of 500 mμ on an SP 500 Spectrophotometer using 1 cm. glass cuvettes. A sample in which ATP was omitted served as a control (validity of the control is discussed in Chapter III)*. Synthetic γ-glutamylhydroxamic acid (GHA) was used as a standard. The colour produced with this ferric reagent obeys Beer's Law over the range 0·0 - 0·6 μmoles hydroxamate/ml. A standard curve is shown in Fig. 2. The optical densities of the samples were read against solution containing 2 ml. of the ferric reagent diluted to 5 ml. with water.

Definition of Unit

One unit of enzyme is defined as that amount which produces, under the standard test conditions, 1 μmole of GHA/hr.

(ii) NADH Oxidation Method

This method, which measures the ADP formed in the enzyme reaction was independently developed in this laboratory. Similar methods have been published by Wellner, Zoukis and Meister (1966); Kingdon, Hubbard and Stadtman (1968), and Liess, Varricchio, Mecke and Holzer (1968).

In this method, the glutamine synthetase is coupled to an

*The reading for the control was substracted from samples.

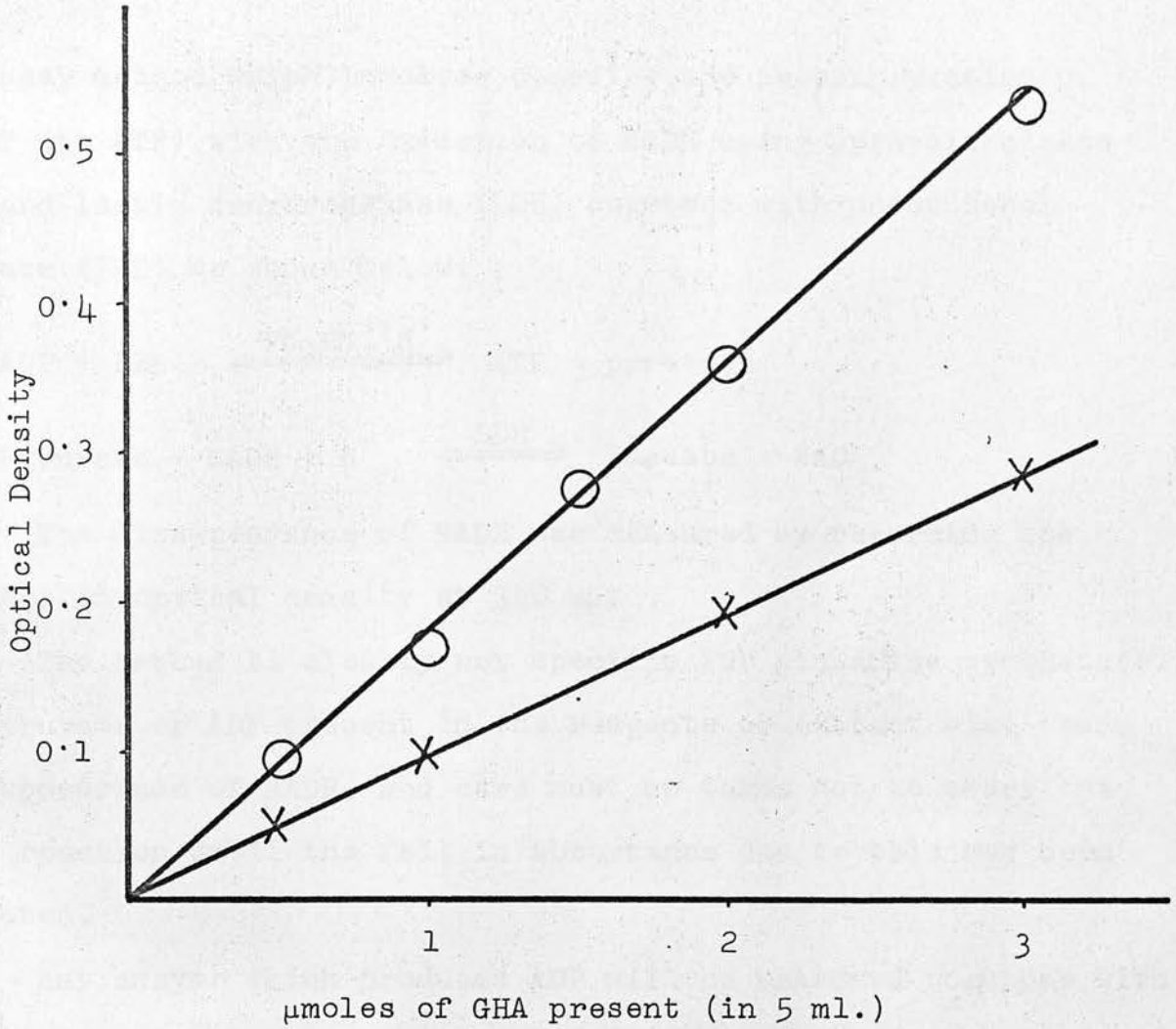


Fig. 2.

Standard hydroxamate (GHA) curve.

○—○ Using reagent of Ottaway and Khalid Iqbal (1969)

X—X Using reagent of Lipmann and Tuttle (1945)

ADP assay method which involves coupling the phosphorylation of ADP (to ATP) with the oxidation of NADH using pyruvate kinase (PK) and lactic dehydrogenase (LDH) together with phosphoenol pyruvate (PEP) as shown below:

(a) ADP + PEP $\xrightleftharpoons{PK, Mg^{++}, K^{+}}$ ATP + pyruvate

(b) Pyruvate + NADH + H^{+} $\xrightleftharpoons{LDH}$ lactate + NAD

The disappearance of NADH was measured by recording the decrease in optical density at 340 mμ.

The method is clearly not specific for glutamine synthetase. Any pyruvate or ADP present in the reagents or extract will cause a disappearance of NADH, and care must be taken not to start the assay reaction until the fall in absorbance due to this has been completed.

Any enzyme which produces ADP will be measured together with glutamine synthetase. In general this interference is negligible, since the substrates are either absent or present in very low concentrations. Considerable interference was, however, experienced with ATP-ase, particularly in the heart and skeletal muscle extracts, since ATP is a common substrate for both enzymes, and the ratio of ATP-ase to glutamine synthetase was much greater than unity in these extracts. Methods of overcoming the difficulty are described in detail later in the text.

Trouble was also experienced with a drifting baseline on the addition of the tissue extract. The rate of drift appeared to be proportional to the concentration of ATP in the reaction mixture,

and the trouble was traced to contamination of the ATP with about 0·1% of AMP. The tissue extracts contain low and variable amounts of adenylate kinase, catalysing the reaction

$$AMP + ATP \rightleftharpoons 2\ ADP$$

and this reaction, running from left to right, was causing the drift. Since it would have been very troublesome to remove either the AMP or the adenylate kinase completely from the system, the expedient was adopted of adding a low concentration of adenylate kinase routinely to the reaction mixture, so that the AMP is removed before the assay begins.

Reagents

0·3 M Imidazole-HCl buffer, pH 7·0

0·3 M Magnesium chloride, pH 7·0

0·25 M 2'-Mercaptoethanol, pH 7·0

0·5 M L-Glutamic acid (monosodium salt), pH 7·0

0·1 M ATP (disodium salt), pH 7·0

0·5 M Hydroxylamine, pH 7·0; a stock solution of M hydroxylamine hydrochloride was neutralised with 2 M KOH solution immediately before use and the volume was adjusted with water.

LDH-PK-MK solutions, 0·2 ml. of lactic dehydrogenase (LDH) (10 mg./ml.), 0·2 ml. of myokinase (MK)(5 mg./ml.), and 0·5 ml. of pyruvate kinase (PK)(10 mg./ml.) were diluted with the imidazole-HCl buffer to a final volume of 3 ml. and this was then dialysed to remove ammonium sulphate from it, in which solution these enzymes were suspended. The dialysis was carried out for about

3 hours in a cold room (ca 5°C) against two changes, each of 100 volumes of 0·001 M EDTA solution, pH 7·0; 0·1 ml. of this LDH-PK-MK solution (containing 24, 25 and 12 units of LDH, PK and MK respectively) was employed per cuvette (1 ml.); this solution was always freshly prepared and kept at about 2°C.

NADH-PEP-KCl-EDTA solution, a solution containing 3·3 mg. NADH, 10·3 mg. PEP, 0·75 ml. of 2 M KCl and 0·05 ml. of 0·1 M EDTA, pH 7·0, per ml. was prepared immediately before use and neutralised with 0·1 M KOH solution.

0·05 ml. of this solution containing approximately 0·3 μmole of NADH, 2 μmoles of PEP, 75 μmoles of KCl and 0·25 μmoles of EDTA, was added to each cuvette (1·0 ml.).

Procedure

A reaction mixture consisting of 60 μmoles (0·2 ml.) of imidazole-HCl buffer, 30 μmoles (0·1 ml.) of magnesium chloride, 25 μmoles (0·05 ml.) of hydroxylamine, 25 μmoles (0·1 ml.) of 2'-mercaptoethanol, 50 μmoles (0·1 ml.) of glutamate, 0·05 ml. of the NADH-PEP-KCl-EDTA solution, 10 μmoles (or different in experiments where enzyme activity was studied at different ATP concentrations)(0·1 ml.) of ATP and 0·1 ml. of the LDH-PK-MK* solution were incubated at 37°C in a semi micro 1 cm. silica cuvette in a Unicam SP 800 Recording Spectrophotometer and the optical density was recorded for 3-5 minutes until it became constant. During this period impurities such as AMP and ADP were removed, with a corresponding fall in concentration of NADH. Then 0·2 ml. of the tissue extract suitably diluted with water was

*It was found that doubling the amount of these ancillary enzymes did not increase the rate of the reaction.

quickly stirred into the cuvette to give a final volume of 1·0 ml. The decrease in the absorbance was recorded for about 3 minutes. The rate of decrease should be linear over almost all this period. The rate of NADH oxidation after addition of the tissue extract to a cuvette in which glutamate was omitted served as a control.

The absorbance was recorded at a wavelength of 340 mμ. The accuracy of the system was frequently checked with a standard solution of ADP.

Similarly the ATP-ase activity was also determined by this method by employing a sample in which both ATP and glutamate were omitted as a control.

Calculations

The difference in the rate of decrease of the absorbance between the sample and the control gave the rate of NADH oxidation due to glutamine synthetase activity, which was multiplied by an appropriate factor to calculate the decrease of absorbance due to 1 g. of the wet tissue/hr. of incubation. This was then divided by the extinction coefficient of NADH at 340 mμ i.e. 6·22 [$Cm.^2/\mu mole$] to give the number of μmoles of NADH oxidised or μmoles of ADP produced in the system per g. of wet tissue/hr. This was equivalent to the glutamine synthetase activity.

Definition of Unit

One unit of enzyme was defined as that amount which produced, under the standard test conditions, 1 μmole of ADP/hr.

(C) PHOSPHATE DETERMINATION

The inorganic phosphate (Pi) content of the tissue extracts used for the estimation of glutamine synthetase activity, were determined by a colorimetric method based on that of Taussky and Shorr (1953).

Reagents

Trichloroacetic acid (TAC), 20% solution (w/v).

0.2 mM KH_2PO_4 solution was prepared by dissolving 27.2 mg. of it in 500 ml.

Phosphate reagent, a stock solution of 10% ammonium molybdate in 10 N H_2SO_4 was prepared. Then 10 ml. of this stock solution was diluted to about 70 ml., and 5 g. of $FeSO_4.7H_2O$ were dissolved in it and volume was made up to 100 ml. This reagent was always prepared fresh each day before use.

Procedure

1 ml. of 20% trichloroacetic acid (TCA) was mixed with 3 ml. of the sample. After standing for about 15 minutes in a refrigerator (ca. $2^{\circ}C$), it was centrifuged for about 10 minutes in an ordinary bench centrifuge in a cold room (about $5^{\circ}C$). 1 ml. of the supernatant obtained was diluted to 5 ml. with water, and then 3 ml. of the phosphate reagent was mixed quickly in it. After standing for 10 minutes the blue colour produced was read against water at a wavelength of 600 mμ on an SP 500 spectrophotometer.

Phosphate contents of the sample were read directly from

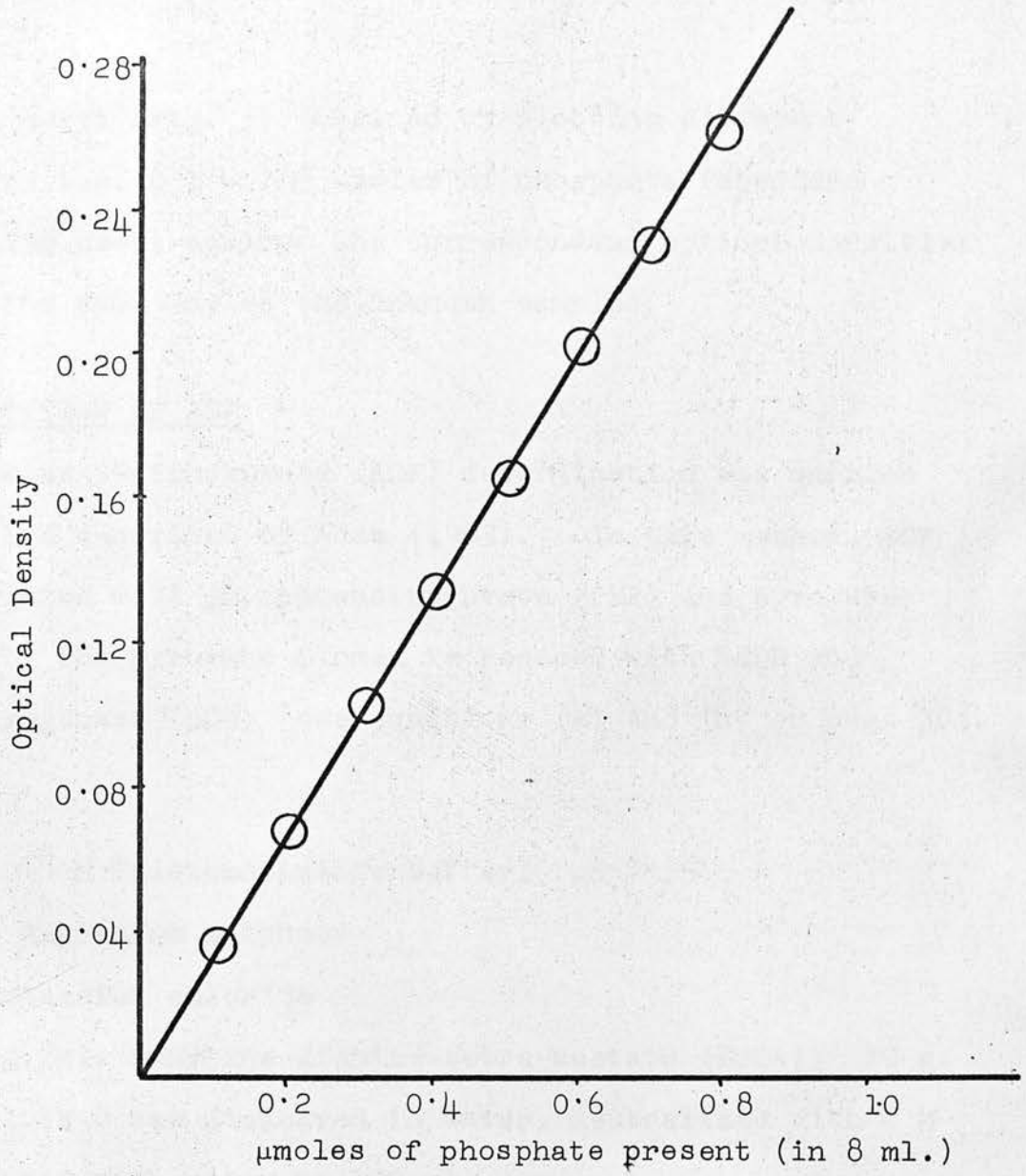


Fig. 3.

Standard curve for phosphate determination.

the standard curve (Fig. 3) obtained by plotting different concentrations i.e. 0·1 - 0·8 μmoles of phosphate (standard KH_2PO_4 solution used) against the corresponding optical densities obtained in the same way as the unknown samples.

(D) DETERMINATION OF ADP

Adenosine-5'-diphosphate (ADP) determination was carried out by a method described by Adam (1963). In this method, ADP is phosphorylated with phosphoenolpyruvate (PEP) and pyruvate kinase (PK). The pyruvate formed is reduced with NADH and lactic dehydrogenase (LDH) [see equations (a) and (b) on page 30].

Reagents

5×10^{-2} M Triethanolamine buffer; pH 7·55

0·5 M Magnesium sulphate

2 M Potassium chloride

100 mg./ml. Ethylene-diamine-tetra-acetate (EDTA); 10 g. of EDTA-$Na_2H_2.2H_2O$ was dissolved in water, neutralized with 2 M NaOH and diluted with water to 100 ml.

4×10^{-2} M Phosphoenolpyruvate (PEP)

10^{-2} M NADH, it was always prepared immediately before use and was neutralised with 10 mM KOH.

10^{-2} M Adenosine triphosphate (ATP)

0·1 mg. protein/ml. Lactic dehydrogenase (LDH), 0·01 ml. of a crystalline suspension (10 mg. protein/ml.) was diluted to 1 ml. with 2·25 M ammonium sulphate solution (pH 6·5).

0·5 mg. protein/ml. Pyruvate kinase (PK), 0·05 ml. of a

crystalline suspension (10 mg. protein/ml.) was diluted to 1 ml. with 2·1 M ammonium sulphate solution (pH 5·5).

M Perchloric acid

2 M Potassium hydroxide.

Procedure

Deproteinization: To the assay mixture (3·0 ml.) in the hydroxamate method after 30 min. incubation at 37°C was added 0·6 ml. of M perchloric acid and after being allowed to stand for about 5-7 minutes in an ice bath, was centrifuged in an ordinary bench centrifuge, placed in a cold room (ca 5°C), for about 10 minutes. The supernatant was decanted off and was neutralised with 2 M potassium hydroxide solution, volume made to 4·0 ml. with water, and again allowed to stand for about 5-7 minutes in an ice bath. The crystalline precipitates of potassium perchlorate were settled at the bottom and 0·5 ml. of the clear supernatant was taken out for estimating ADP in it.

Spectrophotometric measurements: From the above described reagents, the following reaction mixture was prepared and quickly neutralised with 10 mM KOH solution.

0·36 ml. Magnesium sulphate solution

0·76 ml. potassium chloride solution

0·04 ml. EDTA solution

0·40 ml. PEP solution

0·30 ml. NADH solution

0·07 ml. ATP solution

or multiple of the individual volumes.

Then 0·5 ml. of the deproteinized sample, 1·3 ml. of the triethanolamine buffer and 0·15 ml. of the reaction mixture were pipetted successively in a 1 cm. silica cuvette. This cuvette was then placed (25°C) in a recording SP 800 Spectrophotometer. 0·02 ml. of the LDH solution was very quickly mixed in it with a plastic stirrer and optical density was recorded at 340 mμ for about 5 minutes until no further change occurred. This value of optical density was marked as E_1. After this 0·03 ml. of the PK solution was stirred in the cuvette and again optical density recorded for about 10 minutes till no further change. This new value of optical density was marked as E_2.

A reference containing all the reagents except the sample was employed. Working of the method was checked with standard solution of ADP.

Calculations

The optical density difference $E_1 - E_2 = E_{ADP}$ was determined, and the amount of ADP in μmoles present/assay of the hydroxamate method was calculated as under:

$$\frac{E_{ADP} \times V_A \times V_E}{\varepsilon \times d \times V_P} = \text{μmoles of ADP/assay of the hydroxamate method.}$$

where $E_{ADP} = E_1 - E_2$

V_A = Volume of the test mixture in the cuvette (2·0 ml.)

V_E = Total volume of the test sample after deproteinizing (4·0 ml.)

V_P = Volume of the test sample added to the cuvette (0·5 ml.)

ε = Extinction coefficient for $NADH_2$ at 340 mμ which is 6·22 [cm^2/μmole]

d = Light path of the cuvette (1 cm.)

C H A P T E R III

ACTIVITY OF ENZYME IN EXTRACTS MEASURED BY THE HYDROXAMATE METHOD

(A) Control

(B) Preliminary results

(C) Achievement of linearity

(i) Effect of high speed centrifugation

(ii) Effect of dialysis

(D) Optimum concentrations of substrates

(E) Solubilisation of the enzyme by detergents

(F) Amount of the enzyme in tissues

(G) Possible nature of the inhibitor

ACTIVITY OF ENZYME IN EXTRACTS MEASURED BY THE HYDROXAMATE METHOD

This chapter begins with a systematic discussion of the importance of a valid control in the hydroxamate method especially in measuring low levels of enzyme activity. This chapter also includes the most important part of this thesis - an answer to the question whether there is really any glutamine synthetase activity in rat cardiac and skeletal muscle, which depends on the criteria by which the presence of enzyme activity may be established.

The check of the optimum concentrations of substrates and the effect of detergents for more efficient extraction of the enzyme are also reported.

Finally the possibility that inorganic phosphate is the inhibitor responsible for the observed non-linearity of the reaction, and the unsuitability of the hydroxamate method for studying this inhibition, are discussed.

(A) CONTROL

Controls with enzyme and ATP separately omitted have been employed by Pamiljans, Krishnaswamy, Dumville and Meister (1962), and Berl (1966), while Trush (1963) has suggested a sample containing all the reagents but with previously boiled enzyme or a sample containing no ATP as a control.

As the glutamine synthetase activity in the muscle extract was very small, the absorbance of the sample was comparable with that of the control (see Fig. 4) and hence the latter became very

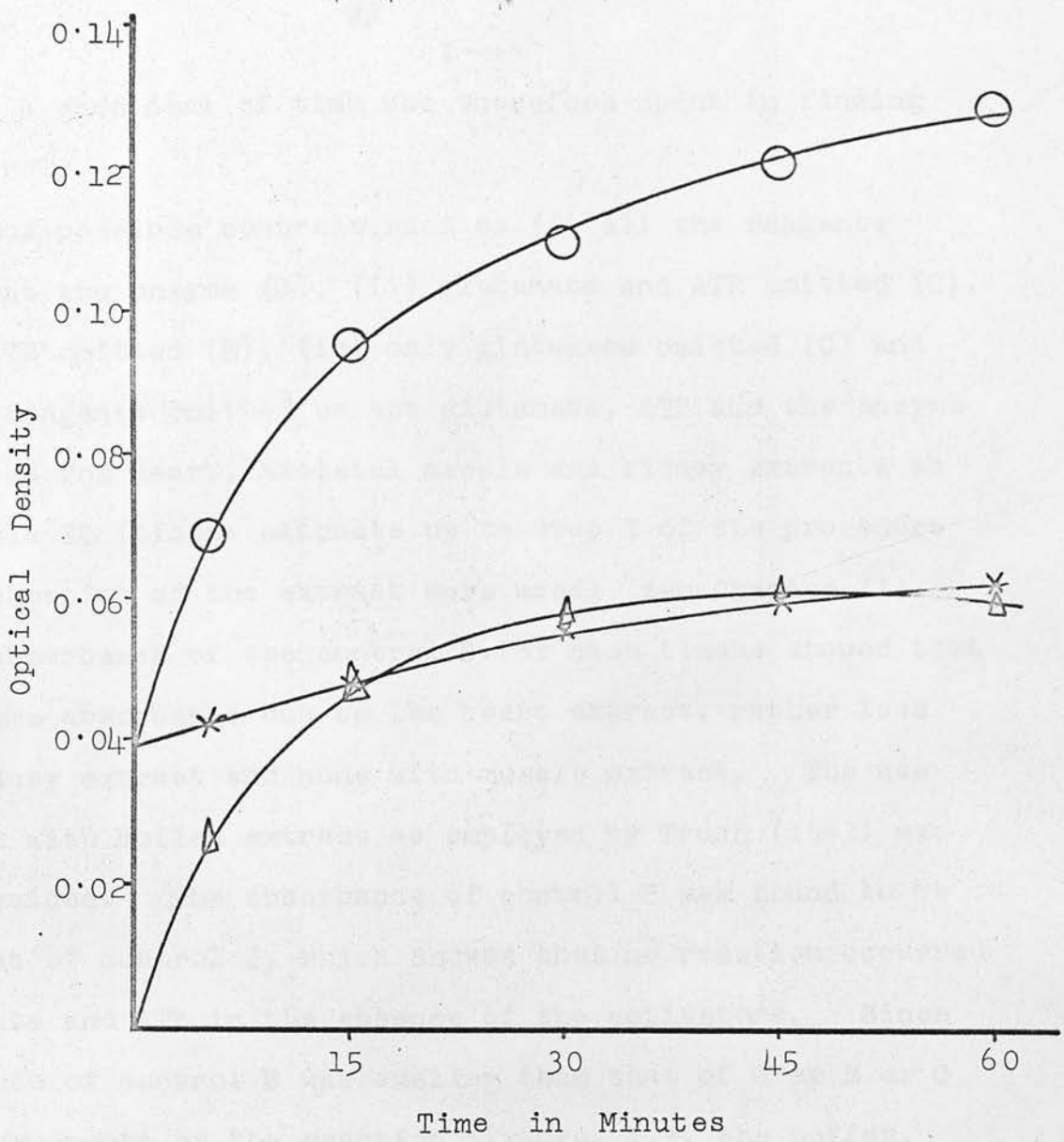


Fig. 4.

Showing that the activity in muscle is comparable to control.

○—○ Represents the optical density both due to sample + control.

×—× Represents the optical density due to control

Δ—Δ Represents the optical density due to sample only.

important. A good deal of time was therefore spent in finding a valid control.

Various possible controls such as (i) all the reagents omitted except the enzyme (B), (ii) glutamate and ATP omitted (C), (iii) only ATP omitted (E), (iv) only glutamate omitted (G) and (v) all the reagents omitted except glutamate, ATP and the enzyme (J) were tried for heart, skeletal muscle and kidney extracts as shown in Table II (tissue extracts up to Step I of the procedure for the preparation of the extract were used; see Chapter II).

The absorbance of the control B for each tissue showed that there was some absorbance due to the heart extract, rather less with the kidney extract and none with muscle extract. The use of a control with boiled extract as employed by Trush (1963) was therefore avoided. The absorbance of control B was found to be equal to that of control J, which showed that no reaction occurred with glutamate and ATP in the absence of the activators. Since the absorbance of control B was smaller than that of C or E or G, the other components of the reaction mixture, i.e. the buffer, hydroxylamine, magnesium chloride and 2'-mercaptoethanol must also be responsible for a part of the colour in a control, in addition to that of the extract itself. Since controls C, E and G were equal, it may be concluded that the omission of either glutamate or ATP will produce a colour which was due only to the extract and to the hydroxylamine, Mg^{++} and mercaptoethanol, and which may reasonably be taken to be the control optical density to be subtracted from the experimental observations. It was not necessary

TABLE II

Composition and optical density measurements of various controls.

Optical density (O.D.) measured per assay at 500 mμ on an SP 500 Spectrophotometer against ferric reagent + H_2O.

Incubations were carried out at 37°C for 10 minutes using non dialysed tissue supernatants.

Reaction mixture	Control					Sample
	B	C	E	G	J	
Imidazole-HCl buffer	x	✓	✓	✓	x	✓
Hydroxylamine	x	✓	✓	✓	x	✓
Glutamic acid	x	x	✓	x	✓	✓
Magnesium chloride	x	✓	✓	✓	x	✓
2'-mercaptoethanol	x	✓	✓	✓	x	✓
Adenosine triphosphate (ATP)	x	x	x	✓	✓	✓
Enzyme	✓	✓	✓	✓	✓	✓
	Optical Densities					
Skeletal muscle	0·00	0·04	0·04	0·04	0·00	0·08
Kidney	0·01	0·05	0·05	-	0·01	0·29
Heart	0·02	0·05	0·05	0·05	0·02	0·07

to omit both glutamate and ATP because the amounts of these substances present in the extract itself were not sufficient to sustain a measurable reaction.

However, in another experiment in which no enzyme was added to the reaction mixture, it was found that addition of glutamate either in the presence or absence of ATP caused a slight increase in optical density (from 0·02 to 0·03), whereas addition of ATP either in the presence or absence of glutamate caused no change in optical density. It was concluded that glutamate was responsible for a part of the observed colour, not due to glutamine synthetase activity, and the omission of ATP would give a better estimate of the enzyme activity. A sample containing all the reagents, but no ATP was therefore employed as a control.

The control was also incubated together with the samples because its optical density also increased with the time of incubation. This may be due to some enzyme similar to a bacterial enzyme reported by Meister, Levintow, Greenfield and Abendschein (1955), and Ehrenfeld, Marble and Meister (1963) which catalyses the synthesis of L-γ-glutamylhydroxamic acid from L-glutamic acid and hydroxylamine without requiring ATP, or to some nonenzymic reaction. If this were to be the case, the use of a control containing all the reagents but with boiled enzyme, as recommended by Trush (1963) would not give a true estimate of glutamine synthetase activity as defined by equation (1) in Chapter I. This was another reason for not employing it as a control.

(B) PRELIMINARY RESULTS

After finding a valid control, I turned to investigating whether the low optical density differences between the sample and the control, when cardiac and skeletal muscle extracts were used (see Table II), were due to glutamine synthetase activity or were some form of artefact. Trush (1963) clearly took these small optical density differences as evidence of enzyme activity in cardiac and skeletal muscle homogenates, but in order to be certain, it is necessary to satisfy certain criteria. For instance, hydroxamate formation should be proportional to incubation time, and to concentration of extract added. Moreover, unless the first criterion, in particular, is satisfied, the rates observed are almost certainly not the maximum rates. For both these reasons, it was necessary to carry out further work on cardiac and skeletal muscle extracts. In order to check both the efficiency of the assay system, and to compare the results of cardiac and skeletal muscle extracts with those of other workers, the enzyme activity in kidney, which is already known (see Chapter I) to contain a high level of the enzyme was also studied.

The enzyme activity was therefore determined in all three tissues by incubating the reaction mixture for different intervals of time. Tissue homogenates (see Chapter II; up to Step I of the procedure for the preparation of the extract) were used, since these experiments were done before I investigated high-speed supernatants.

The enzyme activity was found to be nonlinear with increase in the time of incubation for both muscle and kidney homogenates

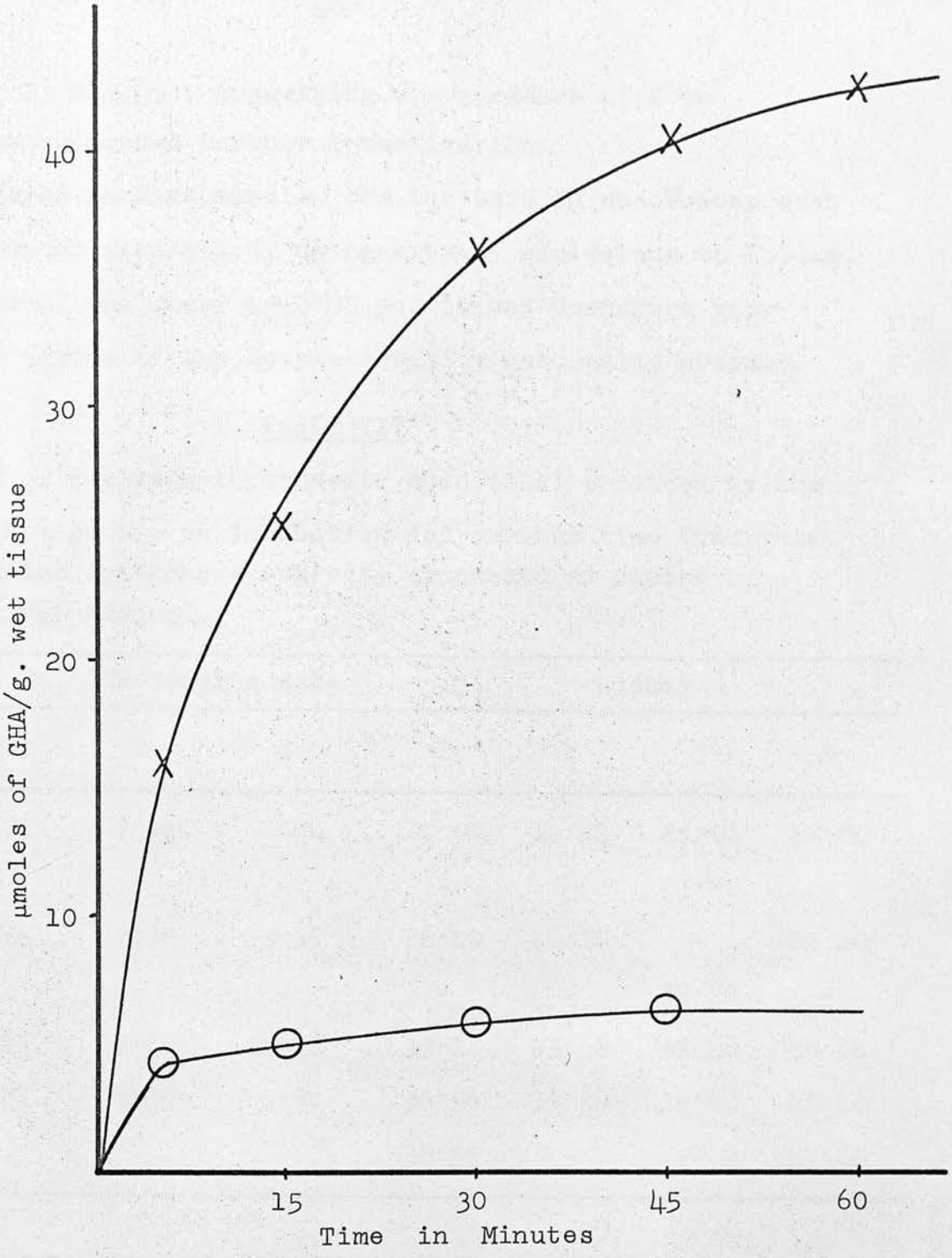


Fig. 5.

Relation of glutamyl hydroxamate (GHA) formed to time of incubation, using tissue homogenates.

X—X Kidney.

O—O Skeletal muscle.

(see Table III, Fig. 5), suggesting the presence of some inhibitor, which needed further investigation.

As regards cardiac muscle, the increase in absorbancy even after one hour of incubation, using extract equivalent to 250 mg. of tissue was of the order of 0·05 and it was therefore very difficult to decide if any enzyme activity was really present.

TABLE III

Amount of γ-glutamylhydroxamic acid (GHA) produced by the total homogenate on incubation for various time intervals. (Glutamine synthetase activity expressed as μmoles of GHA/g. wet tissue).

Time of incubation in min.	Skeletal muscle			Kidney			
	1	2	3	1	2	3	4
5	3·71	7·04	2·24	20·00	14·24	22·00	11·84
10	-	-	-	-	-	23·00	-
15	4·55	7·20	3·36	26·40	16·96	-	23·20
20	-	-	-	-	-	32·20	-
30	5·15	7·84	4·48	33·12	23·68	35·40	39·04
45	5·24	8·88	4·80	36·96	35·84	39·60	44·16
60	-	-	-	38·56	-	-	47·20

In order to cut down the unnecessary amount of work, further investigations on this tissue were postponed until some improvements in the preparation of the extracts and the development of a more sensitive assay method could be achieved. If one could get glutamine synthesis by muscle and kidney extracts to be proportional

to incubation time, and concentration of extract, it would then be profitable to study cardiac muscle extracts again.

(C) ACHIEVEMENT OF LINEARITY

(i) Effect of high speed centrifugation

Reiner and Hudson (1953) reported that high-speed centrifugation of rat kidney homogenates increased the glutamine synthetase activity 3-fold. They found that all the activity was recovered in the supernatant after centrifuging the tissue homogenate for one hour at 40,000 R.P.M. in a Spinco Preparative Ultracentrifuge, using the type 40 rotor.

This observation was confirmed and it was found that the supernatant SII obtained after centrifuging the total homogenate at 30,000 R.P.M. for 45 minutes was more active than supernatant SI, centrifuged at 30,000 R.P.M. for 15 minutes, or supernatant SIII, centrifuged at 40,000 R.P.M. for one hour. This is shown in Table IV. The decrease in enzyme activity due to further centrifugation of the supernatant SII may be due to loss of some unknown activator of glutamine synthetase which is spun down during 1 hour at 40,000 R.P.M.

TABLE IV

Effect of differential spinning of the total homogenate on the glutamine synthetase activity of the tissue.(Enzyme activity expressed in units/g. wet tissue).

Tissue	SI	SII	SIII
Skeletal muscle	8·2	23·8	11·2
Kidney	187·2	198·0	157·5

It was established in another experiment that the residue RII obtained when supernatant SI was further centrifuged, caused inhibition when added to the supernatant SII (see Table V). The supernatant SII was therefore employed in all subsequent assays.

TABLE V

Inhibition of the tissue supernatant (SII) glutamine synthetase by the residue (RII) obtained by high speed centrifugation of the supernatant SI.

The enzyme activity is expressed in units/g. wet tissue.

Tissue	SI	SII	SII + RII
Skeletal muscle	12·2	27·6	16·2
Kidney	122·4	136·2	124·2

The enzyme activity was found to be linear with increase in the time of incubation using kidney supernatant only when small amounts (i.e. extract equivalent to 50 mg. of wet tissue) of the extract were used (see Fig. 6). The activity in this tissue was also found to be linear with increase in the volume of the extract (see Fig. 8).

On the other hand, the glutamine synthetase activity of muscle extracts was still quite nonlinear with increase in the time of incubation (see Fig. 7) and with increase in the amount of the

Fig. 6.

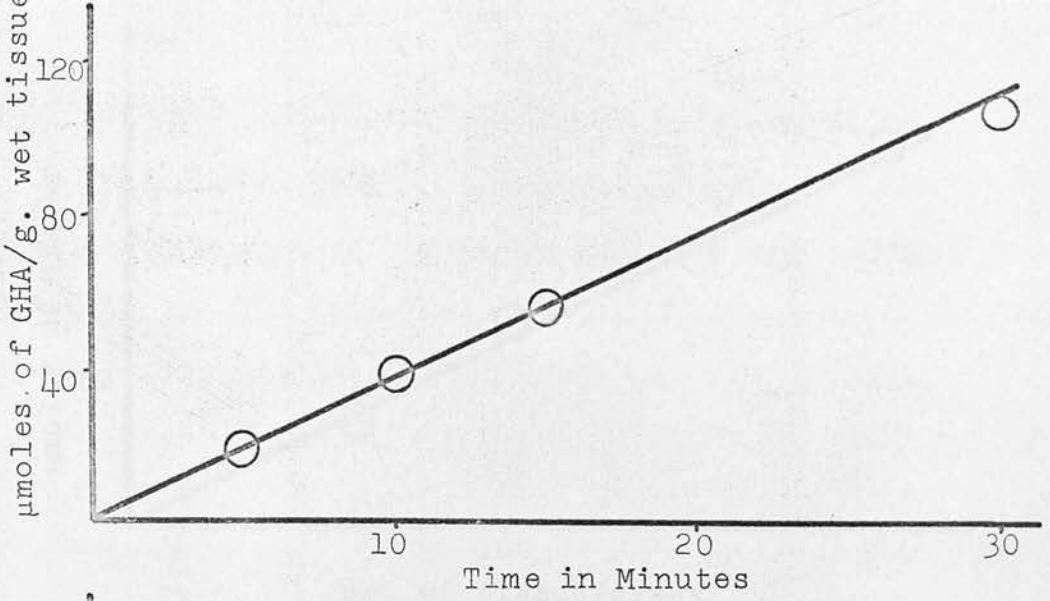
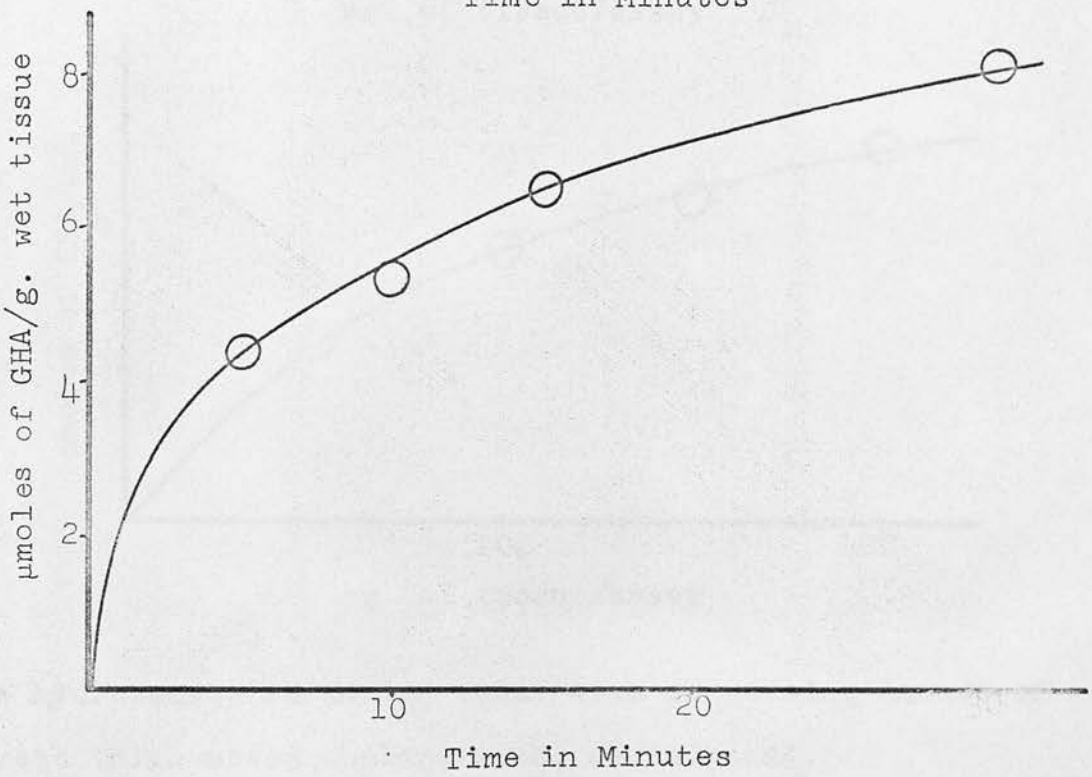


Fig. 7.



Rate of the hydroxamate (GHA) formation with increase in the time of incubation, using high-speed supernatants (undialysed).

Fig. 6. for kidney extracts

Fig. 7. for skeletal muscle extracts.

Fig. 8.

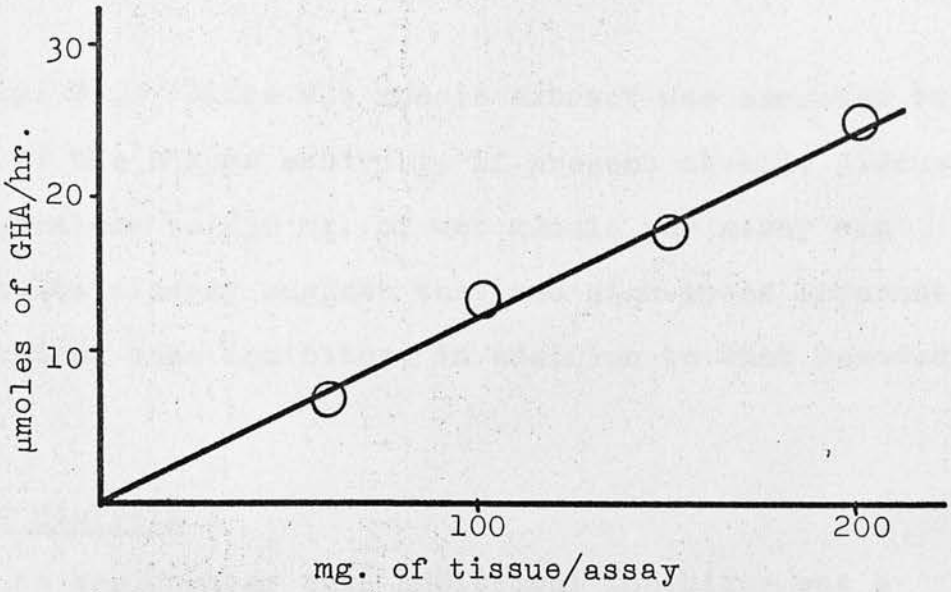
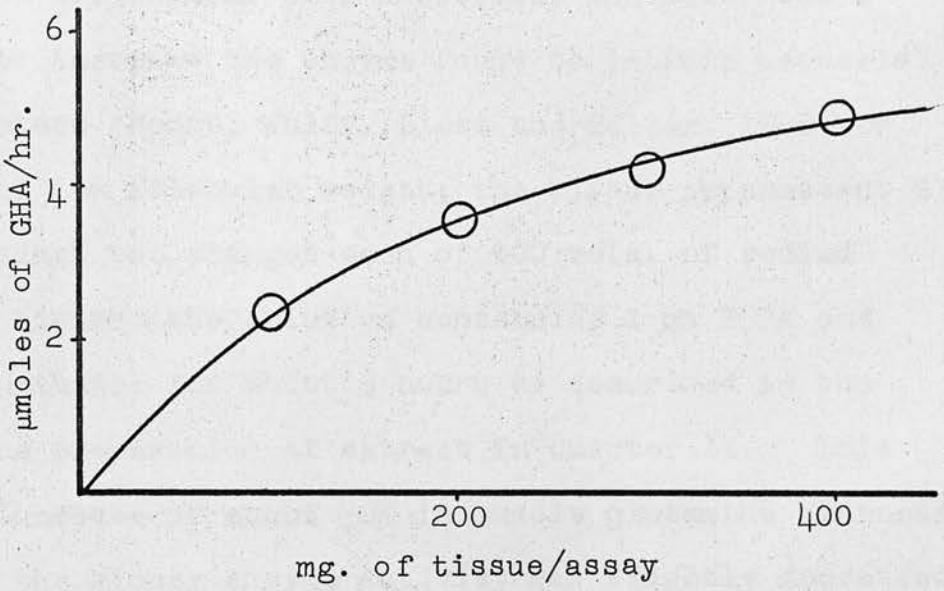


Fig. 9.



Rate of the hydroxamate formation (GHA) with increasing amount of tissue extract (high-speed supernatants, undialysed).
Incubations were carried out for 10 minutes at 37°C.

Fig. 8, using kidney extract.

Fig. 9, using skeletal muscle extract.

extract (see Fig. 9). Since the muscle extract was expected to have low level of the enzyme activity, if present at all, tissue supernatant equivalent to 250 mg. of wet muscle per assay was used. The results clearly suggest that the high-speed supernatant, SII, still contained some inhibitor, in addition to that removed in the residue.

(ii) Effect of dialysis

In order to see whether this additional inhibitor was a protein, like for instance the enzyme found to inhibit bacterial glutamine synthetase (Mecke, Wulff, Liess and Holzer, 1966) or whether it was of low molecular weight, the tissue supernatant SII was dialysed against two changes each of 100 vols. of sodium chloride-sodium bicarbonate solution containing 1 mM EDTA and 5 mM 2'-mercaptoethanol for about 3 hours as described in the procedure for the preparation of extract in Chapter II. This resulted in an increase of about 30% in muscle glutamine synthetase activity, while the kidney enzyme activity was slightly decreased. The most important effect of dialysis was that the activity of the dialysed muscle extract was found to become linear both with time of incubation (see Fig. 10), and with increase in the volume of the extract (see Fig. 11). This result removed any doubts that the colour measured after incubation of muscle extracts with the assay mixture might be due to an artefact, and not due to the enzyme, glutamine synthetase. Only dialysed tissue supernatants, as described in Chapter II, were therefore employed for the determination of the enzyme activity in all the experiments carried

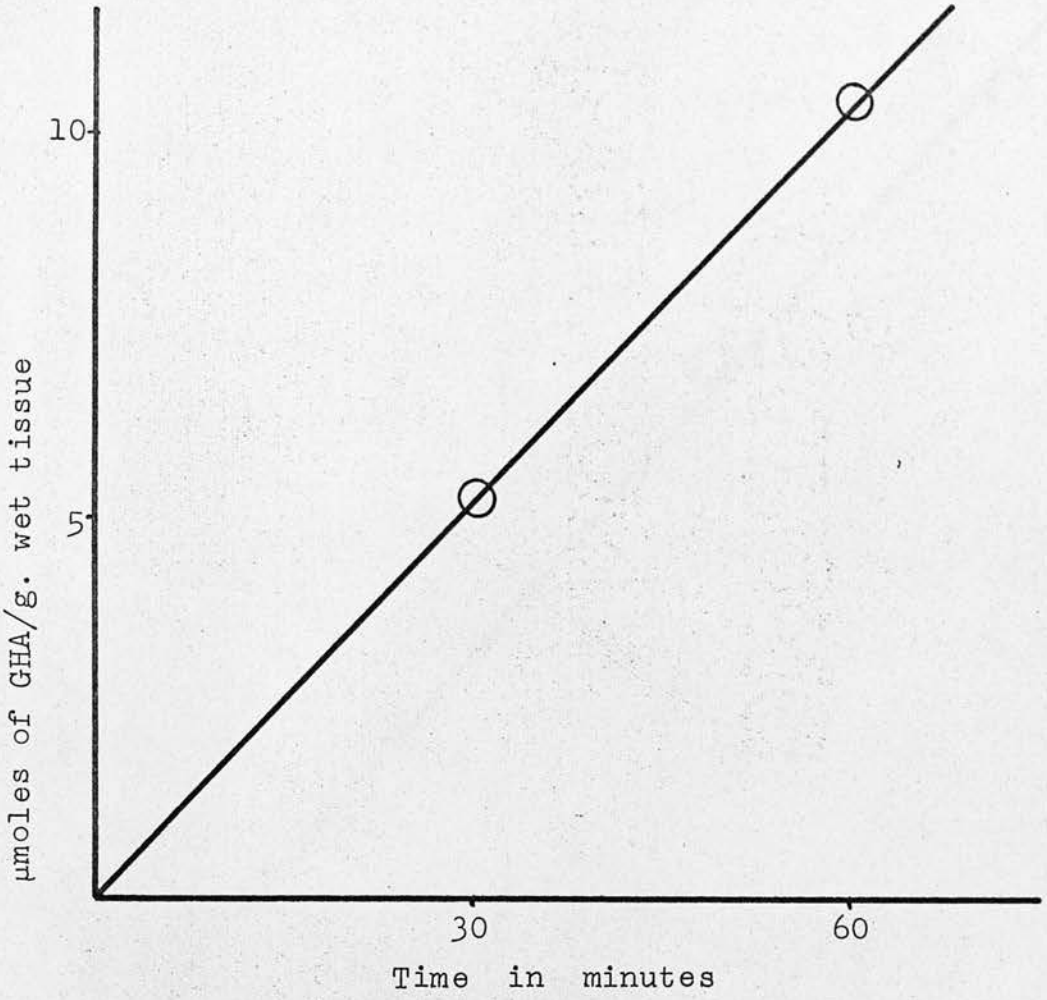


Fig. 10.

Linearity of the muscle glutamine synthetase activity with incubation time.

Dialysed high-speed supernatant was employed.

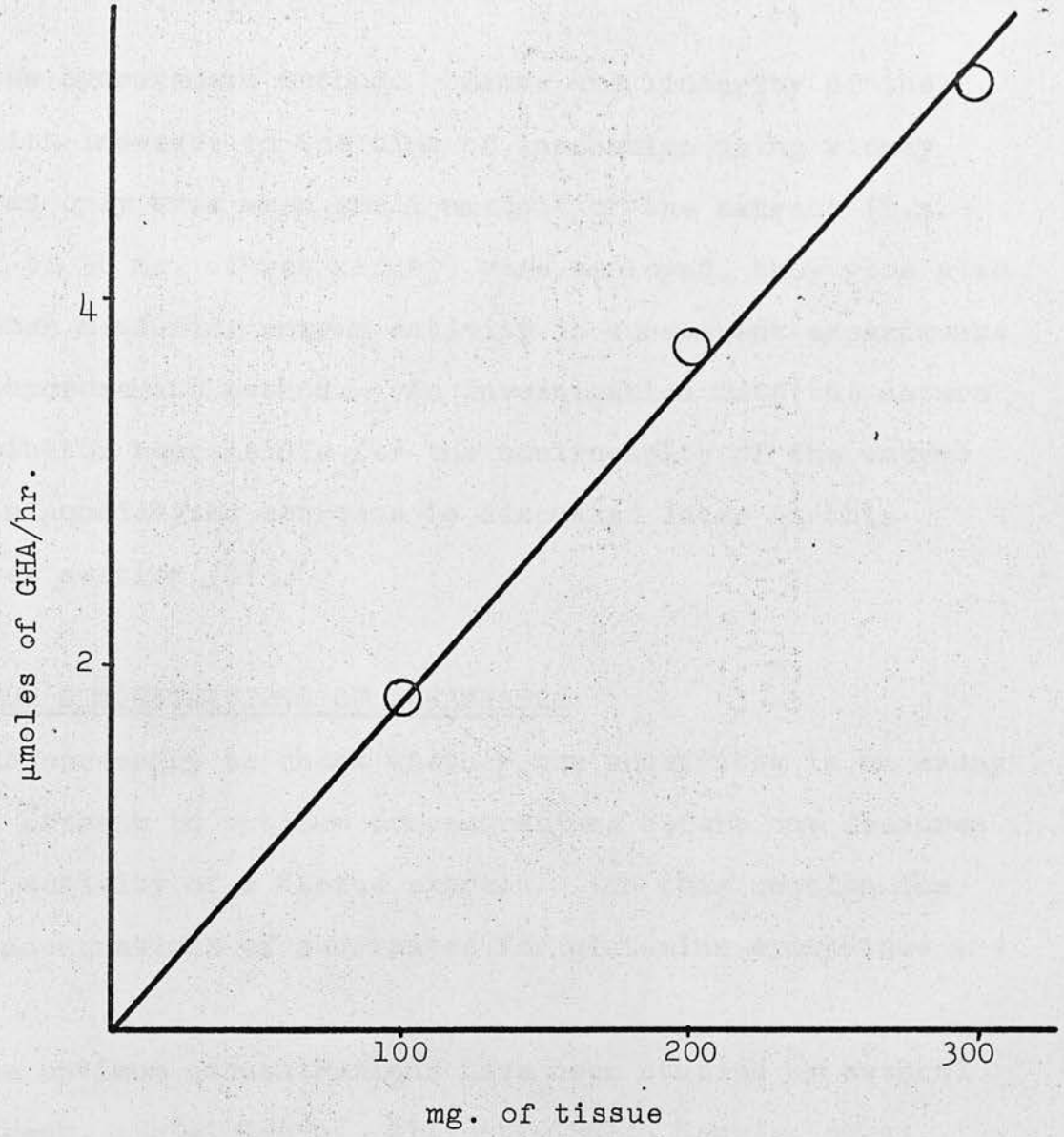


Fig. 11.

Linearity of the muscle glutamine synthetase activity with increasing amount of the tissue extract (dialysed high-speed supernatant).

Incubations were carried out for 10 minutes at 37°C.

out with the hydroxamate method. Since the linearity of the activity with increase in the time of incubation using kidney extracts was only true when small amounts of the extract (i.e. equivalent to 50 mg. of wet kidney) were employed, they were also dialysed when measuring enzyme activity in subsequent experiments using the hydroxamate method. An investigation into the nature of the inhibitor responsible for the nonlinearity of the enzyme activity in nondialysed extracts is discussed later in this chapter [see section (G)].

(D) OPTIMUM CONCENTRATIONS OF SUBSTRATES

It is necessary to check whether the substrates in an assay system are present in optimum concentrations before one measures the enzyme activity of a tissue extract. In this section the optimum concentrations of substrates for glutamine synthetase are reported.

These optimum concentrations have been studied by several workers (Speck, 1949a, 1949b; Elliott, 1951; Baerle, et al., 1957; Pamiljans, Krishnaswamy, Dumville and Meister, 1962; Trush, 1963). There is a good measure of agreement between the results except for ATP and Mg^{++} concentrations (see Chapter I) which thus need confirmation. Different concentrations of ATP have been employed by each worker, perhaps because of different amounts of ADP produced by their preparations (containing ATP-ase as an interfering enzyme) during the reaction.

In view of this, an optimum concentration of ATP was determined as shown in Fig. 12. The enzyme activity reached a plateau

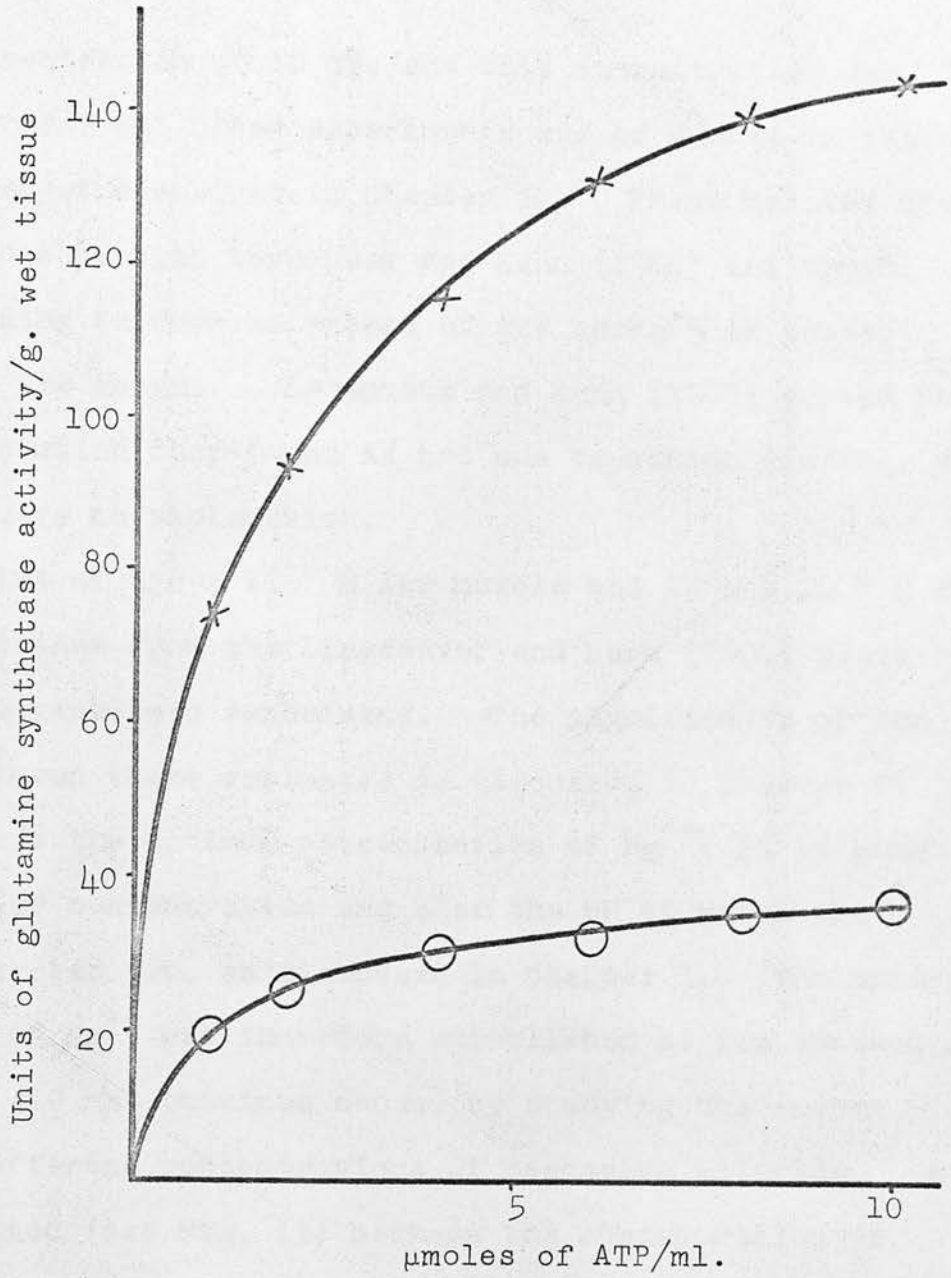


Fig. 12.

Optimum concentration of ATP.

X—X Shows the activity in kidney

○—○ Shows the activity in skeletal muscle

at an ATP concentration of 10 mM, and this concentration was therefore used for all other experiments and is quoted in the hydroxamate method described in Chapter II. These results disagree with those of both Levenbook and Kuhn (1962) and Trush (1963), according to whom an excess of ATP above 5 mM causes inhibition of the enzyme. Levenbook and Kuhn (1962) stated that the inhibition which they found is not due to cation binding, while Trush (1963) gave no explanation.

A Km value of $8{\cdot}8 \times 10^{-4}$ M for muscle and $12{\cdot}4 \times 10^{-4}$ M for kidney was obtained from the Lineweaver and Burk (1934) plots from the above-mentioned experiment. The significance of the difference between these estimates is discussed in Chapter VI.

As regards the optimum concentration of Mg^{++}, it is known to depend on ATP concentration and also the pH at which the reaction is carried out, as discussed in Chapter I. The optimum concentration of Mg^{++} was therefore established at the optimum ATP concentration (10 mM) obtained above, by studying the enzyme activity at different concentrations of magnesium chloride. A graph was plotted (see Fig. 13) between the concentration of magnesium ions and the enzyme activity. The highest activity was found at a Mg^{++} concentration of 15 mM both for muscles and the kidney extracts for 10 mM ATP concentration. Since this value, i.e. 15 mM Mg^{++} was very close to that value of 20 mM quoted by Elliott (1951) and Baerle, et al. (1957) 20 mM Mg^{++} was employed for all other experiments done with the hydroxamate method.

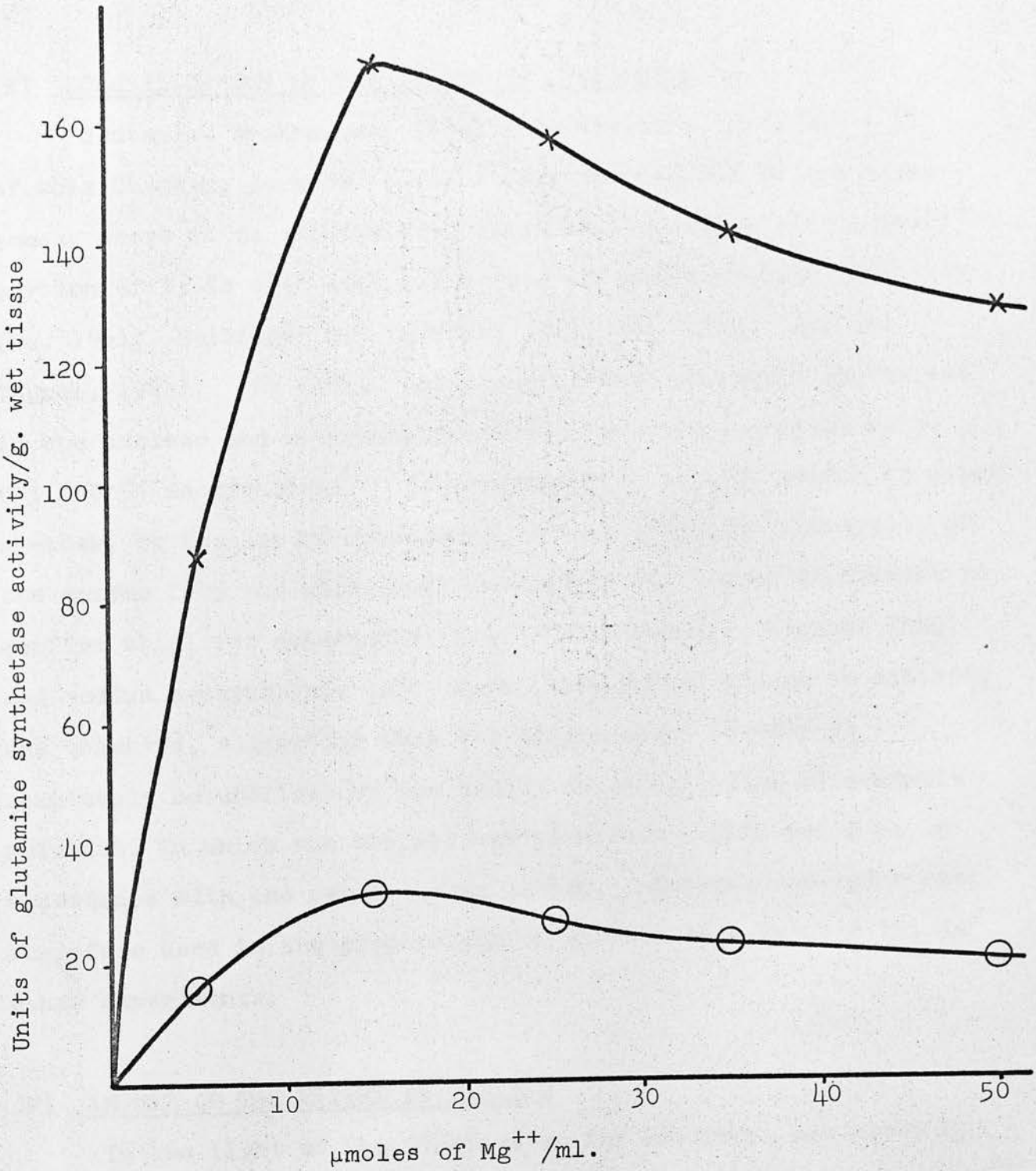


Fig. 13.

Optimum concentration of Mg^{++}.

X—X Shows the activity in kidney

O—O Shows the activity in skeletal muscle

(E) SOLUBILISATION OF THE ENZYME BY DETERGENTS

Glutamine synthetase, as already discussed in section (C) of this Chapter, is known to be mainly distributed in the microsomes, where it is chiefly associated with vesicles, and a small portion of it is also attached to the ribonucleoprotein particles (Wu, 1961; Sellinger and Verster, 1962; Wu, 1963; Hsu and Tappel, 1964). Wu (1961) has reported that glutamine synthetase in the nuclear and microsome fractions can be solubilised with 0·1 - 0·5% deoxycholate. It was therefore thought useful to check whether, by the use of detergents, a more efficient extraction of the enzyme from the microsomes was possible. In an experiment to confirm this, two detergents, i.e. sodium dodecyl sulphate (SDS) and sodium deoxycholate (SDC) were used, but no change in activity was observed, suggesting that the enzyme might already be completely solubilised by the sodium chloride-sodium bicarbonate solution, in which the extract was prepared. This would be in accordance with the report of Wu (1963a). Neither detergent was therefore used in the preparation of extract (see Chapter II) in other experiments.

(F) AMOUNT OF THE ENZYME IN TISSUES

In the light of the findings so far reported, estimates of tissue glutamine synthetase activity were therefore determined in dialysed supernatant fractions of tissue homogenates. The homogenates were prepared from tissue samples obtained from a group of animals and the hydroxamate method was used. The results are given in Table VI.

TABLE VI

Glutamine synthetase activity as units/g. wet tissue ± standard deviation.

The kidney, muscle and heart extracts were not necessarily prepared from the tissues obtained from the same rat.

Kidney	Skeletal muscle	Heart
307·2	33·1	1·2
198·0	23·8	2·7
136·2	27·6	3·6
146·2	10·5	2·4
119·7	31·1	2·0
313·0	9·0	2·0
171·3	12·0	2·4
144·0	21·6	3·7
129·0	7·2	2·0
204·0	9·2	
144·0	33·0	
186·0	10·4	
Average 183·2 ± 65·1	19·1 ± 10·3	2·5 ± 0·8

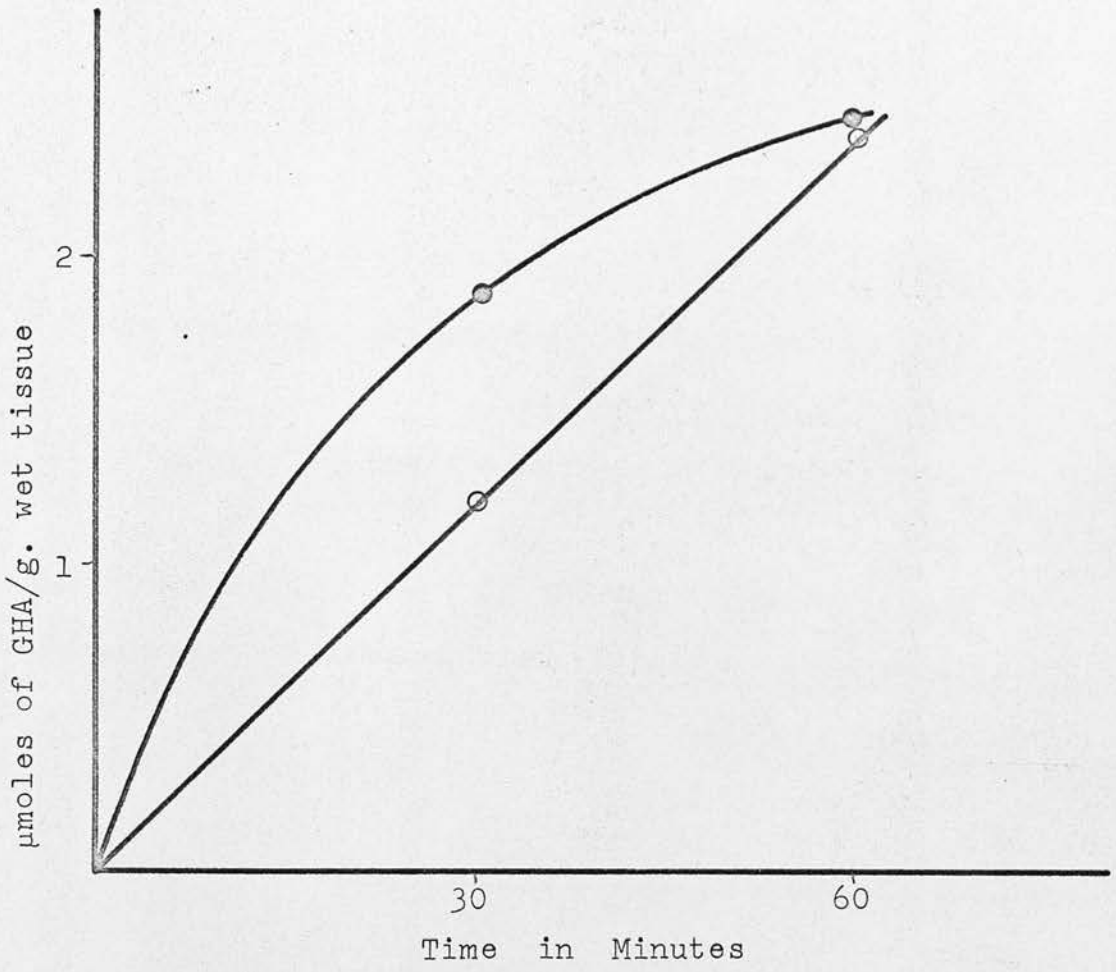


Fig. 14.

Rate of the glutamylhydroxamate synthesis with increase in the incubation time, using heart extracts equivalent to 250 mg. wet tissue/assay.

●—● Nondialysed supernatant

○—○ Dialysed supernatant

Fig. 15.

Rate of the glutamyl hydroxamate synthesis with increase in amount of the heart extract.

Incubations were carried out for 30 minutes at 37°C.

The nondialysed (●—●)(a) and dialysed (O—O)(b) heart supernatants used in this experiment were prepared from different rats.

Fig. 15(a)

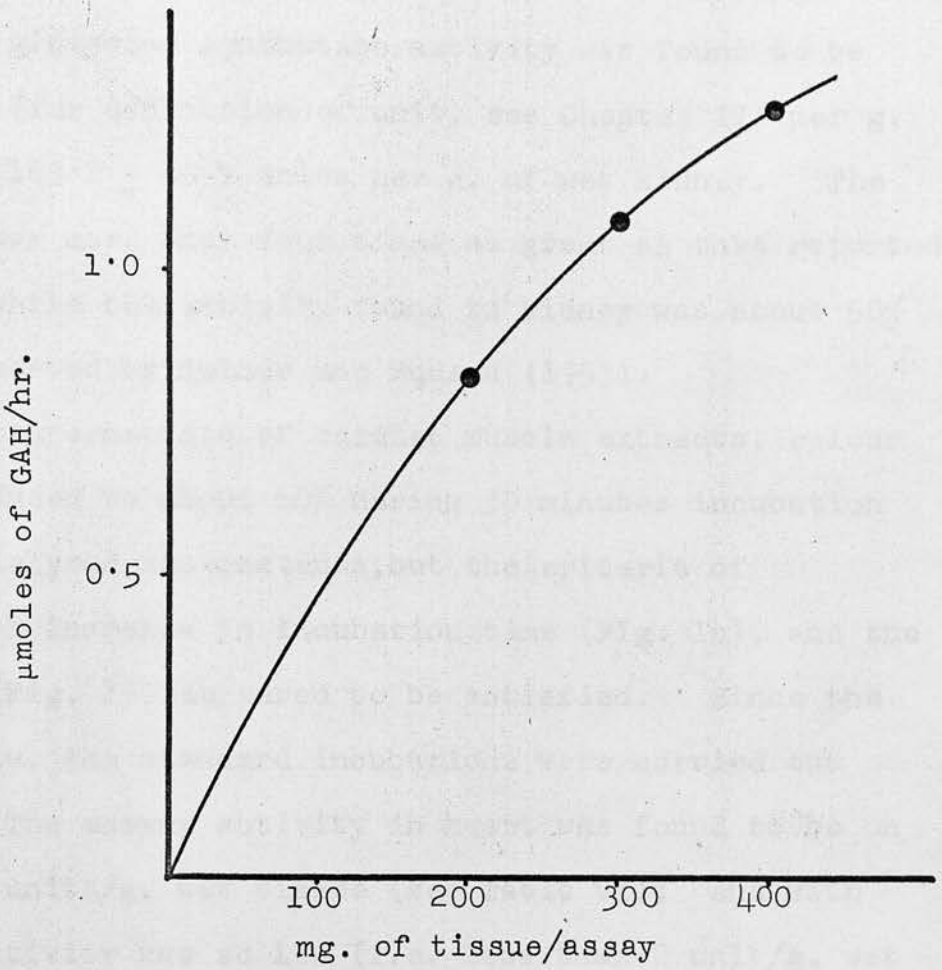
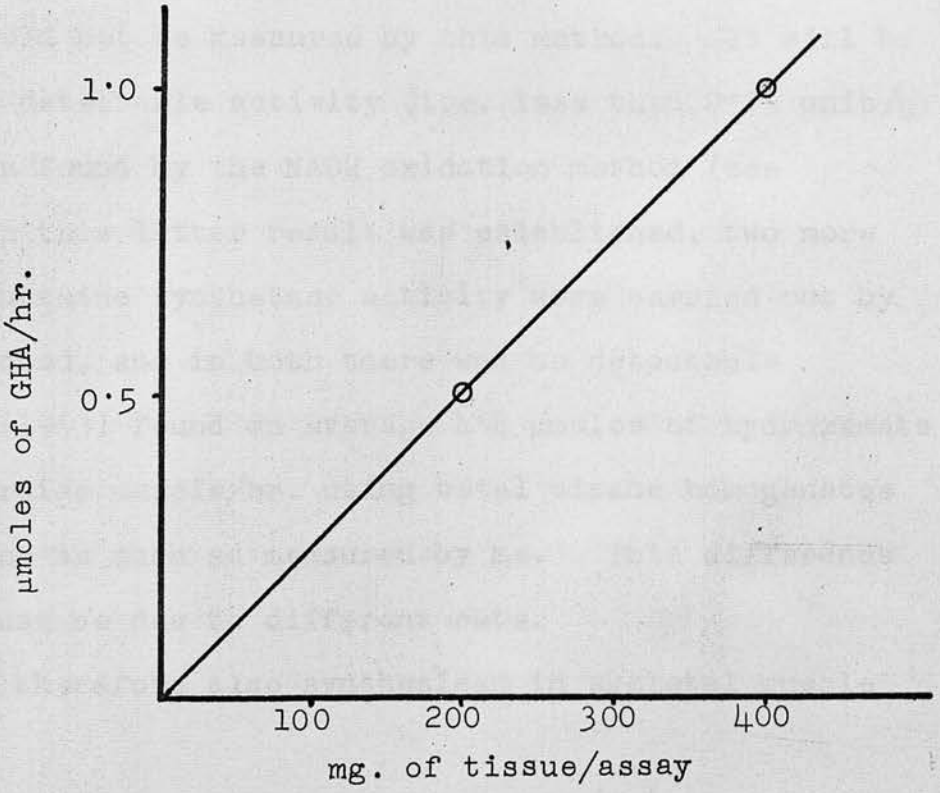


Fig. 15(b)



The average glutamine synthetase activity was found to be 19·1 ± 10·3 units (for definition of unit, see Chapter II) per g. of wet muscle and 183·2 ± 65·1 units per g. of wet kidney. The value for muscle was more than four times as great as that reported by Trush (1963), while the activity found in kidney was about 50% more than that reported by Reiner and Hudson (1953).

In dialysed supernatants of cardiac muscle extracts, colour production was reduced to about 60% during 30 minutes incubation compared with undialysed supernatants, but the criteria of linearity both with increase in incubation time (Fig. 14), and the amount of tissue (Fig. 15) appeared to be satisfied. Since the activity was so low, the standard incubations were carried out for 30 minutes. The enzyme activity in heart was found to be on average 2·5 ± 0·8 units/g. wet tissue (see Table VI); and with 3 extracts, the activity was so low (i.e. less than 2 unit/g. wet tissue) that it could not be measured by this method. It will be seen later that no detectable activity (i.e. less than 0·75 unit/g. wet heart) could be found by the NADH oxidation method (see Chapter IV). When this latter result was established, two more assays of heart glutamine synthetase activity were carried out by the hydroxamate method, and in both there was no detectable activity. Trush (1963) found on average 4·4 μmoles of hydroxamate produced/g. wet cardiac muscle/hr. using total tissue homogenates which is about twice as much as measured by me. This difference in results might just be due to different rats.

Glutamine is therefore also synthesised in skeletal muscle

beside liver, brain and kidney while heart is partly dependent on the blood stream for its supply of glutamine, at least in the rat (Ottaway, 1969).

After establishing the presence of glutamine synthetase in muscle, it seemed worthwhile to investigate the nature of the inhibitor which was responsible for the nonlinearity of the enzyme activity in the nondialysed tissue extracts, since it may be of physiological significance in the regulation of the enzyme in the body, even though this is not strictly related to the main investigation with which this thesis is concerned. The investigation of the inhibitor was limited to the skeletal muscle extracts and for comparison the kidney extracts were also studied in parallel to it.

(G) POSSIBLE NATURE OF THE INHIBITOR

The effect of dialysing the tissue supernatant SII, as found above, clearly indicated that the inhibitor was nonenzymic in nature. A look at various already known inhibitors of glutamine synthetase (as discussed in Chapter I) which may exist physiologically in the animal body suggests Ca^{++} as a possibility; the Ca^{++} content of rat kidney and lean somatic muscle is known to be 3 and 1·5 µg atoms/g. wet tissue respectively (Long, 1961). This could explain the inhibition produced by adding increasing amounts of extract (Fig. 9) and the increase in the enzyme activity by dialysis of the muscle extract. But had the inhibition in the muscle enzyme been due to Ca^{++} alone, the inhibition would have not been progressive with time

(see Fig. 7), i.e. the true course of the inhibited reaction would still be linear. This nonlinearity of the enzyme activity, when using nondialysed muscle extract, could therefore be only due to a product or products of some enzyme, even glutamine synthetase itself, which accumulated during the incubation causing progressive inhibition with time. Looking at this hypothesis that the inhibitor is an enzymic product, I considered ADP. ATP-ase is known to be present in both muscle and kidney, and is activated by Ca^{++} (Du Bois and Potter, 1943; Martonosi, 1968; Martonosi, Donley and Halpin, 1968). Since ATP is a common substrate both for glutamine synthetase and ATP-ase, its hydrolysis results in both a constant decrease in the concentration of ATP, and at the same time accumulation of ADP and inorganic phosphate (Pi). It may therefore be responsible for the progressive inhibition with time of incubation (Fig. 7).

The amount of ADP which accumulates during 30 minutes incubation in the assay system using nondialysed tissue supernatant was determined by the method given in Chapter II and was not found to be enough in either nondialysed muscle [viz. 3 μmole/assay (250 mg.) per 30 minutes incubation], or kidney (viz. 0·9 μmole/assay (50 mg.)/30 min. incubation] supernatants to account for the progressive inhibition of glutamine synthetase activity with time (Fig. 7). This low rate of ADP accumulation suggested that the ADP produced in the system might be converted to AMP and ATP by adenylate kinase present in the tissue supernatants. Elliott (1951) has reported that

$$2\ ATP \xrightleftharpoons{\text{ATP-ase; G.synth.}} 2\ ADP + 2Pi$$

$$2\ ADP \xrightleftharpoons{\text{Adenylate kinase}} AMP + ATP$$

AMP is only slightly inhibitory and that this inhibition may be due to the formation of ADP from it by adenylate kinase. These results therefore suggested that inorganic phosphate accumulating in the reaction system during incubation may be responsible for the progressive inhibition with time. Gothoskar, Raina and Ramakrishnan (1960) have reported that phosphate at a concentration of 90 mM or over completely inhibits chicken heart glutamine synthetase activity, while Pamiljans, Krishnaswamy and Meister (reported by Meister, 1962) failed to find any phosphate inhibition of the enzyme in mammalian tissues. This needed further investigations. The amount of inorganic phosphate which accumulates during 30 minutes incubation both due to ATP-ase and glutamine synthetase was, therefore, determined in an experiment using non-dialysed supernatants, by a method based on that of Taussky and Shorr (1953) and described in Chapter II. It was found to be 22·1 µmoles/assay (250 mg.) in nondialysed muscle and 9·3 µmoles/assay (50 mg.) in nondialysed kidney supernatants, while the corresponding values in dialysed muscle and kidney supernatants were 9·7 and 7·1 respectively. In addition to this the amount of endogenous inorganic phosphate present in the undialysed supernatants, when measured by the same method, was found to be 5·0 µmole/assay (250 mg.) in muscle and 1·3 µmole/assay (50 mg.) in kidney. This endogenous amount of inorganic phosphate together with that which

accumulates as a result of both ATP-ase and glutamine synthetase activities in nondialysed muscle extract could therefore be the cause of progressive inhibition of glutamine synthetase activity, if phosphate is, in fact, an inhibitor of the mammalian enzyme. The reason for linear activity in the case of kidney even in the nondialysed supernatants might be that the amount of extract used (i.e. equivalent to 50 mg. of wet tissue) was only 1/5th of that used for assays with muscle supernatants and consequently as shown above, both the amount of endogenous inorganic phosphate, and the accumulation of Pi during incubation, were not enough to inhibit the enzyme significantly (see Fig. 16). In the light of these results, it was decided to study glutamine synthetase activity in the presence of added inorganic phosphate.

When glutamine synthetase activity was studied in the presence of 5 mM phosphate (disodium hydrogen phosphate solution, neutralised to pH 7·2 was employed) using dialysed tissue supernatants, about 25% decrease in the enzyme activity of muscle supernatant and 10% decrease in kidney supernatant was found (see Table VII) suggesting that the inorganic phosphate does inhibit the enzyme. This agrees with the inhibition of the enzyme from chicken heart found by Gothoskar, Raina and Ramakrishnan (1960). The reason for the greater inhibition of the enzyme in the muscle extracts was not clear at this point.

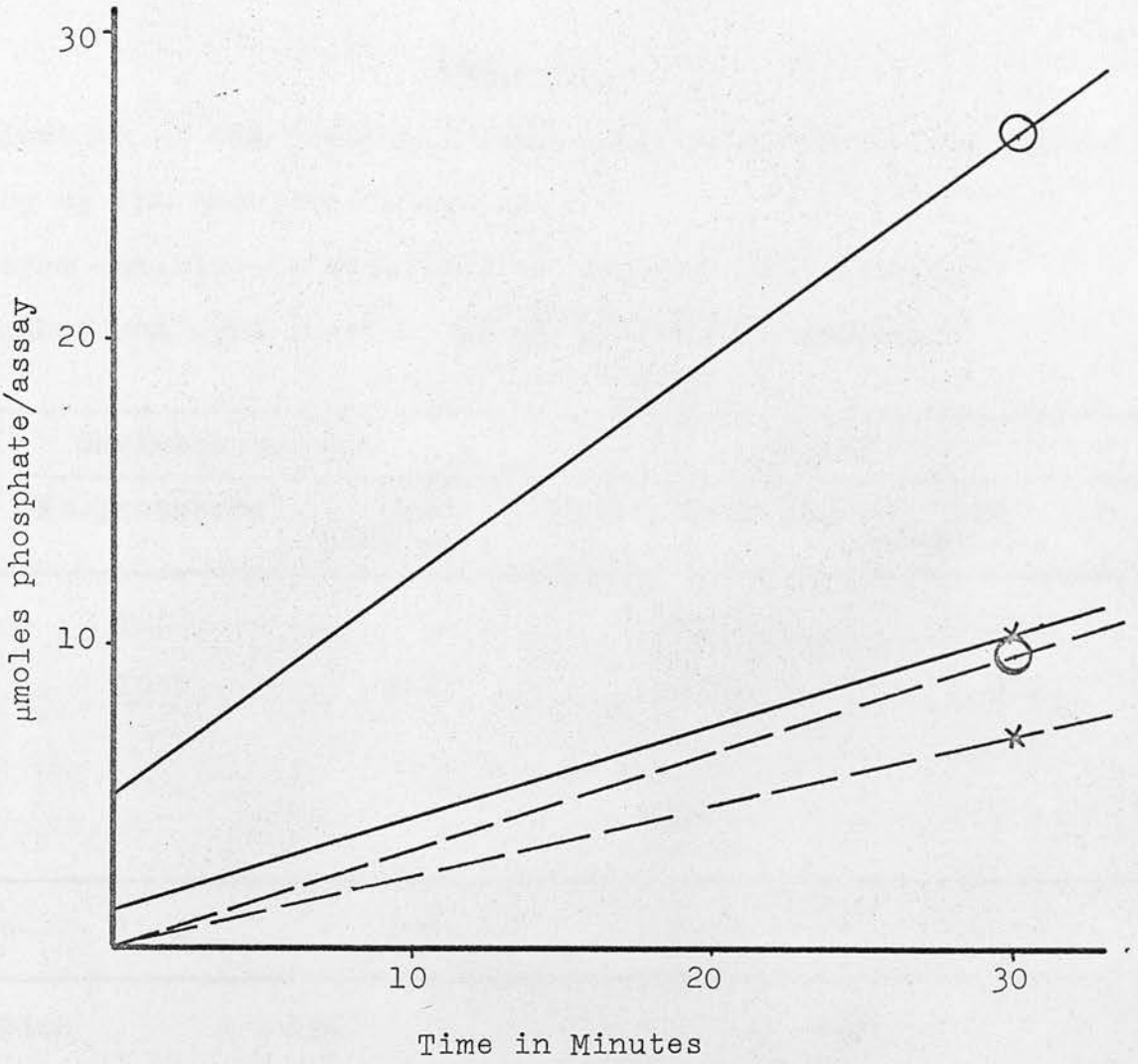


Fig. 16.

Showing the theoretical accumulation of inorganic phosphate per assay during 30 minutes incubation at 37°C; assuming both ATP-ase and glutamine synthetase activities to be linear.

○——○ Undialysed skeletal muscle
○- - -○ Dialysed skeletal muscle
×——× Undialysed kidney
×- - -× Dialysed kidney

TABLE VII

Determination of the phosphate inhibition of glutamine synthetase activity by the hydroxamate method.

The enzyme activity is expressed as units/g. wet tissue.

The incubations were carried out at 37°C for 30 minutes.

Expt. No.	Skeletal muscle		Kidney	
	No.phosphate	5mM phosphate	No phosphate	5mM phosphate
1	10·0	7·4	110·0	100·0
2	10·7	8·2	165·0	143·0
3	-	-	120·0	106·0
4	-	-	205·0	187·0
Average	10·4	7·8	150·0	134·0
Inhibition		25%		10%

Further study of the inorganic phosphate inhibition of the enzyme needed to be carried out in an assay system essentially free from any other inhibitor, for otherwise it would not have been possible to identify the type of inhibition. In the hydroxamate method ADP constantly accumulates. Hence it is not a suitable method to study the effect of the phosphate (Pi) on the rate of the enzyme reaction, unless some way can be introduced of removing the ADP from the assay system as soon as it is formed.

Attempts to do this by adding creatine phosphate (CP) (10 μmoles) and creatine phosphokinase (CPK) (100 μg.) were unsuccessful, perhaps because the enzyme was inhibited in the reaction mixture. Moreover, even if the ADP can be prevented from accumulating, Pi will still accumulate in considerable quantities during the time required for an accurately measurable amount of glutamyl-hydroxamate to be produced. This would mean that the concentration of inorganic phosphate (Pi) at the end of the incubation will be different from that added at the beginning, which would make estimation of the inhibitor constant, for example by Lineweaver-Burk plots, impossible. For these reasons it is necessary to choose both a more sensitive assay method and one in which ADP does not accumulate.

C H A P T E R IV

PHOSPHATE INHIBITION OF THE ENZYME REACTION, STUDIED BY THE NADH OXIDATION METHOD

(A) NADH Oxidation method

(B) Interference by ATP-ase and its removal from the extract

(C) Measurement of both ATP-ase and glutamine synthetase in the same cuvette

(D) Determination of inorganic phosphate inhibition of glutamine synthetase

(E) Check of heart extracts for glutamine synthetase activity by the NADH oxidation method

PHOSPHATE INHIBITION OF THE ENZYME REACTION STUDIED BY THE NADH OXIDATION METHOD

As discussed in the previous Chapter, the hydroxamate method is unsuitable for accurate study of the effect of inorganic phosphate (Pi) on the glutamine synthetase reaction. In this Chapter the development of a new method is discussed. The removal of most of the ATP-ase present in the crude enzyme extracts, which interferes in the assay has also been described. An economic method of measuring residual ATP-ase and glutamine synthetase activity in the same cuvette is also reported. The main part of the Chapter is concerned with establishing the effect of inorganic phosphate (Pi) on the rate of the enzyme reaction, which clearly showed it to be an inhibitor.

This Chapter also includes a further test for the existence of glutamine synthetase in cardiac muscle by the NADH oxidation method using ammonium sulphate treated extracts (see section B).

(A) NADH OXIDATION METHOD

The details of the NADH oxidation method are given in Chapter II. It is not only several times more sensitive than the hydroxamate method but also has the great advantage that ADP does not accumulate in the assay system. Moreover, the initial rate of the enzyme reaction can conveniently be studied. This was not possible with the hydroxamate method owing to its relatively low sensitivity. Measures of the glutamine synthetase activity by the NADH oxidation method were found to be comparable with the hydroxamate method only in the case of muscle extracts,

while when using kidney extracts the former method measured, on average, only about half of the activity, as compared with that found by the hydroxamate method. The values of the enzyme activity measured by the NADH oxidation method are given in Table VIII, and those found by the hydroxamate method are given

TABLE VIII

Determination of glutamine synthetase activity in dialysed supernatants by the NADH oxidation method ± standard deviation. The activity is expressed as units/g. wet tissue.
The muscle and kidney extracts were prepared from tissues obtained from different rats.

	Skeletal muscle	Kidney
	24·78	82·40
	18·23	113·40
	22·10	87·09
	14·90	86·71
	23·88	93·26
Average	20·78 ± 4·14	92·57 ± 12·27

in Table VI (Chapter III). These results suggest that the kidney enzyme,unlike the muscle enzyme may be inactivated (of course, partly) by some component or components of the assay mixture used in the NADH oxidation method. This shows a different behaviour of the enzyme from the two sources, i.e. muscle and the kidney.

(B) INTERFERENCE BY ATP-ASE AND ITS REMOVAL FROM THE EXTRACT

In spite of the increased sensitivity of the NADH oxidation method, the rate of change in optical density due to the glutamine synthetase reaction itself was in most cases very small because of the high ATP-ase activity and comparatively very low glutamine synthetase activity of the extracts. It could not be increased by adding more extract because NADH disappearance was then too fast to be accurately measured (see Fig. 17). This was true even with saturating concentrations of ATP, but with low ATP concentrations the rate of the reaction could not be accurately measured at all. Hence it was necessary to devise a method of inhibiting or removing the ATP-ase present in the dialysed tissue supernatants.

Weinbach (1954, 1956, 1957), and Weinbach and Bowen (1958) have demonstrated that pentachlorophenol (PCP) in concentrations of 5×10^{-4} M and higher, inhibits the ATP-ase of damaged mitochondria as well as that of soluble preparations of mitochondria. These workers also reported pentabromophenol (PBrP) to be a stronger inhibitor than PCP. The effects of both these substances on the ATP-ase activity of tissue extract were investigated. A 0·5 M PCP solution in ethanol and a 0·2 M solution in 2'-methoxy-ethanol were employed. About 40% of the ATP-ase and 45% of the glutamine synthetase activities were inhibited by PCP (5×10^{-3} M) in muscle extract, while the corresponding inhibition found in kidney extract was 60% of ATP-ase and 15% of glutamine synthetase activities. Pentabromophenol (2×10^{-3} M) was found to be less

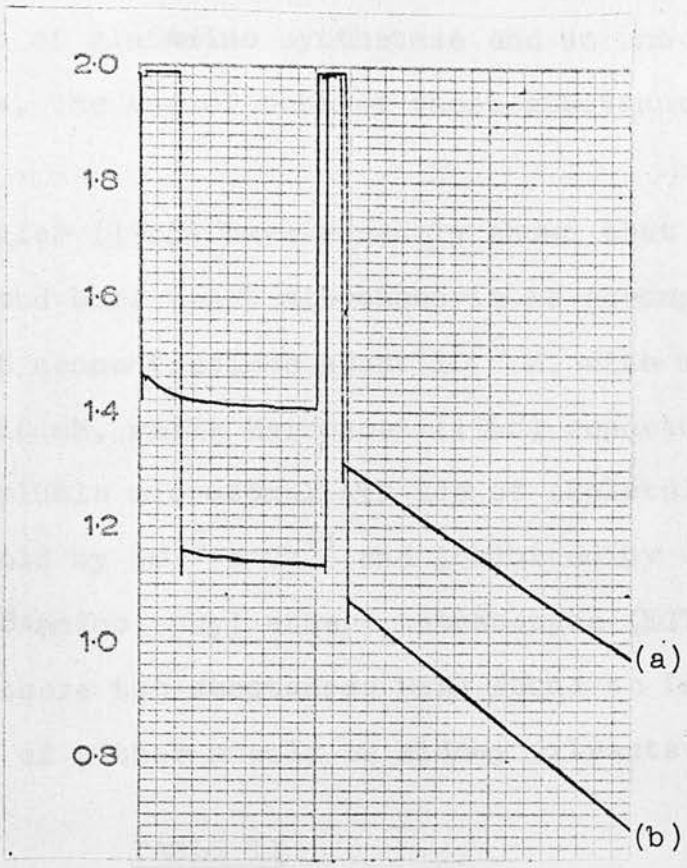


Fig. 17.

Actual recording of glutamine synthetase assay done by the NADH oxidation method, showing fast rate of NADH disappearance when a dialysed tissue supernatant (i.e. the one not treated with ammonium sulphate) is employed.

Extract equivalent to 25 mg. of wet skeletal muscle/assay was used.

(a) Control (assay mixture without glutamate, i.e. ATP-ase activity).

(b) Sample (assay with complete assay mixture, i.e. ATP-ase + glutamine synthetase activity).

effective than PCP with both kinds of extract (see Table IX). Due to the inhibition of glutamine synthetase and an uncomplete inhibition of ATP-ase, the use of both of these substances was abandoned.

Vigers and Ziegler (1968) have recently shown that ATP-ase from both rat liver and beef heart mitochondria is strongly inhibited by azide at concentrations about 10^{-5} M, with maximal inhibition at about 10 mM, while Martonosi (1968) reported that the Mg^{++}-moderated soluble microsomal ATP-ase of skeletal muscle is activated 10-20 fold by 10^{-5} M Ca^{++} and inhibited by 0·5 mM ethylene glycolbis (β-amino ethyl ether) tetracetate (EGTA). However, neither of these two substances were found to inhibit the ATP-ase activity of either muscle or kidney extracts.

TABLE IX

Effect of pentachlorophenol (PCP) and pentabromophenol (PBrP) on ATP-ase and glutamine synthetase (G. synth.) activities.

The activities measured by the NADH oxidation method as units/g. wet tissue.

Treatment	Skeletal muscle		Kidney	
	ATP-ase	G. synth.	ATP-ase	G. synth.
No treatment	34·7	12·7	81·0	88·0
PCP (5×10^{-3} M)	20·9	7·0	33·6	75·2
PBrP (2×10^{-3} M)	30·1	9·3	55·6	72·0

As these attempts to inhibit the ATP-ase were unsuccessful, I turned to removing it physically. This was more successful, using differential precipitation with ammonium sulphate.

The procedure used for investigating this was as follows. The tissue supernatant obtained by centrifuging the total homogenate (up to Step II of the procedure for the preparation of extract; see Chapter II)was treated with solid ammonium sulphate to give various concentrations (see Table X). The extract was shaken frequently and kept in a refrigerator for about 15 minutes, after which it was centrifuged in an ordinary bench centrifuge for about 10-15 minutes and the supernatant was discarded. The residue was redissolved in ice cold potassium chloride-potassium bicarbonate solution (0·15 M KCl + 0·005 M $KHCO_3$) so as to be equivalent to 1 g. of wet tissue per ml. This was then dialysed against two changes each of 100 vols. of potassium chloride-potassium bicarbonate solution containing 1 mM EDTA and 5 mM mercaptoethanol overnight in a cold room (ca 5°C). The preparation obtained by precipitation with an ammonium sulphate concentration of 1·5 M for muscle and 1·8 M for kidney extracts contained only about 10% of the original ATP-ase in muscle extracts, and about 30% in kidney extracts. The recovery of glutamine synthetase activity in these precipitated fractions was found to be about 60% for muscle and 70% for kidney of that measured in the corresponding untreated extracts (see Table X). Moreover, as this NADH oxidation method was used in the present investigation to study the phosphate inhibition of the enzyme activity, NH_2OH

TABLE X

Effect of ammonium sulphate treatment on the ATP-ase and glutamine synthetase activities of the centrifuged extracts.

The fractions having no ammonium sulphate treatment are the ordinary dialysed supernatants. The activities measured by the NADH oxidation method are expressed as units/g. wet tissue.

Ammonium sulphate treatment	Skeletal muscle		Kidney	
	ATP-ase	Glutamine synth.	ATP-ase	Glutamine synth.
No treatment	36·5	11·6	69·0	125·0
1·5 M (ppt. fraction)	4·5	7·0	21·9	60·5
1·5 M (supt. fraction)	18·5	1·2	-	-
1·8 M (ppt. fraction)	-	-	23·0	86·0
1·8 M (supt. fraction)	-	-	37·0	4·6
2·0 M (ppt. fraction)	5·2	7·3	22·7	83·6
4·0 M (ppt. fraction)	6·0	4·0	-	-

instead of NH_4^+ (which forms $MgNH_4PO_4$ precipitates as discussed in Chapter II), was used. In equimolar concentrations (100 mM) the rate of the reaction is only about 60% (muscle) and 50% (kidney) of that found when using NH_4^+ (see Table XI). In practice, a NH_2OH concentration of 25 mM was found to be better than 100 mM* for it measured about 75% (muscle) and 60% (kidney)

TABLE XI

Comparison of glutamine synthetase activity measurements by the NADH oxidation method using NH_4^+ (as NH_4Cl) and NH_2OH. The activity is expressed as units/g. wet tissue. (Ammonium sulphate treated extracts were used).

Substrate	Skeletal muscle	Kidney
100 mM NH_4^+	9·6	114·6
100 mM NH_2OH	5·9	56·0
25 mM NH_2OH	7·3	69·3

of the activity found when using 100 mM NH_4^+ (see Table XI). Hence, 25 mM NH_2OH was used in all other experiments with the NADH oxidation method. This means that the glutamine synthetase activity left in the ammonium sulphate treated extracts was only about 45%, in muscle, and 42%, in kidney, of that originally present as measured by the most efficient NADH oxidation method, i.e. that using NH_4^+.

*This decrease in activity when using NH_2OH in concentrations higher than 25 mM in the NADH oxidation method may be due to the inhibition of some ancillary enzyme or enzymes used in the assay system for NH_2OH does not inhibit the glutamine synthetase activity (Elliott, 1953).

In the course of this work it was found that NH_4^+ is a strong inhibitor of ATP-ase activity in dialysed extracts not treated with ammonium sulphate (see Fig. 18).

In the removal of ATP-ase by the above described technique which was used in all the studies reported in this and the subsequent Chapter, it was found that the sum of glutamine synthetase activity in the two fractions (precipitates and the supernatant) obtained was less by about 15-30% than that existing in the untreated extract (see Table X) suggesting that the precipitation permanently inactivated some of the enzyme. Gothoskar, Raina and Ramakrishnan (1960) have found that sulphate inhibits the chicken-heart glutamine synthetase activity; these workers reported 73% inhibition of the enzyme with 89 mM or higher concentration of sulphate (as ammonium sulphate). This suggested an experiment in which the activity found when using NH_4Cl and $(NH_4)_2SO_4$ was compared. About 15-30% less glutamine synthetase activity was found in the presence of SO_4^{--} compared with Cl^-. This suggests that SO_4^{--} inhibits the enzyme. Of course, this is a different kind of inhibition from that caused by precipitation of the enzyme by ammonium sulphate treatment. However, the observation is important because SO_4^{--} is an analogue of HPO_4^{--}, for example both activate glutaminase as well as the γ-glutamyl transfer reaction, and is further evidence that inorganic phosphate binds to glutamine synthetase, and does not merely form γ-glutamyl phosphate, for which there would be no sulphate analogue.

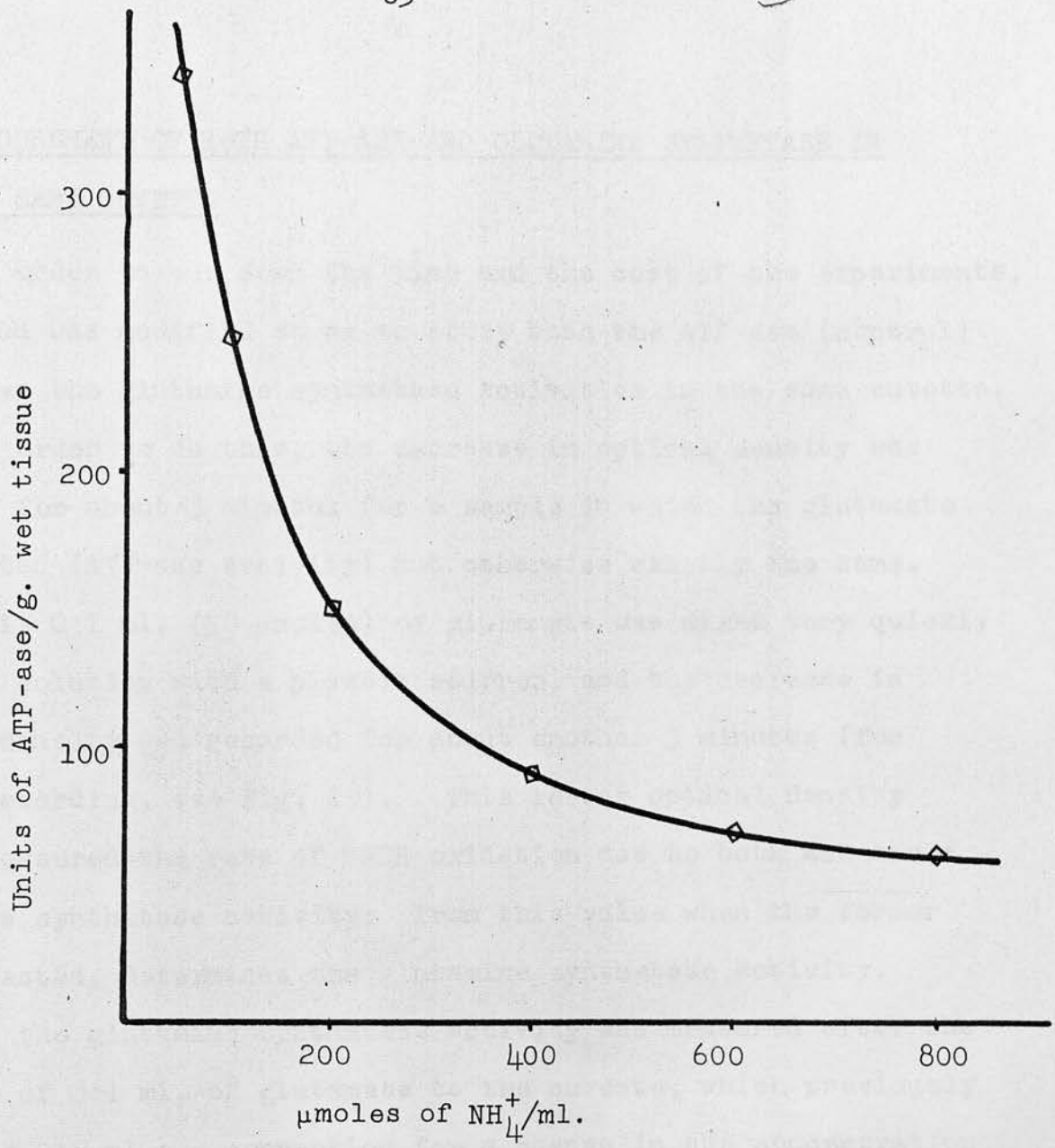


Fig. 18

Inhibition of ATP-ase activity by NH_4^+.

(ammonium chloride solution (pH 7·0) was used)

The ATP-ase activity was measured by the NADH oxidation method.

The extract used was dialysed kidney supernatant.

(C) MEASUREMENT OF BOTH ATP-ASE AND GLUTAMINE SYNTHETASE IN THE SAME CUVETTE

In order to cut down the time and the cost of the experiments, the method was modified so as to study both the ATP-ase (control) as well as the glutamine synthetase activities in the same cuvette.

In order to do this, the decrease in optical density was recorded for about 3 minutes for a sample in which the glutamate was omitted (ATP-ase activity) but otherwise exactly the same. After this 0·1 ml. (50 μmoles) of glutamate was mixed very quickly into the solution with a plastic stirrer, and the decrease in optical density was recorded for about another 3 minutes (for actual recording, see Fig. 19). This latter optical density change measured the rate of NADH oxidation due to both ATP-ase + glutamine synthetase activity; from this value when the former is subtracted, determines the glutamine synthetase activity.

As the glutamine synthetase activity was measured after the addition of 0·1 ml. of glutamate to the cuvette, which previously contained 0·9 ml., a correction for a change in the concentration of ATP-ase in the extract as well as of the substrate ATP, was necessary because of the change in volume of the system.

The rate of optical density change due to ATP-ase activity (i.e., the decrease in optical density recorded before adding the glutamate) was multiplied by a factor of 9/10 to correct for the change in enzyme concentration. The values so obtained were then plotted against the corresponding actual concentrations of

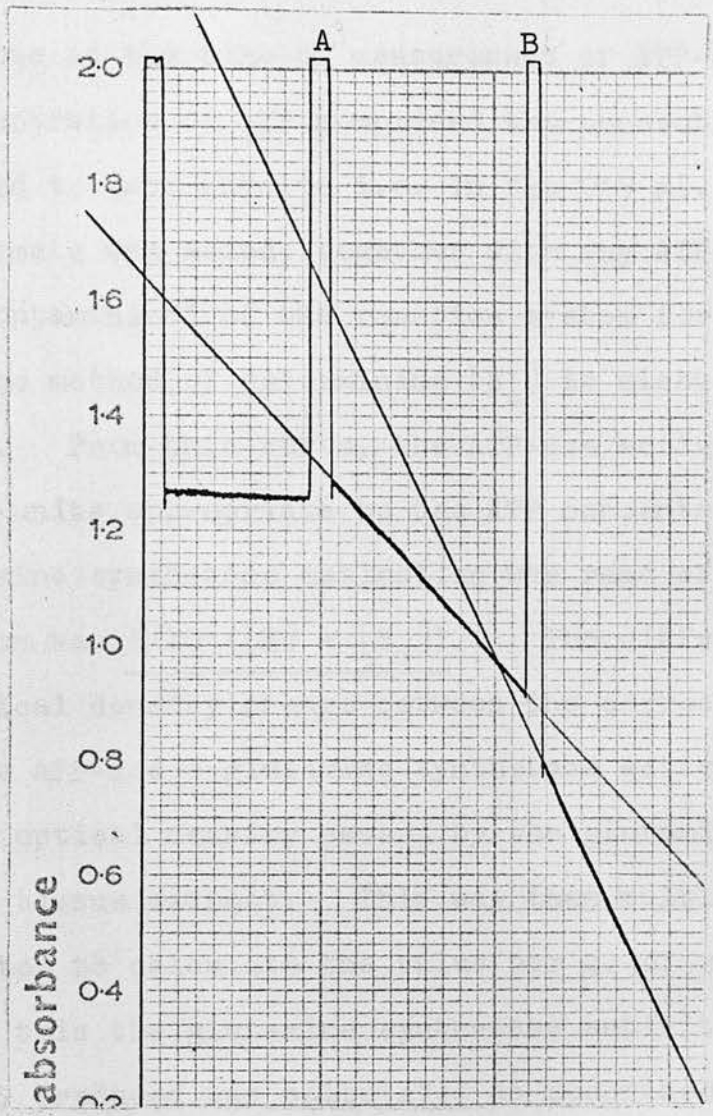


Fig. 19.

Actual recording of measurement of both ATP-ase and glutamine synthetase in the same cuvette by the NADH oxidation method.

Ammonium sulphate treated muscle extract equivalent to 200 mg. of wet tissue/assay was employed.

A and B in the figure represents the points where extract (i.e. ATP-ase activity) and glutamate (i.e. ATP-ase + glutamine synthetase activity) were added to the assay mixture.

ATP in the cuvette at the time of measurement of ATP-ase activity. The actual concentration of ATP comprised the concentration of the ATP [S] added to each cuvette i.e. in the 0·9 ml., present before the glutamate was added, together with any ATP [S_o] present as a "contaminant" of the reaction system for each experiment. The method of determining [S_o] is discussed later in this Chapter. From this curve, the ATP-ase activity (V) in optical density units appropriate to the ATP concentration in the ATP-ase + glutamine synthetase estimation was read off. This ATP concentration was 9/10 ([S] + [S_o]). The difference in the rate of optical density change between the corrected ATP-ase activity and the ATP-ase + glutamine synthetase activities gave the decrease in optical density caused by the glutamine synthetase activity of the tissue extract. This was then multiplied by an appropriate factor to calculate the value per g. of wet tissue/hour; and from this the glutamine synthetase activity expressed as μmoles of ADP produced was calculated as described earlier (see Chapter II).

(D) DETERMINATION OF INORGANIC PHOSPHATE INHIBITION OF GLUTAMINE SYNTHETASE

The initial rate of the enzyme reaction was determined at various concentrations of ATP both in the absence of Pi and in the presence of 5 mM and 20 mM phosphate (0·2 M sodium phosphate, pH 7·0 was employed for the purpose) using extracts partially

purified by $(NH_4)_2SO_4$ treatment. To avoid any precipitation due to $MgNH_4PO_4$ in the assay system, hydroxylamine was employed instead of NH_4^+ (see Chapter II).

The amount of tissue extract added was equivalent to 50 mg. of wet kidney or 200 mg. of muscle per assay. This gave a high rate of reaction in the controls, so that there was still a measurable rate in the phosphate inhibited assays.

An increase in NADH disappearance after addition of glutamate, which must be assumed to be due to glutamine synthetase activity was found even without any addition of ATP to the assay mixture (see Fig. 20). This must have been due to an impurity, probably ADP, in the reagents used, possibly in the NADH, or nucleotide too tightly bound to be completely removed by dialysis of the tissue extract, or both. Any ADP added would be converted to ATP by the PEP and PK present in the assay system, and the concentration of the latter would of course be maintained during the measurement. Had the NADH been oxidised non-specifically by O_2, it would not be affected by addition of glutamate. Hence, this increase in NADH disappearance at zero ATP addition was almost certainly due to glutamine synthetase activity.

To determine this unknown concentration of nucleotide, the following calculations were carried out.

Let the unknown concentration of ATP be S_o.

Then the rate of ATP-ase in the absence of added ATP

$$V_o = \frac{AS_o}{B + S_o} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

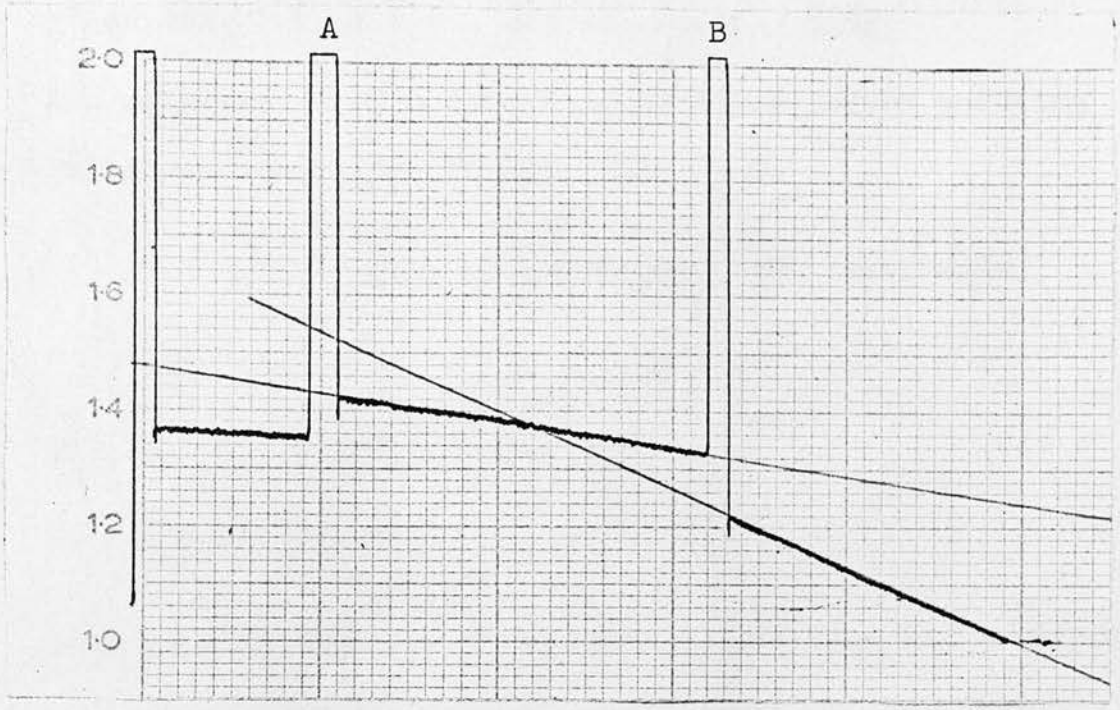


Fig. 20.

Showing both ATP-ase and glutamine synthetase activity in the absence of any added ATP [S_o].

Actual recording of measurement of both ATP-ase and glutamine synthetase in the same cuvette by the NADH oxidation method.

Ammonium sulphate treated extract equivalent to 200 mg. of wet muscle/assay was used.

A and B in the figure indicates the points of addition of extract (giving ATP-ase activity) and glutamate (giving ATP-ase + glutamine synthetase activity) to the assay system.

$$\text{or } AS_o = V_o(B + S_o) \quad \ldots\ldots\ldots\ldots\ldots \quad (1a)$$

The observed rate of ATP-ase activity V, after addition of a nominal concentration of ATP equal to S.

$$V = \frac{A(S + S_o)}{B + S_o + S} = \frac{AS + V_o(B + S_o)}{(B + S_o) + S} \quad \ldots\ldots \quad (2)$$

$$V(B + S_o) + VS = AS + V_o(B + S_o)$$

$$\text{or } (V - V_o)(B + S_o) = AS - VS$$

$$\frac{V - V_o}{S}(B + S_o) = A - V$$

Thus by plotting $\frac{V - V_o}{S}$ against V, (see Fig. 21) a straight line should be obtained whose intercept on the V axis should be $A(= V_{max})$, and whose slope gives $(B + S_o)$.

Then from equation (1a)

$$S_o = \frac{V_o(B + S_o)}{A}$$

Since $(B + S_o)$ is known, $B (= K_m)$ can also be obtained. For the experiments with phosphate B was replaced by $B' = B\ (1 + i/K_i)$, from which K_i could also be obtained. These values refer, of course, only to the ATP-ase of the extracts, and are therefore not of great interest in present circumstances.

The data for one of the experiments and the calculation of the value of $[S_o]$ is shown in Table XII (Fig. 21 corresponds to the data given in this Table). Table XIII shows the values of $[S_o]$ obtained in the experiments given in this and the next

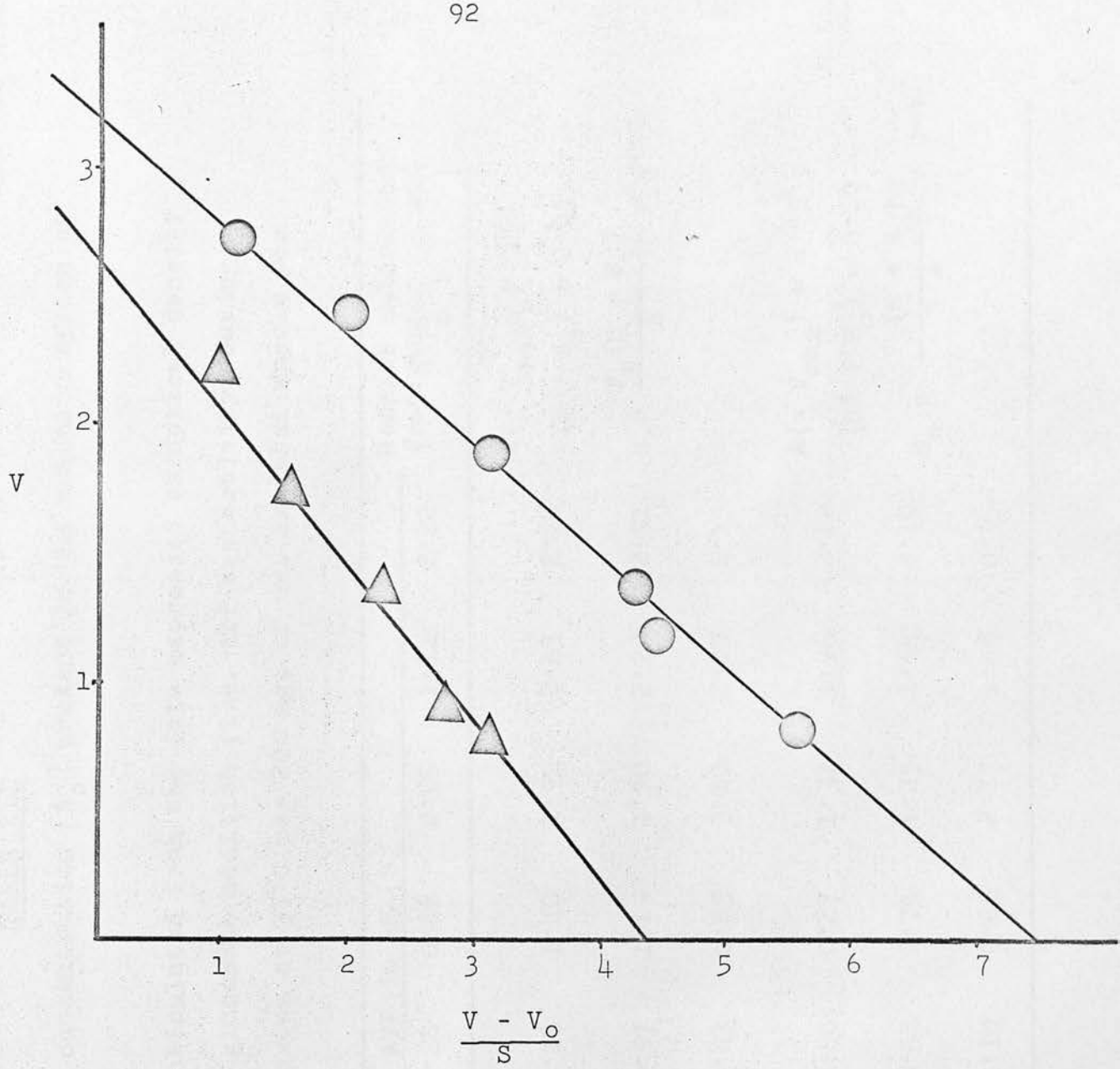


Fig. 21.

Determination of the unknown substrate concentration [S_o] present in the reaction system at zero ATP addition.

For values of V and $\frac{V - V_o}{S}$, see Table XII.

(This figure corresponds to Table XII).

○—○ For no added phosphate

△—△ For 5 mM phosphate

TABLE XII

Determination of unknown ATP concentration [S_o] present in the reaction system at zero ATP addition.

The values given below were calculated from the data expressed as optical density measurements obtained before glutamate addition (i.e. ATP-ase activity) during incubation of the reaction system at 37°C per 200 mg. of wet skeletal muscle per 0·9 ml. per 400 seconds.

Calculations	Added ATP in mM [S]							Results calculated from graph; and [S_o]
	0·00	0·11	0·22	0·28	0·55	1·11	2·22	
No phosphate:								$A(= V_{max}) = 3·20$
$V \longrightarrow$	0·21 (V_o)	0·82	1·18	1·40	1·91	2·43	2·72	$(B + S_o) = 0·43$
$V - V_o$	-	0·61	0·97	1·19	1·70	2·22	2·51	$S_o = \frac{V_o(B + S_o)}{A} = 0·03$
$\frac{V - V_o}{[S]}$	-	5·54	4·41	4·25	3·10	2·00	1·13	
5 mM phosphate:								$A(= V_{max}) = 2·65$
$V \longrightarrow$	0·12 (V_o)	0·51	0·80	0·87	1·37	1·76	2·22	$(B' + S_o) = 0·60$
$V - V_o \longrightarrow$	-	0·39	0·68	0·75	1·25	1·64	2·10	$S_o = \frac{V_o(B + S_o)}{A} = 0·03$
$\frac{V - V_o}{[S]}$	-	3·55	3·10	2·68	2·27	1·48	0·95	

TABLE XIII

The value of the unknown ATP concentration, $[S_o]$ present in the reaction system at zero ATP addition. The values are given in mM.
(These values are calculated for 0·9 ml. i.e. from the ATP-ase activity).

	Skeletal muscle	Kidney
	0·02	0·01
	0·03	0·00
	0·04	0·01
	0·03	0·01
	0·03	0·02
	0·03	0·01
	0·05	-
	0·03	-
	0·03	-
	0·02	-
Average	0·03	0·01

Chapter. They are pretty constant, averaging 0·03 mM for muscle, and 0·01 mM for kidney. These values are comparable with the 0·1 mM, which was the lowest nominal concentration of ATP used, and therefore the correction was a necessary one.

6 out of 11 experiments when using muscle and 10 out of 19 when using kidney extracts gave linear plots of $\frac{V-V_o}{S}$ against V, while the rest gave nonlinear plots especially with phosphate.

Inorganic phosphate (Pi) was found to inhibit the glutamine synthetase activity of both kidney and muscle extracts. The data, after making the necessary corrections (as described above) both for the unknown concentration of the substrate $[S_o]$ at zero ATP addition and the change in volume on addition of glutamate in the assay system, are shown in Table XIV for skeletal muscle and Table XV for kidney. The estimates of K_m are given in Table XVI for the muscle and Table XVII for kidney enzymes. The estimates were computed by a program due to Cleland (1967) which determines the parameters of the Michaelis Menten equation by a least squares method.

All the data given in Tables XIV and XV when plotted graphically [Lineweaver Burk plots (1934)] suggested inorganic phosphate to be a competitive inhibitor of the muscle enzyme (see Fig. 22) and a non-competitive inhibitor of the kidney enzyme (see Fig. 23). Figures 22 and 23 are only illustration.

Since the inhibition with the muscle extracts appeared to be competitive, values of K_i for each concentration of the

Fig. 22.

Phosphate inhibition of the muscle glutamine synthetase activity.

(a) Plots of the enzyme activity at various concentrations of the substrate.

(b) The lineweaver-Burk plots of (a).

●——● Shows the activity of the control, i.e. the one without any added phosphate.

■——■ Shows the activity in the presence of 5 mM phosphate.

▲——▲ Shows the activity in the presence of 20 mM phosphate.

Fig. 22 (a)

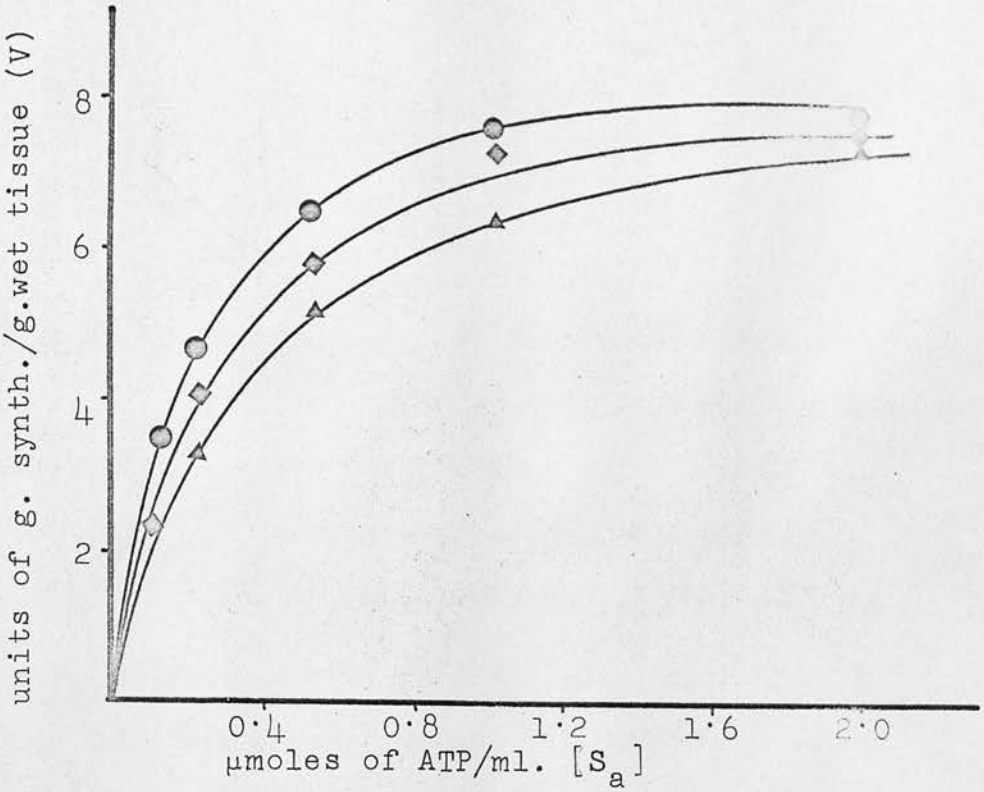


Fig. 22 (b)

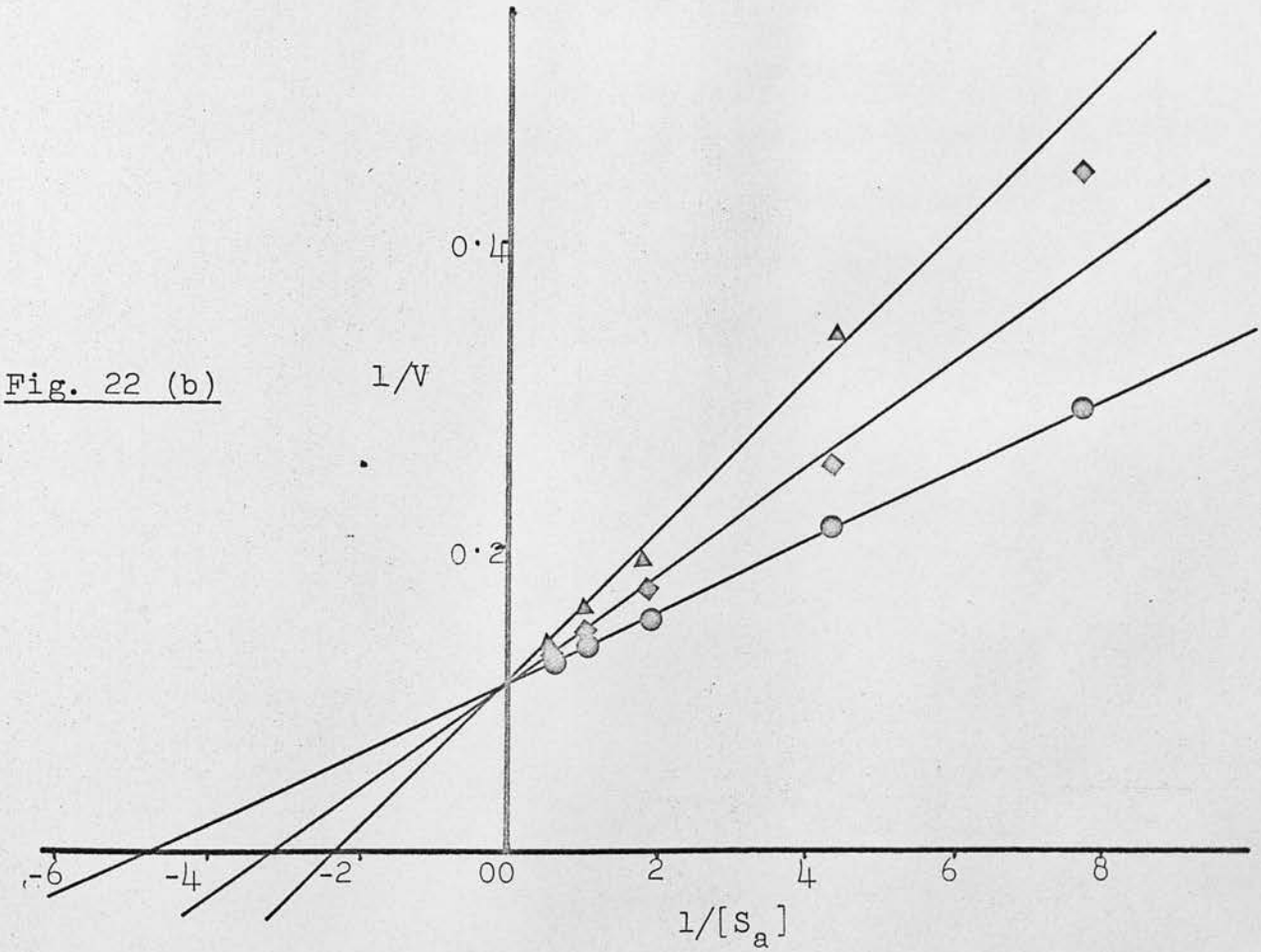


Fig. 23.

Phosphate inhibition of the kidney glutamine synthetase activity.

(a) Plots of the activity at various concentrations of the substrate.

(b) The Lineweaver-Burk plots of (a).

●——● shows the activity of the control, i.e. the one without any phosphate.

■——■ shows the activity in the presence of 5 mM phosphate.

▲——▲ shows the activity in the presence of 20 mM phosphate.

Fig. 23(a)

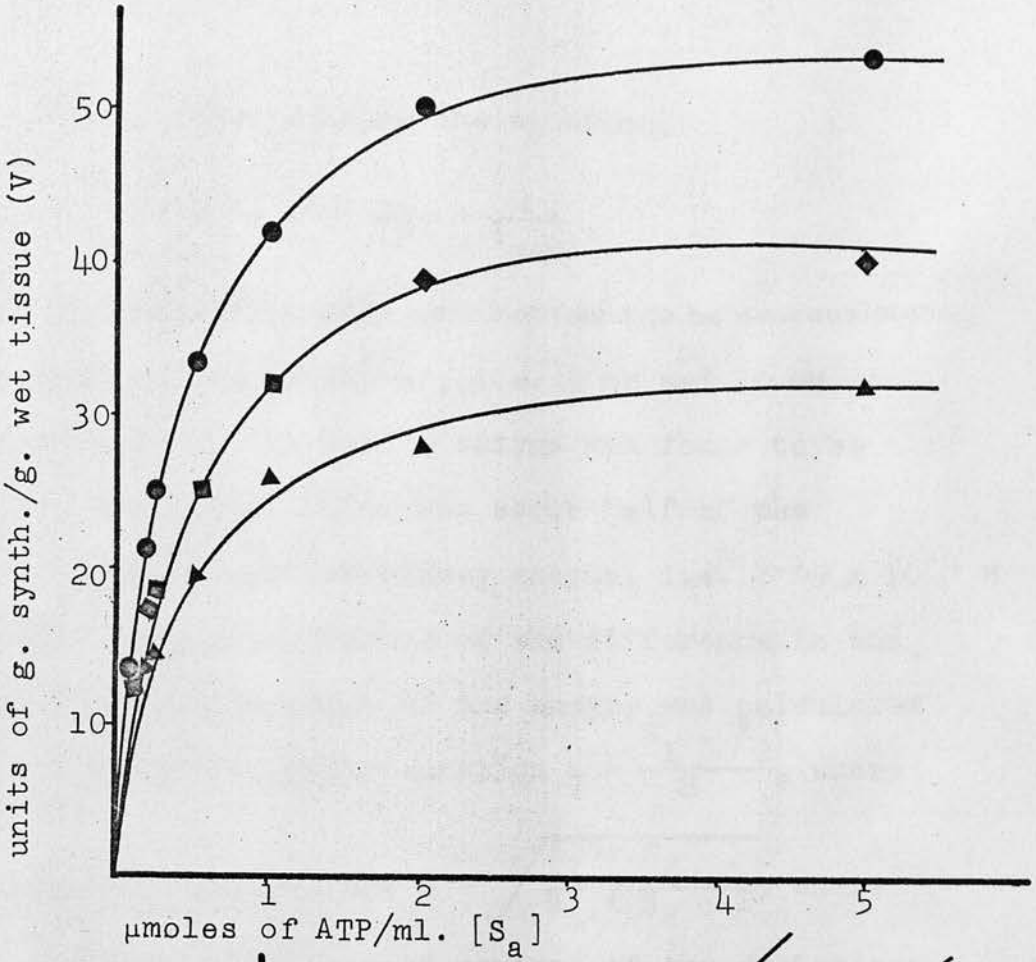
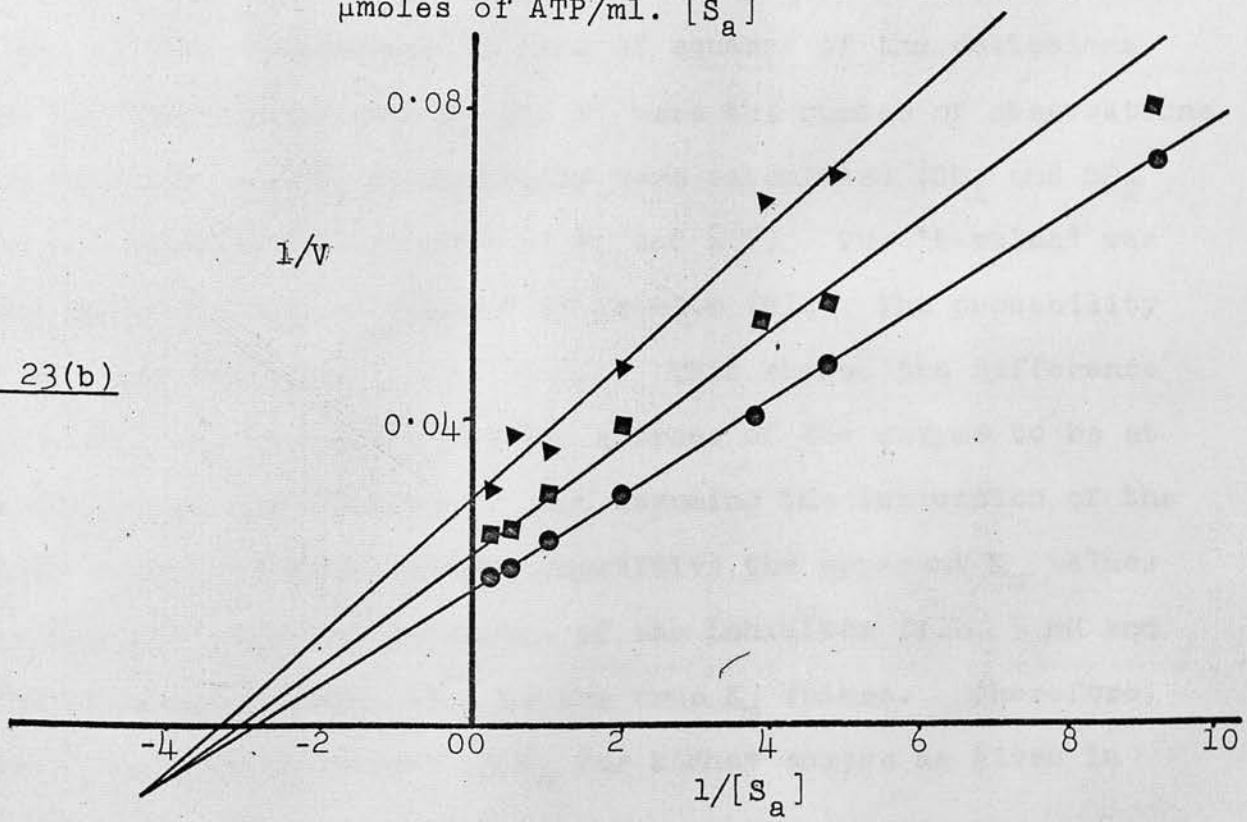


Fig. 23(b)



inhibitor (Pi) was calculated using the equation,

$$\frac{1}{V} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i}\right) \frac{1}{[S]} + \frac{1}{V_{max}} .$$

These values of K_i (see Table XVI) were not found to be nonconsistent for the two phosphate concentrations, i.e. 5 mM and 20 mM.

The mean K_m of the muscle enzyme was found to be $1{\cdot}62 \times 10^{-4}$M (see Table XVI) which was about half of the corresponding value obtained for kidney enzyme, i.e. $2{\cdot}99 \times 10^{-4}$ M (see Table XVII). The significance of the difference in the mean K_m values of the two sources of the enzyme was calculated by the "student t-test" using the equation $t = \frac{\bar{x}_1 - \bar{x}_2}{S}$, where $\bar{x}_1$ and $\bar{x}_2$ are the mean values, and $S = \sqrt{\frac{S_1 + S_2}{N_1 + N_2 - 2}}$, where S_1 and S_2 are the respective sums of squares of the deviations from the mean value, and N_1 and N_2 were the number of observations from which $\bar{x}_1$ and $\bar{x}_2$ respectively were calculated (SD_1 and SD_2 were the standard deviations of $\bar{x}_1$ and $\bar{x}_2$). The 't-value' was found to be $2{\cdot}15$ at 6 degrees of freedom (F). The probability (P) was therefore $0{\cdot}10 > P > 0{\cdot}05$. This showed the difference in the mean K_m values of the two sources of the enzyme to be at the border of significance. But assuming the inhibition of the kidney enzyme to be pure non-competitive the apparent K_m values obtained for each concentration of the inhibitor (i.e. 5 mM and 20 mM phosphate) would also be the true K_m values. Therefore, when all the seven values of K_m for kidney enzyme as given in

TABLE XIV

Showing the data for the experiments done to study the phosphate inhibition of the muscle glutamine synthetase activity. $[S_a]$ is the actual ATP concentration $[S + S_o]$ as μmoles/ml., and V (containing no added phosphate), VP_5 (containing 5 mM added phosphate) and VP_{20} (containing 20 mM added phosphate) show the corresponding values of the glutamine synthetase activity expressed as units/g. wet tissue.

[The saline injected control group experiments are marked (S)]

Expt. No.	Enzyme Activity	$[S_a]$ 0·03	0·13	0·23	0·28	0·53	1·03	2·03	5·03
1	V	3·26	8·97	10·78	-	12·30	13·75	-	-
	VP_5	-	6·52	9·26	-	10·78	11·58	13·38	-
	VP_{20}	-	-	5·93	6·58	8·61	11·14	13·53	-
2	V	1·45	4·41	6·22	6·95	8·25	8·47	10·56	-
	VP_5	0·94	2·97	-	4·27	-	7·66	-	9·19
	VP_{20}	0·80	-	3·47	3·62	5·57	6·30	-	-
3	V	3·18	8·25	-	-	11·30	12·23	12·73	-
	VP_5	2·24	6·30	-	-	9·84	10·56	12·15	-
	VP_{20}	-	-	-	-	8·46	8·90	9·77	13·89
4 (S)	V	0·72	3·47	4·56	-	6·73	7·45	7·90	-
	VP_5	0·65	2·24	4·05	-	5·79	7·45	7·52	-
	VP_{20}	0·07	-	3·26	-	5·21	6·30	7·67	-
5 (S)	V	1·29	5·28	6·66	-	10·27	11·87	12·52	-
	VP_5	0·94	3·91	5·57	-	8·75	10·72	11·29	-

TABLE XV

Showing the data for the experiments done to study the phosphate inhibition of the kidney glutamine synthetase activity.

For the description of $[S_a]$, V, VP_5 and VP_{20} see the previous table (Table XIV).

Expt. No.	Enzyme Activity	$[S_a]$							
		0·01	0·11	0·21	0·26	0·51	1·01	2·01	5·01
1	V	2·02	13·60	21·70	25·46	33·28	41·96	51·22	52·96
	VP_5	-	12·42	18·24	18·82	25·46	33·00	39·64	40·00
	VP_{20}	-	-	13·90	14·48	21·42	27·80	26·04	32·12
2 (S)	V	-	21·42	33·28	-	49·78	59·32	70·36	-
	VP_{20}	-	14·76	22·86	-	30·68	35·88	44·28	-
3 (S)	V	-	17·94	28·66	-	40·80	50·06	53·54	-
	VP_{20}	-	-	18·24	-	25·76	31·54	35·98	38·48

TABLE XVI

Showing the value of K_m obtained by computing the data given in Table XIV, as described in the text, for the experiments done using the skeletal muscle enzyme, and the values of K_i calculated from it with each concentration of the inhibitor (Pi). The standard deviations for each constant are also given.

Expt. No.	K_m (x 10^{-4} M)	K_i (x 10^{-3} M)	
		5 mM Phosphate	20 mM Phosphate
1	0·93	11·56	5·78
2	1·82	6·32	23·75
3	0·86	8·78	4·46
4 (S)	2·11	10·14	17·96
5 (S)	2·37	16·93	-
Average	1·62 ± 0·69	10·75 ± 3·96	12·99 ± 9·40

TABLE XVII

Showing the value of K_m obtained by computing the data given in Table XV, as described in the text, from the experiments done with the kidney enzyme, and the value of K_i calculated from it for each concentration of the inhibitor (Pi).

Standard deviation for each constant are also given.

Expt. No.	No Phosphate	5 mM Phosphate		20 mM Phosphate	
	K_m (x 10^{-4} M)	K_m (x 10^{-4} M)	K_i (x 10^{-3} M)	K_m (x 10^{-4} M)	K_i (x 10^{-3} M)
1	3·50	3·15	25·91	2·94	42·92
2 (S)	3·01	-	-	2·54	49·62
3 (S)	2·47	-	-	2·72	30·17
Average	2·99 ± 0·51	3·15	25·91	2·73 ± 0·20	40·90 ± 9·88

Table XVII are taken into consideration, and the significance of the difference of their mean K_m with that of the mean value of K_m for muscle enzyme is calculated as above, a 't-value' of 2·46 with 11° of freedom (F) is obtained. The probability read from the tables is found to be $P < 0{\cdot}05$, which is significant. This suggests the enzyme from the two sources i.e. muscle and kidney to be non-identical.

The mechanism proposed by Meister (1962, 1965, 1968) gives a rate equation, which, when simplified by assuming absence of saturation with glutamate and hydroxylamine, and absence of all products other than inorganic phosphate (Pi), predicts uncompetitive inhibition. To obtain a mechanism predicting non-competitive inhibition, in the case of the phosphate inhibition of kidney enzyme, a dead-end EI (enzyme-phosphate) complex was added to the mechanism.

A rate equation of the form

$$\frac{1}{V} = \left(\frac{a}{K'E_o} + \frac{c[I]}{K'E_o}\right) \frac{1}{A} + \left(\frac{b}{K'E_o} + \frac{d[I]}{K'E_o}\right)$$

can be derived from this mechanism in a similar way as explained on p. 129-130. In this equation $[A]$ and $[I]$ are the concentrations of ATP and inhibitor (phosphate) respectively and E_o is the concentration of the enzyme at the beginning of the reaction; K', a, b, c and d are complex constants compounded from the rate constants of individual reactions from specific stages in the mechanism.

The values of K_i for the kidney enzyme (see Table XVII) when calculated using this equation simplified as

$$\frac{\text{slope (inhibited)}}{\text{slope (control)}} = \left(1 + \frac{[I]}{K_i}\right),$$

was not found to be very consistent for 5 mM and 20 mM phosphate. Nevertheless, the large apparent value of K_i for the kidney enzyme compared with that for the muscle enzyme, especially at 20 mM phosphate, agrees with slighter inhibition of the kidney enzyme found using the hydroxamate method (see Chapter III).

Interestingly enough the ATP-ase in the two sources of enzyme

was also different. A K_m value of $2 \cdot 7 \times 10^{-3}$ M for muscle and 5×10^{-3} M for kidney ATP-ase was also found.

On average, a V_{max} value of 11·95 for muscle and 66·01 for kidney was found in these experiments. These values when multiplied by an appropriate factor (i.e. $\frac{100}{60}$ for muscle and $\frac{100}{70}$ for kidney), as discussed earlier in this Chapter (see section B), to calculate activity in the tissue supernatants not treated with $(NH_4)_2SO_4$, give an enzyme activity of 19·9 in muscle and 94·3 units in kidney per g. wet tissue. These values when compared with those found and given in Chapter III (see Table VI) i.e. 19·4 in muscle and 183·2 in kidney by the hydroxamate method show that only about half of the kidney activity unlike the muscle activity, is found with the NADH oxidation method when using NH_2OH. The significance of these results have already been discussed earlier in this Chapter (see section A).

(E) CHECK OF HEART EXTRACTS FOR GLUTAMINE SYNTHETASE ACTIVITY BY THE NADH OXIDATION METHOD

As mentioned earlier in Chapter III, enzyme activity though found in some heart supernatants by the hydroxamate method could not be measured in such extracts by the NADH oxidation method. Ammonium sulphate-treated extracts were prepared from cardiac muscle using the conditions employed for skeletal muscle. The enzyme activity was determined in both fractions, i.e. the precipitate and the supernatant fraction. For each extract the

tissue was obtained from 6-8 animals. In four such extracts, no enzyme activity could be measured by the NADH oxidation method, using extract equivalent to 500 mg. of wet tissue per assay.

Failure to measure any enzyme activity in the ammonium sulphate treated extracts might only mean that the enzyme from heart (or because there was only a low concentration of it anyway) is particularly sensitive to $(NH_4)_2SO_4$, or perhaps is more sensitive when present in very low concentration. Nevertheless, the enzyme activity in heart, as already discussed in Chapter III, was measured in some of the extracts by the hydroxamate method, suggesting that the activity in this organ is present in very low levels in rat.

C H A P T E R V

EFFECT OF GROWTH HORMONE ON GLUTAMINE SYNTHETASE

EFFECT OF GROWTH HORMONE ON GLUTAMINE SYNTHETASE

As already discussed in detail in Chapter I, growth hormone may play an important role in the metabolism of glutamine in animal tissues. The effect of growth hormone on glutamine synthetase activity was therefore studied as part of the present investigation, by investigating the effect of bovine growth hormone (BGH) both *in vivo*, to see whether there was any increase in activity per unit mass of tissue, and *in vitro* on the rate of the enzyme reaction. Both muscle and kidney extracts were used. In order to see whether the hormone has any effect on the binding sites of the enzyme attacked by phosphate, the study of the effect of growth hormone was also extended to the phosphate inhibition of the enzyme. A search for enzyme activity was also made in the cardiac muscle of growth hormone-treated rats.

A group of female albino rats, each weighing about 225 g. was taken; the weight of each rat was recorded daily at about the same time (11 a.m. to 12 noon) for about a week and then half of them were injected subcutaneously with 1 mg. of purified bovine growth hormone (dissolved in 0·4 ml. saline) per rat per day for 5-7 days while the other half were injected similarly with 0·9% saline and kept as a control group. Both groups of rats had free access to food and water and were kept in the same place side by side. The process of weighing was started about a week before giving injections, so as to minimize any reaction of these animals to handling. The growth hormone treated rats were found

to grow on average by 2-3 g. per rat per day while the corresponding growth rate in the control group was less than 0·5 g. (see Fig. 24).

Each animal was killed about 2-3 hours after the final injection; its heart, both kidneys and about 2·5 g. of its leg muscle were removed very quickly one after another and the partially purified (ammonium sulphate treated) extract of each tissue was prepared as described in the previous chapter. A set of three rats was employed for each experiment so as to get enough tissue extract.

The data, after making the necessary corrections (as described in the previous chapter) both for the unknown concentration of the substrate $[S_o]$ at zero ATP addition and the change in volume on addition of glutamate in the assay system, are shown in Tables XVIII (for skeletal muscle) and XIX (for kidney) for the extracts prepared from the growth hormone treated rats, while the corresponding data for the control group are given in the previous chapter in Tables XIV (for skeletal muscle) and XV (for kidney). The values of K_m computed and the calculated K_i, as described in the previous chapter are shown in Tables XVI (for skeletal muscle) and XVII (for kidney) for the control group of rats, and those of the growth hormone treated are given in Tables XX (for skeletal muscle) and XXI (for kidney). On average, a V_{max} value of 13·17 for muscle and 62·99 for kidney enzyme was found.

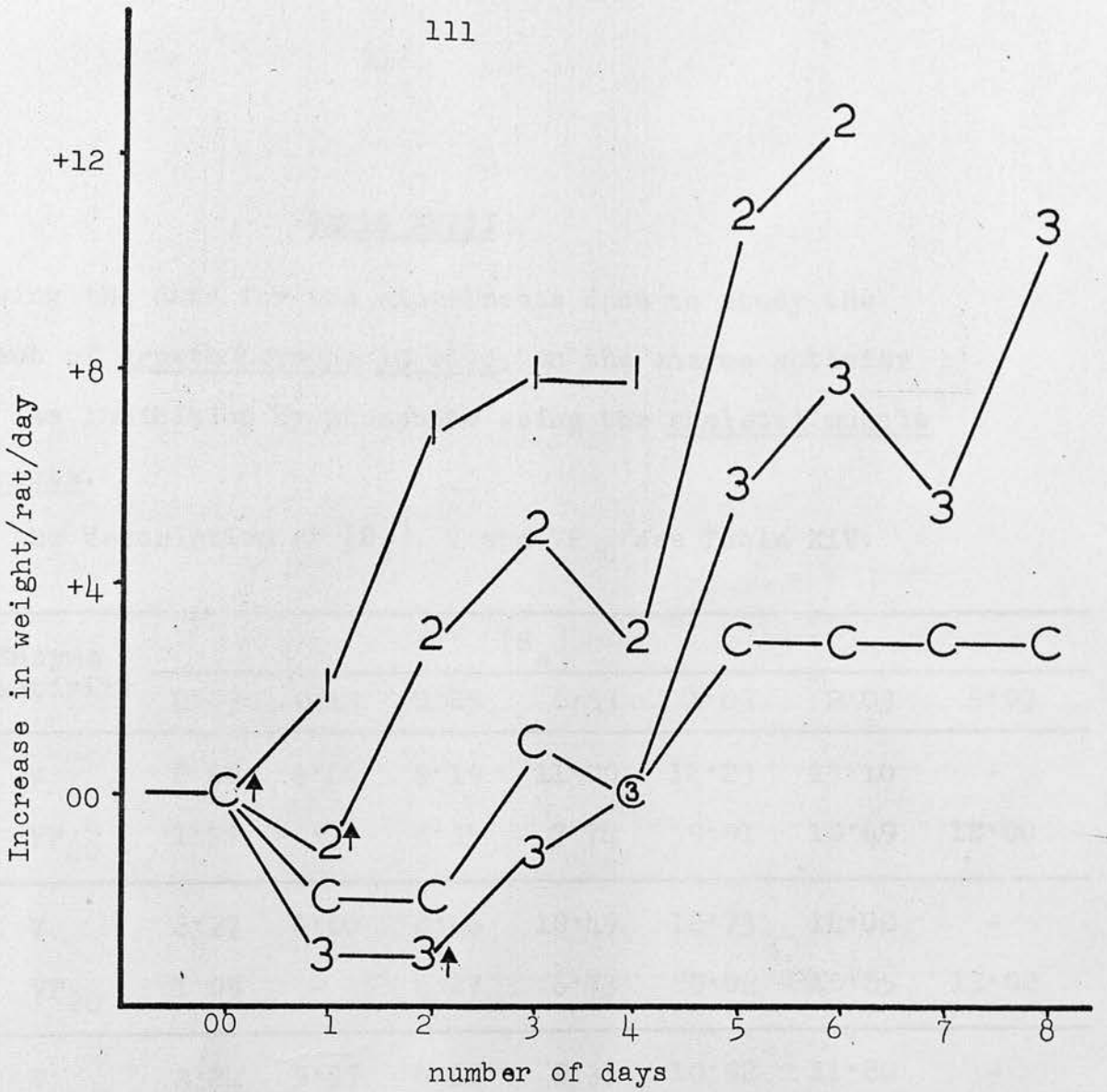


Fig. 24.

Effect of growth hormone on the growth rate of rats.

C represents control group rats

1, 2 and 3 represents three growth hormone injected groups rats

↑ indicates the start of growth hormone injections

TABLE XVIII

Showing the data for the experiments done to study the effect of growth hormone in vivo, on the enzyme activity and its inhibition by phosphate using the skeletal muscle extracts.

For the description of $[S_a]$, V and VP_{20} see Table XIV.

Expt. No.	Enzyme activity	$[S_a]$						
		0·03	0·13	0·23	0·53	1·03	2·03	5·03
1	V	2·68	6·66	9·19	11·00	12·23	13·10	-
	VP_{20}	1·59	-	5·35	7·74	9·91	10·49	12·60
2	V	2·17	6·00	8·46	10·49	12·73	14·00	-
	VP_{20}	1·09	-	4·27	6·73	9·04	10·85	13·00
3	V	2·24	5·57	6·58	9·33	10·92	11·80	-

TABLE XIX

Showing the data for the experiments done to study the effect of growth hormone in vivo, on the kidney enzyme activity and its inhibition by phosphate.

For the description of $[S_a]$, V and VP_{20} see Table XIV.

Expt. No.	Enzyme activity	$[S_a]$						
		0·01	0·11	0·21	0·51	1·01	2·01	5·01
1	V	-	14·18	20·54	35·88	43·40	51·22	-
	VP_{20}	-	-	15·62	23·44	27·58	31·84	33·58
2	V	0·28	15·20	24·16	39·28	44·18	57·12	-
	VP_{20}	0·14	_	15·48	25·62	31·02	34·44	38·20

TABLE XX

Showing the value of K_m obtained by computing the data given in Table XVIII, as described in the text, for the experiments done using the skeletal muscle enzyme of the growth hormone injected rats, and the values of K_i calculated from it for 20 mM phosphate (Pi).

Expt. No.	K_m (x 10^{-4} M)	K_i (x 10^{-3} M) at 20 mM Phosphate
1	1·30	12·62
2	1·96	10·29
3	1·84	-

TABLE XXI

Showing the value of K_m obtained by computing the data given in Table XIX, as described in the text, for the experiments done using the kidney enzyme of the growth hormone injected rats, and the value of K_i calculated from it for 20 mM phosphate (Pi).

Expt. No.	No Phosphate	20 mM Phosphate	
	K_m (x 10^{-4} M)	K_m (x 10^{-4} M)	K_i (x 10^{-3} M)
1	3·79	2·68	95·1
2	3·69	3·22	49·75

Growth hormone was found to have no significant effect on the enzyme activity of either muscle or kidney extract, and no enzyme activity could be detected in the growth hormone treated cardiac muscle extracts. The type of phosphate inhibition of the enzyme was also not affected by growth hormone treatment. This is shown in Figs. 25 and 26 for growth hormone-treated muscle and kidney enzymes respectively.

The rate of the enzyme reaction was also observed to be unaffected by in vitro addition of growth hormone in all the tissues studied; in vitro growth hormone studies were carried out by adding 10 µg. of BGH to the assay system directly in a cuvette. These findings of the effect of growth hormone differ from those of Wu (1964a). They suggest that an increase in the rate of protein synthesis may not in itself cause an increase in the glutamine synthetase activity at least of muscle and kidney - a different behaviour of these enzyme sources from that found in liver but similar to brain (Wu, 1960, and Bauer Wu, 1964a, 1964b). It might also suggest that the method of causing a change in the rate of the protein synthesis used by Wu (1964a) was important. It also suggests that the apparent mobilisation of glutamine into blood by growth hormone reported by Bartlett (1949) does not depend on prior synthesis or activation of glutamine synthetase. Wu (1964b) also found that the concentration of free glutamine in liver after regeneration remains almost the same as that of a normal liver, but glutamine synthetase does not increase with increase in

Fig. 25

Effect of growth hormone on the phosphate inhibition of the muscle glutamine synthetase activity.

(a) Plots of the activity at various concentrations of the substrate.

(b) The Lineweaver-Burk plots of (a).

●—● shows the activity of the control, i.e. the one without any added phosphate.

▲—▲ shows the activity in the presence of 20 mM phosphate.

Fig. 25(a)

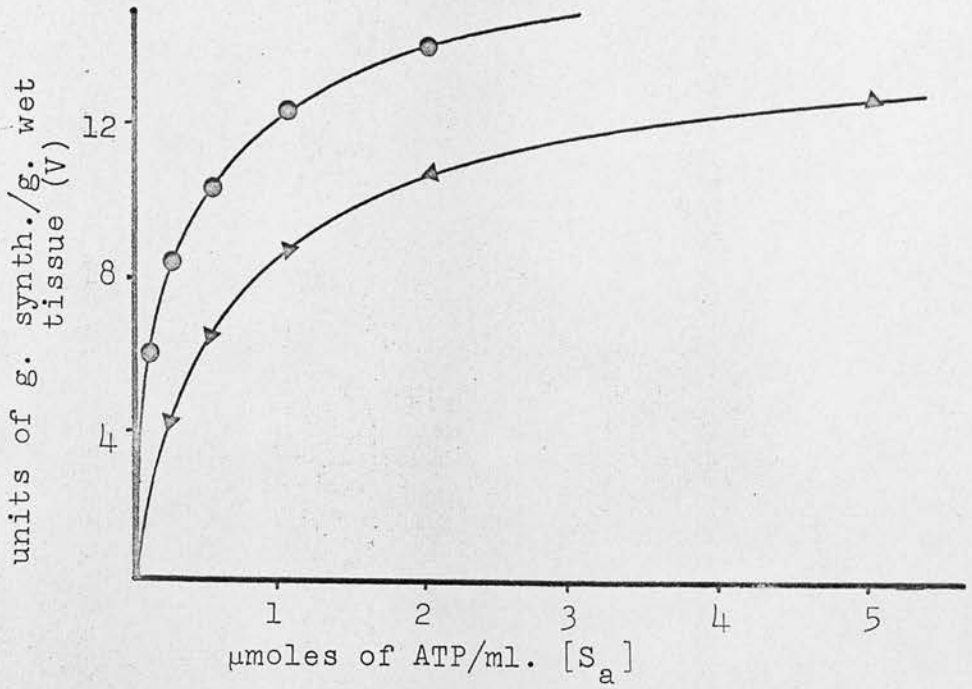


Fig. 25(b)

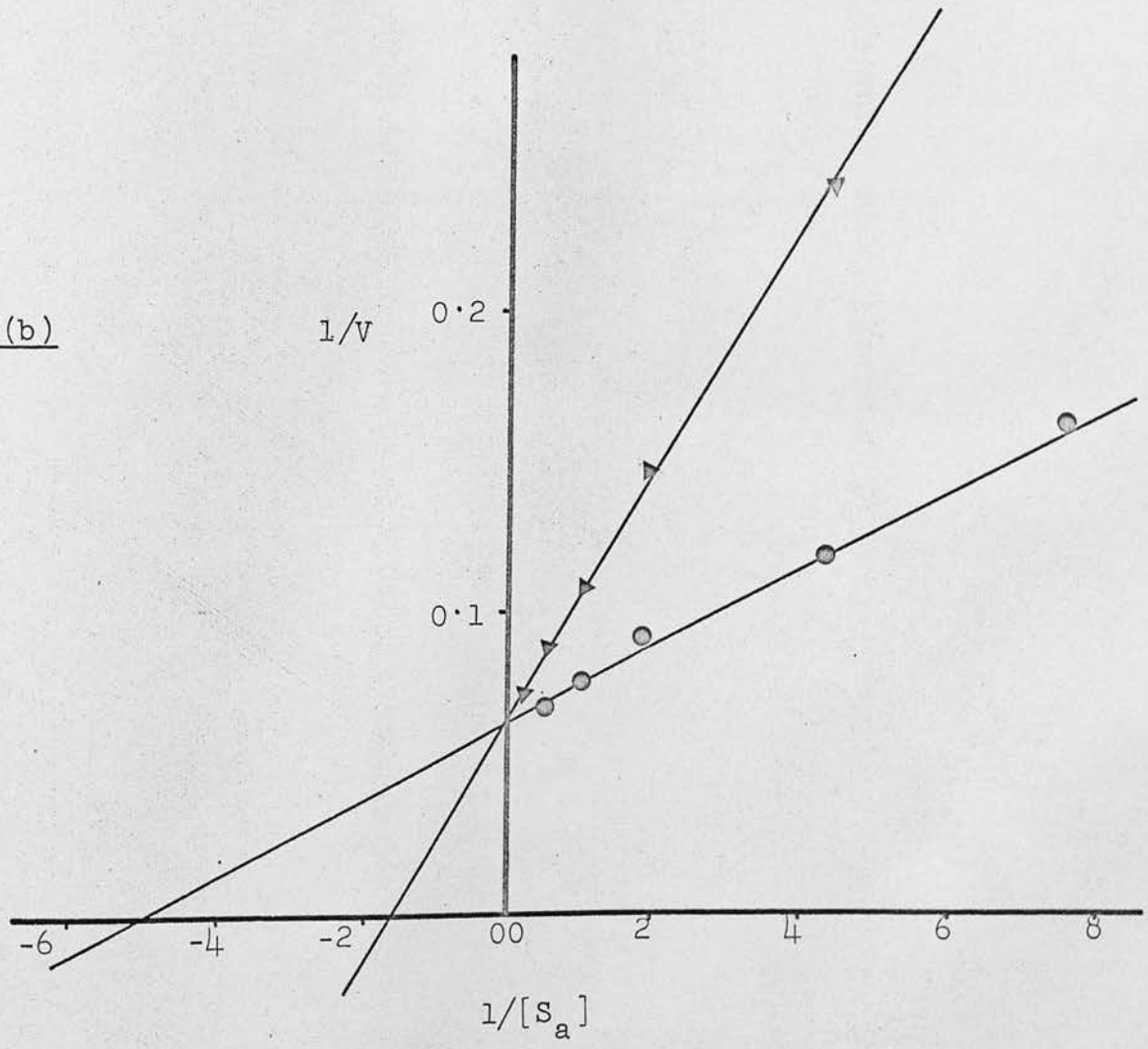


Fig. 26

Effect of growth hormone on phosphate inhibition of the kidney glutamine synthetase activity.

(a) Plots of the activity at various concentrations of the substrate.

(b) The Lineweaver-Burk plots of (a).

●—● shows the activity of the control, i.e. the one without any added phosphate.

▲—▲ shows the activity in the presence of 20 mM phosphate.

Fig. 26(a)

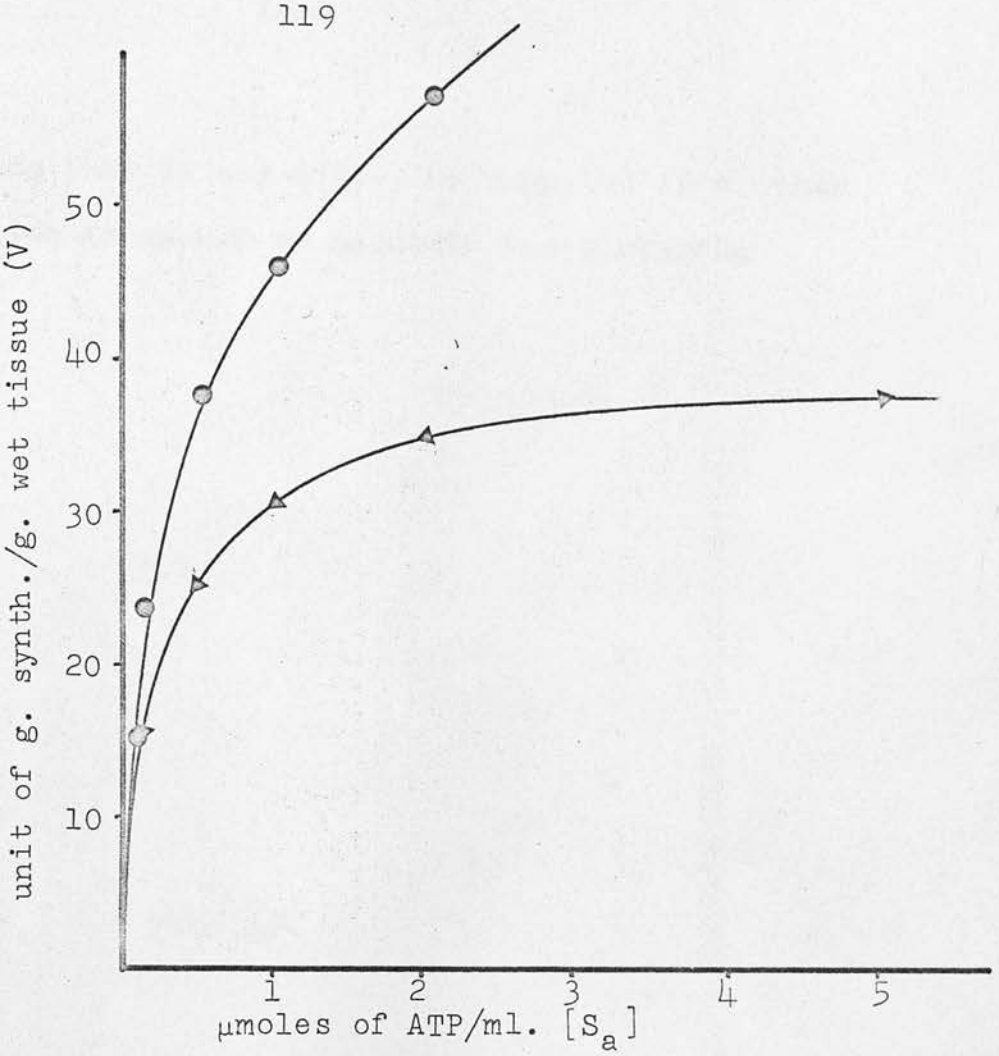
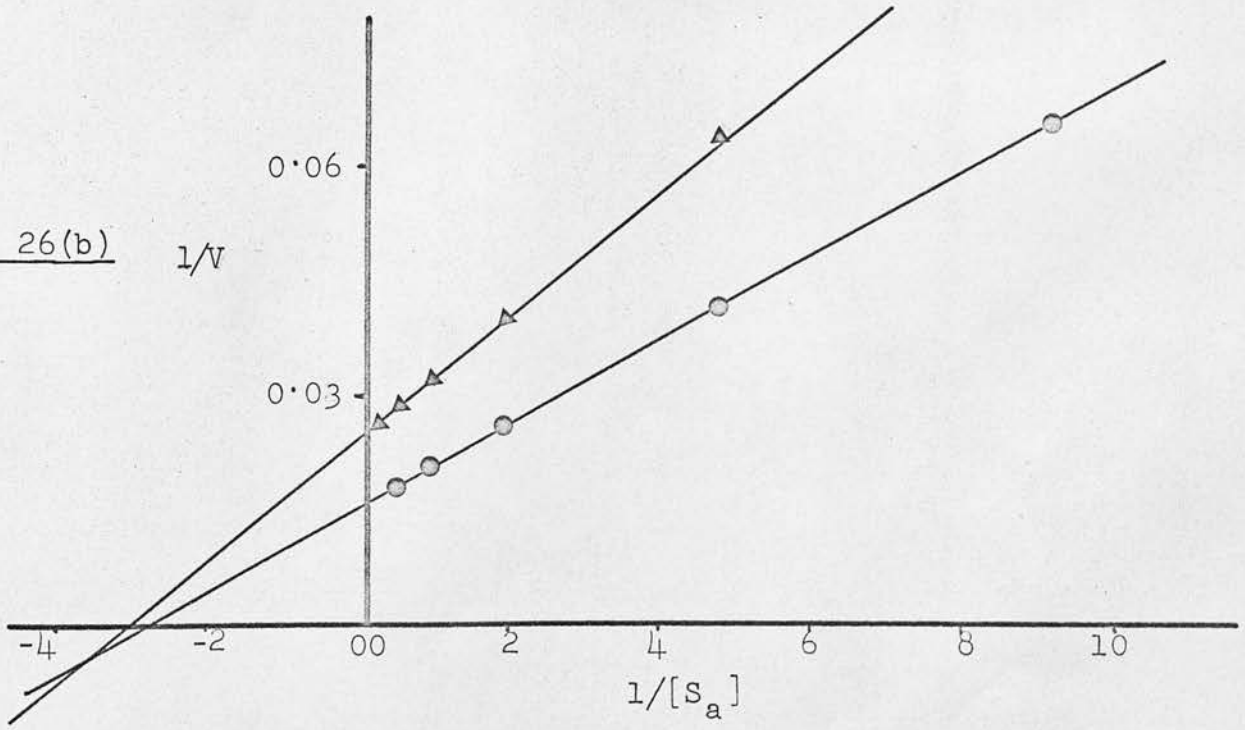


Fig. 26(b)



glutamine suggesting that it may either be supplied from other tissues or the enzyme is enough to maintain the glutamine concentration.

C H A P T E R VI

DISCUSSION

DISCUSSION

It is unsatisfactory to put forward results of the measurement of the activity of an enzyme without

(i) employing an appropriate tissue extract preparation. This must satisfy the criteria of linearity both with increase in incubation time and with the volume of the extract added, because these are properties by which an enzyme reaction differs from a nonenzymic reaction.

(ii) checking the validity of the analytical method or methods used. A good deal of time was spent in satisfying both of these requirements (see Chapter III), and comparison of my results with those of others is perhaps the best way of demonstrating the efficiency of the methods which I used.

Since kidney glutamine synthetase activity has been investigated by a number of workers (Reiner and Hudson, 1953; Baerle, et al., 1957; Richterich and Goldstein, 1958; Wu, 1963b; Wu, 1964a) and it was included in the present studies so that the results of the investigation of the enzyme activity in skeletal and cardiac muscle could be compared with it, it is best first to compare my results for kidney with those of others.

A mean value of 183·2 ± 65·1 units glutamine synthetase activity per g. of wet tissue was found in kidney (see Chapter III, section F) by the hydroxamate method using dialysed high-speed supernatants (see Chapter II, section 2A) and 92·6 ± 12·3 units per g. wet tissue when using the NADH oxidation method. The almost 50% lower activity found by the latter method may be

because the enzyme in the NADH oxidation method was extracted in KCl-$KHCO_3$ solution, while $NaCl$-$NaHCO_3$ solution was used in the hydroxamate method. This suggests that the kidney enzyme may be sensitive to K^+ ions (the activity found in the muscle extracts was the same with both the methods). Alternatively, some reagent or reagents of the NADH oxidation method inhibited the kidney enzyme. Both reasons may of course be true. It is interesting to note here that Reiner and Hudson (1953) found 133 units of enzyme activity/g. wet kidney by the hydroxamate method using high-speed supernatants of homogenates extracted from the tissue with KCl-$KHCO_3$. This value is about 70% of that which I found, using the same method, but extracting with sodium salts. This discrepancy may possibly be due to their use of KCl-$KHCO_3$ for extracting the enzyme. If so, this would explain why the activity of kidney extracts appeared to be about 50% lower when using the NADH oxidation method. This is supposing that the correction for loss in the ammonium sulphate precipitation step is always good (see Chapter IV, section B). Another reason for the difference between the results of Reiner and Hudson (1953) and those reported here may, however, be that the high-speed supernatant used by these workers was centrifuged at 40,000 R.P.M. for sixty minutes, while I centrifuged at 30,000 R.P.M. for 45 minutes. By experiment, the supernatant from the latter was found to contain about 20% more activity than the former (see Chapter III, section C). Reiner and Hudson (1953) also reported that the activity was not linear with differing amounts of extract,

while the preparation used for these studies did satisfy this criterion of linearity (see Chapter III, section D).

Richterich and Goldstein (1958) found 30 ± 6 units glutamine synthetase activity as μM ammonia taken up per g. wet kidney using tissue homogenates, while Wu (1963b) reported 49·6 units of enzyme activity (as μmoles of GHA/hr.) per g. of wet kidney. The activity which can be measured in homogenates is only about 1/3rd to 1/4th of that of high-speed supernatants (Reiner and Hudson, 1953; see also Chapter III, section C). Despite this, the activity measured by Richterich and Goldstein (1958) was much lower than that found in these studies. This may be because these workers assayed the enzyme activity by measuring the disappearance of ammonia from the assay system. Wu's (1963b) results on the other hand, when multiplied by an appropriate factor (3 - 4 times) to make them comparable to a high-speed supernatant, give a value much the same as found in the present investigation. The differences between my results and those of previous workers may also be due to difference in the rats used. Nevertheless, this comparison suggests that both the enzyme preparation, i.e. the dialysed high-speed supernatant, and the assay method (i.e. the hydroxamate method) used in the present investigation were satisfactory.

In skeletal muscle, I found on average, 19·1 ± 10·3 units (see Chapter III, section D) and 20·8 ± 4·1 units (see Chapter IV, section A) glutamine synthetase per g. of wet tissue by the hydroxamate method and the NADH oxidation method respectively. These values are more than four times as great as that reported by

Trush (1963) (4·6 units/g. wet tissue using the hydroxamate method). This difference was probably because he measured the activity in tissue homogenates while I used dialysed supernatants, which I found to be more active than the homogenates (see Chapter III, section C). Wu (1963b) failed to find any activity both in skeletal and cardiac muscle extracts. This is undoubtedly because he employed tissue homogenates which are less active than the supernatants. In addition to this the minimum activity he could measure by the method he used was 10 units/g. wet tissue.

The actual rate of glutamine synthesis in muscles in vivo will be only a fraction of the maximum activity (about 20 units/g. wet tissue) using substrates in saturating concentrations. This is because while the concentration of ATP (ca 5 mM) (see White, Handler and Smith, 1964) is almost saturating, and that of glutamate (2·34 μmoles/g. wet tissue, Herbert, Coulson and Hernandez, 1966) is not inconsiderable, only a very small quantity of ammonia is available and it is this which limits the rate of glutamine synthesis in this tissue. The ammonia mainly comes from the hydrolysis of adenylic acid by adenylic acid deaminase (see White, Handler and Smith, 1964), and from the hydrolysis of glutamine by glutaminase (Ottaway, 1969). Some ammonia also came from the oxidation of glutamic acid by glutamate dehydrogenase (Pette, Klengenberg and Bucher, 1962; Wergedal and Harper, 1964). No precise figures exist for the actual concentration of ammonia in muscle, and without them it is very hard to say anything about actual rate of glutamine synthesis in

this tissue and to suggest whether muscle gets a part of its glutamine through the blood from the liver, or whether some of the glutamine synthesised in muscle is supplied to other tissues by way of the blood. Nevertheless, these results do show that glutamine can be synthesised in skeletal muscle.

With regard to activity in cardiac muscle, Doell and Felts (1959) found that glutamine is formed from labelled glutamic acid in isolated perfused rabbit heart. Trush (1963) later reported on average an enzyme activity of 4·4, 5·6 and 5·4 units/g. wet tissue in rat, rabbit and cat heart respectively by the hydroxamate method using tissue homogenates. However, I found only about half of this activity, i.e. 2·5 $\pm$ 0·8 units/g. wet heart by the hydroxamate method (see Chapter III, section F), when using dialysed high-speed supernatants although I would have expected to find 3 to 4 times as much activity as in homogenates. In 3 out of 12 cardiac muscle extracts I failed to detect any activity (see Chapter III, section F). In addition to this, I could not detect any activity at all, using the NADH oxidation method, either in ordinary dialysed supernatants or even in ammonium sulphate-treated extracts (see Chapter IV, section E). It may be, of course, that the enzyme was sensitive to extraction by KCl-$KHCO_3$ solution, as has been suggested for the kidney enzyme; or that the enzyme was inhibited by treatment with ammonium sulphate. However, none of these possibilities seems to apply with equal force to all three sets of experiments. I am forced to conclude that in the rats that I used, the glutamine synthetase activity in heart muscle was at the limit of detectability.

The results reported at least confirm that glutamine is synthesised in skeletal muscle as well as in brain, liver and kidney. Some glutamine may be synthesised by heart muscle in the rat, but this organ must be largely dependent for its glutamine on the liver (Trush, 1963; Wu, 1964b) via the blood stream. This probably also applies to mammals other than the rat. In the cardiac muscle of birds, on the other hand, the activity is known to be quite high. Gothoskar, Raina and Ramakrishnan (1960) found 88·9 units/g. wet chicken-heart by the hydroxamate method. Trush (1963) reported 47·2 units/g. wet tissue for pigeon heart, while Wu (1963b) found 27·6, 34·4, 63·3 and 45·6 units of enzyme/g. wet tissue in chicken, pigeon, duck and goose heart respectively. Gothoskar, Raina, Tate and Ramakrishnan (1960) have pointed out that chick heart cells in tissue culture do not require glutamine as a growth factor, in contradistinction to rat heart cells. In the light of this and the results of this investigation, one may suppose that the mammalian heart cells are unable to synthesise enough glutamine to meet their requirements for cellular nucleic acid synthesis for instance, and are therefore dependent for their supply of glutamine on other tissues.

SUITABILITY OF THE NADH OXIDATION METHOD TO STUDY THE PHOSPHATE INHIBITION OF GLUTAMINE SYNTHETASE

Since the enzyme activity in muscle extracts was found to be the same by both the hydroxamate method and the NADH oxidation

method, while the corresponding values for kidney extracts were, as already discussed earlier in this Chapter, found by the latter method to be only about half of that measured by the former method, it seems to be not only unnecessary but disadvantageous to employ the NADH oxidation method simply to measure the maximum activity of tissue extracts. But for the purposes of studying phosphate inhibition of the enzyme, the NADH oxidation method, as discussed in Chapter III, section G and Chapter IV, section A, does have some advantages. In the first place, ADP does not accumulate. Secondly the NADH oxidation method is several times more sensitive than the hydroxamate method and the initial rate of the reaction can be conveniently studied. As the sensitivity of the hydroxamate method is low, a considerable time must elapse before an accurately measurable amount of glutamylhydroxamate is produced, and in this interval, since the sources of glutamine synthetase always contained ATP-ase, ATP was continuously disappearing and inorganic phosphate (and ADP) appearing. Consequently the concentration at the end of the incubation would necessarily be notably different from that added at the beginning. This would make the estimation of the inhibitor constant, for example by Lineweaver-Burk plots, impossible.

MECHANISM OF ACTION OF THE ENZYME AND COMPARISON OF MUSCLE GLUTAMINE SYNTHETASE WITH THAT OF THE KIDNEY ENZYME

Both inorganic phosphate and sulphate were found to inhibit both muscle and kidney glutamine synthetase (see Chapter IV). These results agree with those of Gothoskar, Raina and Ramakrishnan

(1960) reported for chicken-heart glutamine synthetase. On the other hand, Pamiljans, Krishnaswamy and Meister (as reported by Meister, 1962) failed to find phosphate inhibition of the glutamine synthetase of mammalian tissues. The mechanism of action of the enzyme proposed by Krishnaswamy, Pamiljans and Meister (1962), Meister, Krishnaswamy and Pamiljans (1962), and Meister (1962, 1965, 1968) which is discussed in Chapter I (see Fig. 1 on p.13-14) is based on the assumption that inorganic phosphate does not inhibit the enzyme. This is not correct in the light of my own findings. As the phosphate inhibition of the muscle enzyme was found to be competitive (see Chapter IV), a mechanism which will predict competitive inhibition must exist. The mechanism proposed by Meister and his associates (see Fig. 1 on p. 13-14) gives a rate equation which predicts in the absence of saturating concentrations of substrates uncompetitive inhibition. On the other hand, when glutamate and hydroxylamine (or NH_3) are used in saturating concentrations, the reaction from step II to step VI (see Fig. 1) is practically irreversible, and consequently no inhibition by phosphate would be observed. This is shown by a rate equation which can be derived by the method of King and Altman (1956) from the mechanism shown in Fig. I (see Chapter I, page 13-14). The rate equation obtained is:

$$V = \frac{[E_o]K_1K_3K_5K_7K_9[A][G][N]}{K_3K_5K_7K_9[G][N] + K_1K_3K_5K_9[G][N][A] + K_1K_3K_5K_7[G][N][A] + K_2K_5K_7K_9[N] + K_1K_5K_7K_9[A] + K_1K_3K_7K_9[G][A]}$$

(Where [A], [G] and [N] are the concentrations of ATP, glutamate

and hydroxylamine (or ammonia) respectively; $[E_o]$ is the amount of enzyme at the beginning of the reaction; K_1 and K_2 are the rate constants for forward and backward reactions from step I to step II (Fig. 1) respectively; K_3, K_5, K_7 and K_9 are the constants for the forward reactions from step II—III—IV—(or V)—VI—I respectively).

Few studies have been made of the complex kinetics of three-substrate enzymes. It is well known, however, from experimental observations that when one of the substrates of a two-substrate enzyme is used at saturating concentration, the kinetics with respect to the second substrate are much simplified. Reiner (1964) has made theoretical use of this and as a first approach, it has seemed reasonable to extend the idea to glutamine synthetase which is a three-substrate enzyme. Thus all terms not containing $[G][N]$ i.e., saturating substrates are ignored and the equation reduces to

$$V = \frac{E_o K_1 K_3 K_5 K_7 K_9 [A][G][N]}{K_3 K_5 K_7 K_9 [G][N] + K_1 K_3 K_5 K_9 [G][N][A] + K_1 K_3 K_5 K_7 [G][N][A]}$$

taking $K_1 K_7 K_9 = K'$, $K_7 K_9 = a$, and $(K_1 K_9 + K_1 K_7) = b$, the above equation can be simplified to

$$V = \frac{E_o K'[A]}{a + b[A]}$$

which can be reduced to one of the type (see Cleland, 1963)

$$\frac{1}{V} = \left(\frac{a}{K'E_o}\right) \frac{1}{[A]} + \frac{b}{K'E_o} \quad \ldots\ (i)$$

(All other rate equations were similarly worked out; see pages 105, 131, 134).

where [A] stands for ATP concentration, and it is clear that there is no term corresponding to inhibition by phosphate.

The addition of a dead-end EP (enzyme-phosphate) complex to this scheme gives a mechanism which predicts competitive inhibition with respect to ATP when the other substrates are saturating (see Fig. 27). This scheme gives a rate equation of the form

$$\frac{1}{V} = \frac{1}{[A]}\left(\frac{c}{K'E_o} + \frac{d[P]}{K'E_o}\right) + \left(\frac{x+y}{K'E_o}\right) \quad \ldots\ (ii)$$

according to which any change in the phosphate concentration will change the slope of the Lineweaver-Burk plot but the y intercept will remain the same. These are the criteria of competitive inhibition. This scheme also takes account of the competitive inhibition of ATP by ADP found by Gothoskar, Raina and Ramakrishnan (1960) in cardiac muscle enzyme. This latter evidence, which is supported by a number of workers (see Chapter I), and

the related evidence that in the reverse reaction the divalent cation and the nucleotide bind first, rules out a mechanism in which the positions of ADP and phosphate are reversed, giving a dead-end enzyme-ADP complex. The form of the rate equation obtained from this mechanism is the same as (ii), i.e. both these mechanisms are theoretically possible.

As regards the kidney enzyme, for which phosphate was a noncompetitive inhibitor, the mechanism proposed for the muscle enzyme (Fig. 27) cannot be true. The possibility that phosphate may also form another dead-end complex, i.e. enzyme-ATP-phosphate, in addition to the EP complex postulated in the mechanism shown in Fig. 27, cannot be true because the reaction from step II to step VI is practically irreversible when using saturating concentrations of glutamate and hydroxylamine in which situation almost all the enzyme is as EAG complex, and hardly any EAP complex is formed. The rate equation given by such a scheme would therefore be of the same type as described for the muscle enzyme, i.e. competitive at saturating substrate concentrations.

A possible mechanism of the kidney enzyme which also accounts for the competitive inhibition of ATP by ADP in the kidney enzyme as reported by Baerle, et al. (1957), is that shown in Fig. 28(a). This gives a rate equation of the form:

$$\frac{1}{V} = \left(\frac{B + C[P]}{K' E_o}\right) \cdot \frac{1}{[A]} + \left(\frac{D + F[P]}{K' E_o}\right) \quad \ldots\ldots \text{(iii)}$$

According to this equation, any change in phosphate concentration will, in the Lineweaver-Burk plots, change both the slope and the

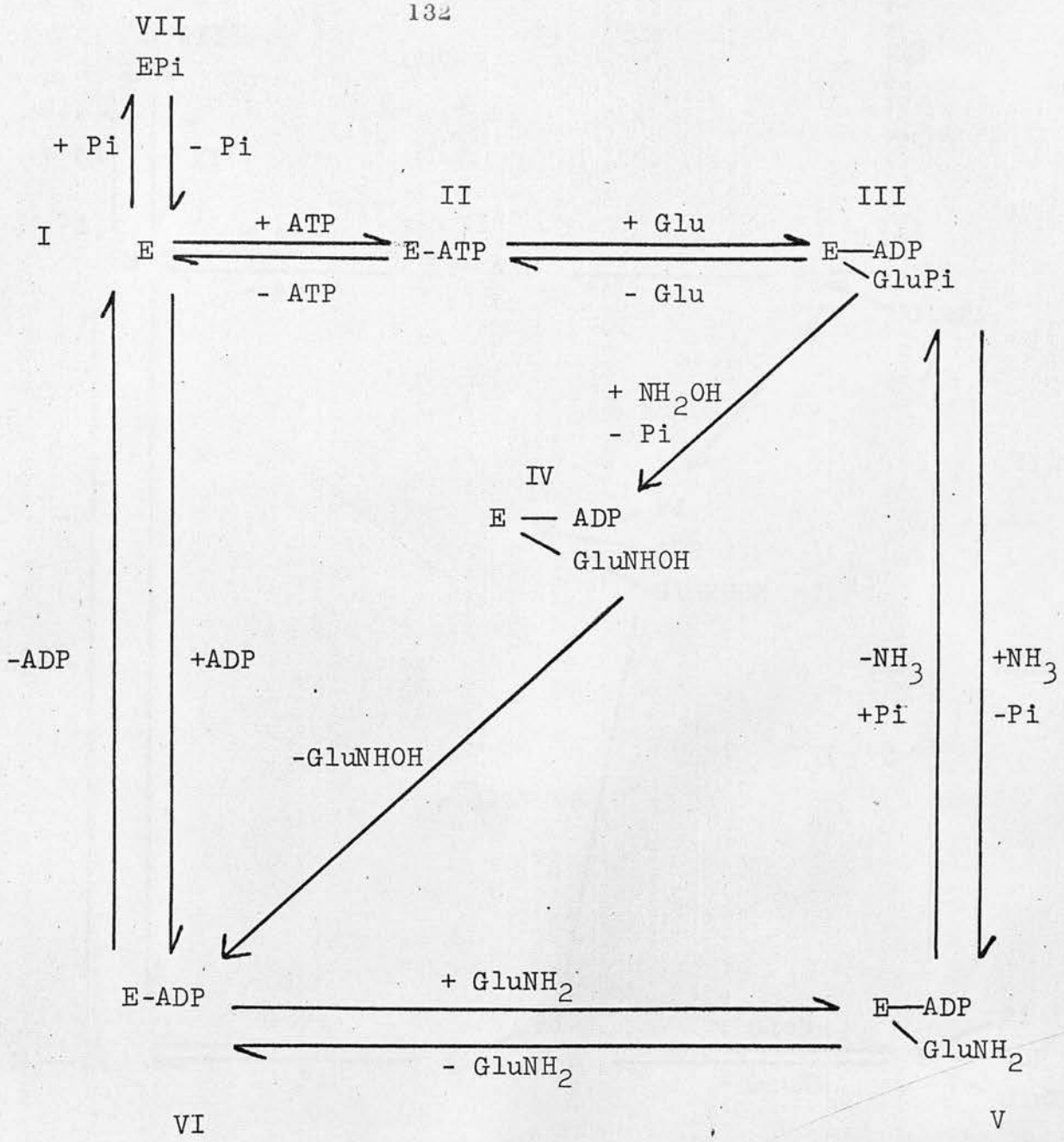


Fig. 27

Mechanism of action of muscle glutamine synthetase.

Glutamine synthesis: I $\xrightarrow{VII}$ II $\longrightarrow$ III $\longrightarrow$ V $\longrightarrow$ VI $\longrightarrow$ I

Hydroxamate formation: I $\xrightarrow{VII}$ II $\longrightarrow$ III $\longrightarrow$ IV $\longrightarrow$ VI $\longrightarrow$ I

For E, Glu, Pi, GluPi, $GluNH_2$, GluNHOH see Fig. I on page 13-14.

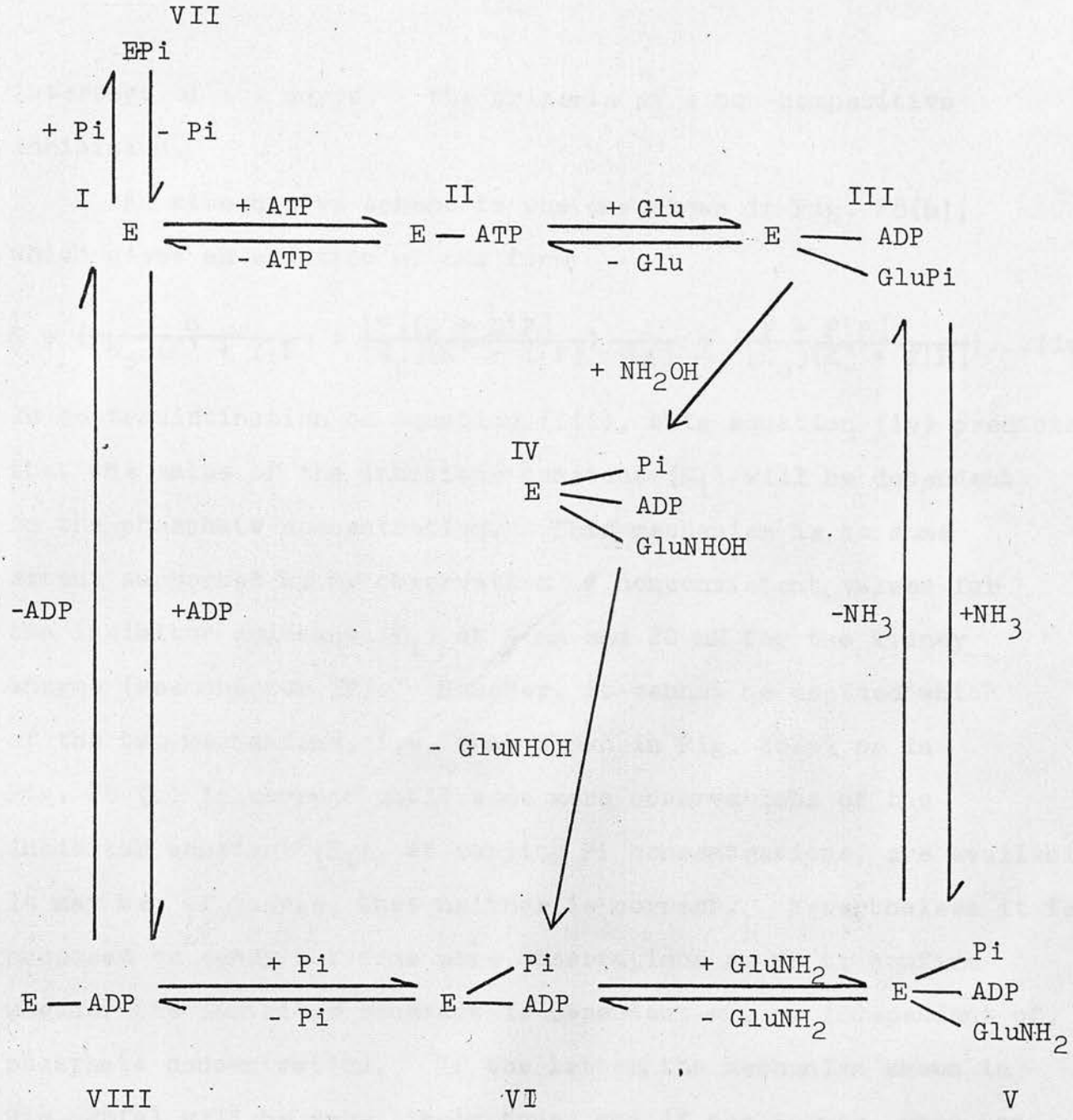


Fig. 28(a)

Mechanism of action of kidney glutamine synthetase.

Glutamine synthesis: $I \rightarrow II \rightarrow III \rightarrow V \rightarrow VI \rightarrow VIII \rightarrow I$ (VII)

Hydroxamate formation: $I \rightarrow II \rightarrow III \rightarrow IV \rightarrow VI \rightarrow VIII \rightarrow I$ (VII)

For E, Glu, GluPi, GluNHOH, $GluNH_2$ and Pi, see Fig. I, page 13-14.

intercept of the curve -- the criteria of a non-competitive inhibition.

An alternative scheme is the one shown in Fig. 28(b), which gives an equation of the form

$$\frac{1}{V} = \left(\frac{B}{[E_o](K' + Y[P]}\right) + \left(\frac{[P](C + D[P]}{[E_o](K' + Y[P]}\right)\frac{1}{[A]} + \left(\frac{F + H[P]}{[E_o](K' + Y[P]}\right) \quad \ldots(iv)$$

In contradistinction to equation (iii), this equation (iv) predicts that the value of the inhibitor constant (K_i) will be dependent on the phosphate concentration. This mechanism is to some extent supported by my observation of nonconsistent values for the inhibitor constant (K_i) at 5 mM and 20 mM for the kidney enzyme (see Chapter IV). However, it cannot be decided which of the two mechanisms, i.e. that shown in Fig. 28(a) or in Fig. 28 (b) is correct until some more observations of the inhibitor constant (K_i), at varying Pi concentrations, are available. It may be, of course, that neither is correct. Nevertheless it is proposed to carry out some more observations so as to confirm whether the inhibitor constant is dependent on, or independent of, phosphate concentration. If the latter, the mechanism shown in Fig. 28(a) will be very likely true, and if the former, then the mechanism shown in Fig. 28(b) may be true. The scheme proposed in Fig. 28(b) will only be confirmed if a plot of $\frac{I' - I}{[P]}$ against I' is a straight line, where I' and I are the Y intercepts in the presence and the absence of any phosphate concentration [P] respectively. This relationship can be obtained by simplifying the Y intercept

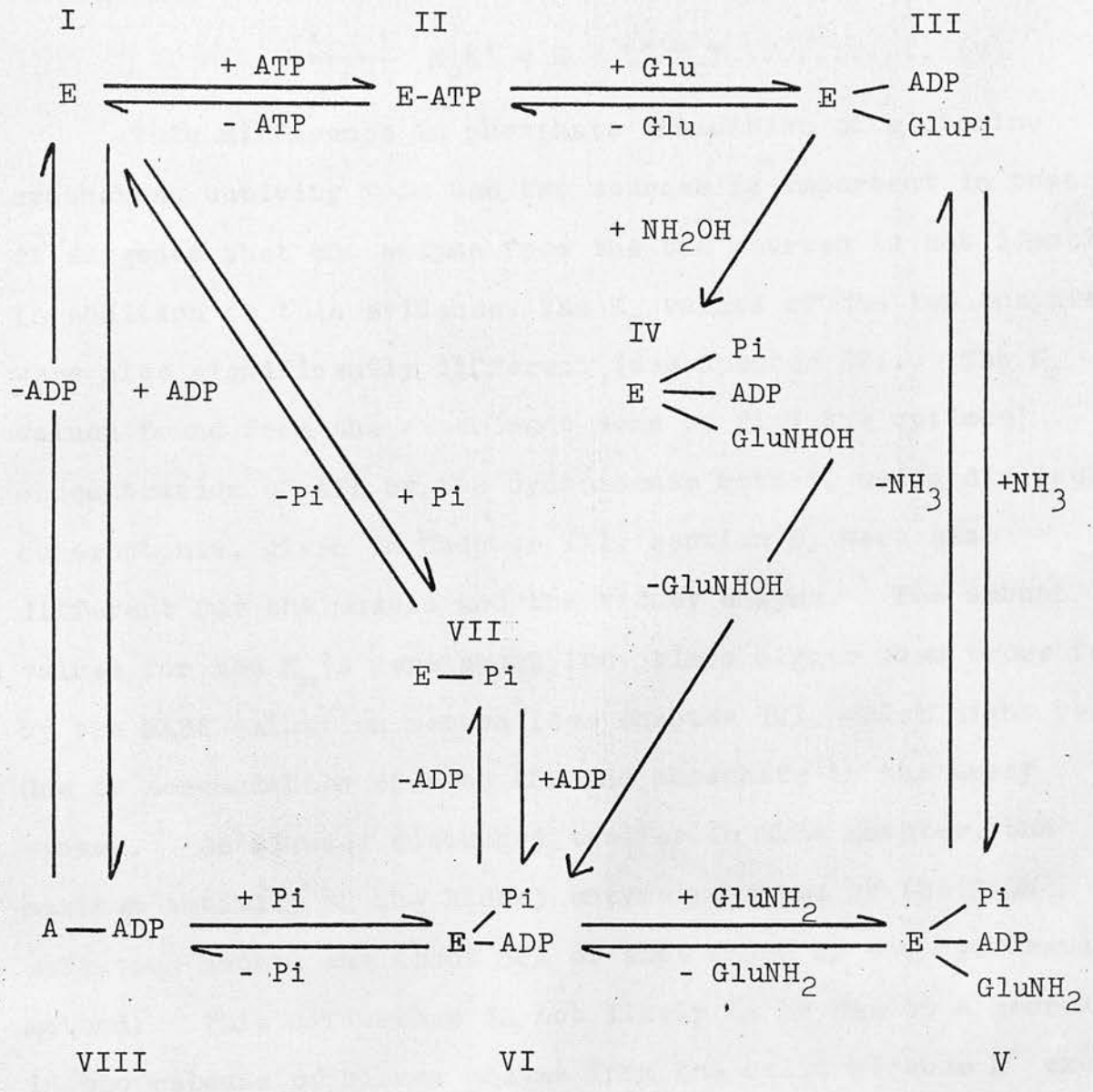


Fig. 28(b)

Mechanism of action of kidney glutamine synthetase.

Glutamine synthetase: I → II → III → V → VI → VII / VIII → I

Hydroxamate formation: I → II → III → IV → VI → VII / VIII → I

For E, Glu, GluPi, GluNHOH, $GluNH_2$ and Pi see Fig. 1 on page 13-14.

$\frac{F + H[P]}{E_o(K' + Y[P])}$ in equation (iv) to an equation

$$\frac{I' - I}{[P]} E_o K' = H - I' E_o Y \quad \ldots\ldots\ldots \quad (v)$$

This difference in phosphate inhibition of glutamine synthetase activity from the two sources is important in that it suggests that the enzyme from the two sources is not identical. In addition to this evidence, the K_m values of the two enzymes were also significantly different (see Chapter IV). The K_m values found from the experiment done to find the optimum concentration of ATP by the hydroxamate method, using dialysed supernatants, given in Chapter III, section D, were also different for the muscle and the kidney enzyme. The actual values for the K_m's were about four times bigger than those found by the NADH oxidation method (see Chapter IV), which might be due to accumulation of both ADP and phosphate in the assay system. As already discussed earlier in this chapter, the maximum activity of the kidney enzyme measured by the NADH oxidation method was about 50% of that found by the hydroxamate method. This difference is not likely to be due to a decrease in the release of kidney enzyme from the cells because K^+ extracts the enzyme as efficiently as does Na^+ (Wu, 1963a). Anyway, whatever is the reason, this suggests the kidney enzyme to be different from the muscle enzyme. All these findings suggest that the kidney enzyme was different from the muscle enzyme. No previous report of an isoenzyme of glutamine synthetase in animal tissue has appeared, although it is clear that the bacterial

enzyme studied by several workers mainly by Holzer and his associates, and Stadtman and his collaborators (Holzer, Schutt, Masek and Mecke, 1968; Kingdon, Hubbard and Stadtman, 1968), which differs from that of the animal tissues in many properties (Ravel, Humphreys and Shive, 1965; Woolfolk and Stadtman, 1967; Kingdon and Stadtman, 1967; Liess, Varrichio, Mecke and Holzer, 1968) exists in different forms.

GROWTH HORMONE AND GLUTAMINE SYNTHETASE

Growth hormone, unlike estradiol, thyroxine, cortisone and hydrocortisone (Moscona and Piddington, 1966; Piddington, 1967; Piddington and Moscona, 1967) was found to cause no increase in glutamine synthetase activity per unit mass of tissue in vivo. An in vitro addition of growth hormone also did not effect the kinetics of the enzyme reaction. A similar lack of effect of growth hormone on metabolism of glutamine and other amino acids have been observed by other workers. For instance, Bertlett and Gaebler (1949a) failed to find any effect of growth hormone on liver glutaminase II activity of hypophysectomised and the normal rats. These workers (Bertlett and Gaebler, 1949b) also found that growth hormone which increased the activity of liver glutaminase I of hypophysectomised rats, did not have any effect on the enzyme activity of the normal rats. It is also interesting to mention here that Kostyo and Knobil (1965) have recently found that hypophysectomy decreases and growth hormone

stimulates leucine-2-C^{14} incorporation in the diaphragm of fed and fasted rats but growth hormone treatment which doubled the rate of gain of body weight in normal rats, did not alter significantly the incorporation of leucine by the diaphragm. A detailed discussion of the experimental results of growth hormone treatment has already been given in Chapter V. The well-established effects of this hormone on nitrogen retention in animals do not seem to depend on changes in the rate of synthesis of glutamine.

REFERENCES

REFERENCES

Adam, H. (1963) in "Methods of Enzymatic Analysis", ed. by Bergmeyer, H.U., 1st edition, Academic Press, N.Y., London, p. 573-577.

Baerle, R.R., Goldstein, L. and Dearborn, E.H. (1957) Enzymologia 18, 327.

Bartlett, P.D. (1949) Fed. Proc. 8, 182.

Bartlett, P.D. and Graebler, O.H. (1949a) J. Biol. Chem. 181, 523.

Bartlett, P.D. and Graebler, O.H. (1949b) J. Biol. Chem. 181, 529.

Berl, S. (1966) Biochemistry, 5, 1966.

Boyer, P.D., Koeppe, O.J. and Luchsinger, W.W. (1956) J. Amer. Chem. Soc. 78, 356.

Boyer, P.D., Mills, R.C. and Fromm, H.J. (1959) Arch. Biochem. Biophys. 81, 249.

Bujard, E. and Leuthardt, F. (1947) Helv. Physiol. et Pharmacol. Acta, 5, C39.

Cleland, W.W. (1963) Biochim. Biophys. Acta, 67, 104,173.

Cleland, W.W. (1967) Adv. Enzym. 29, 1.

de Duve, C., Baudhuin, P. and Wattiaux, R. (1962) Adv. Enzym. 24, 291.

Doell, R.G. and Felts, J.M. (1959) Am. J. Physiol. 197, 138.

Du Bois, K.P. and Potter, V.R. (1943) J. Biol. Chem. 150, 185.

Eagle, H. (1955) Science, 122, 501.

Ehrenfeld, E., Marble, S.J. and Meister, A. (1963) J. Biol. Chem. 238, 3711.

Elliott, W.H. (1948) Nature, 161, 128.

Elliott, W.H. (1951) Biochem. J. 49, 106.

Elliott, W.H. (1953) J. Biol. Chem. 201, 661.

Elliott, W.H. (1955) in "Methods In Enzymology", ed. by Colowick, S.P. and Kaplan, N.O., Academic Press, New York, (1955), Vol. II, p. 337-42.

Elliott, W.H. and Gale, E.F. (1948) Nature, 161, 129.

Friedberg, F. and Greenberg, D.M. (1948) Arch. Biochem, 17, 193.

Gothoskar, B.P., Raina, P.N. and Ramakrishnan, C.V. (1960) Biochim. Biophys. Acta, 37, 477.

Gothoskar, B.P., Raina, P.N., Tate, S.S. and Ramakrishnan, C.V. (1960) J. Natl. Cancer Inst. 24, 573.

Greenberg, J. and Lichtenstein, N. (1959) J. Biol. Chem. 234, 2337.

Herbert, J.D., Coulson, R.A. and Hernandez, T. (1966) Comp. Biochem. Physiol. 17, 583.

Hjalmarson, A. (1968) Acta Endocrin. (Copenhagen) Suppl. 126, 1.

Holzer, H., Schutt, H., Masek, Z. and Mecke, D. (1968) Proc. Natl. Acad. Sci., U.S. 60(2), 721.

Hsu, L. and Tappel, A.L. (1964) J. Cellular Comp. Physiol. 64(2), 265.

Kagan, H.M., Manning, L.R. and Meister, A. (1965) Biochemistry, 4, 1063.

Kagan, H.M. and Meister, A. (1966) Biochemistry, 5, 725.

Kagan, H.M. and Meister, A. (1966) Biochemistry, 5, 2423.

Khedouri, E. and Meister, A. (1965) J. Biol. Chem. 240, 3357.

King, E.L. and Altman, C. (1956) J. Phys. Chem. 60, 1375.

Kingdon, H.S., Hubbard, J.S. and Stadtman, E.R. (1968) Biochemistry, 7(6), 2136.

Kingdon, H.S. and Stadtman, E.R. (1967) Biochem. Biophys. Research Commun., 27, 470.

Kipnis, D.M. and Reiss, E. (1960) J. Clin. Invest. 39, 1002.

Kirk, D.L. (1965) Proc. Natl. Acad. Sci. 54, 1345.

Kirk, D.L. and Moscona, A.A. (1963) Develop. Biol. 8, 341.

Knobil, E. and Hotchkiss, J. (1964) Ann. Rev. Physiol. 26, 47.

Knox, W.E., Auerbach, V.H. and Lin, E.C.C. (1956) Physiol. Revs., 36, 164.

Korner, A. (1959) Biochem. J. 73, 61.

Korner, A. (1965) Rec. Progr. in Hormone Res. 21, 205.

Kostyo, J.L. (1964) Endocrinology, 75, 113.

Kostyo, J.L. (1968) Ann. New York Acad. Sci. 148(2), 389.

Kostyo, J.L. and Knobil, E. (1965) Endocrinology 65, 395.

Kowalsky, A., Wyttenbach, C., Langer, L. and Koshland, D.E. Jr. (1956) J. Biol. Chem. 219, 719.

Krebs, H.A. (1935) Biochem. J. 29, 1951.

Krishnaswamy, P.R., Pamiljans, V. and Meister, A. (1960) J. Biol. Chem. 235, PC39.

Krishnaswamy, P.R., Pamiljans, V. and Meister, A. (1962) J. Biol. Chem. 237, 2932.

Lakatna, D.J., Van Pilsum, J.F. and Ungar, F. (1966) Acta Endocr. (Copenhagen) 52, 210.

Levenbook, L. and Kuhn, J. (1962) Biochim. Biophys. Acta 65, 219.

Levintow, L. and Meister, A. (1953) J. Amer. Chem. Soc. 75, 3039

Levintow, L. and Meister, A. (1954) J. Biol. Chem. 209, 265.

Levintow, L., Meister, A., Hogeboom, G.L. and Kuff, E.L. (1955) J. Amer. Chem. Soc. 77, 5304.

Liberti, J.P., Colla, J.C., Pilsum, J.F.V. and Ungar, J. (1966) Arch. Biochem. Biophys. 113, 718.

Lichenstein, N., Ross, H.E. and Cohen, P.P. (1953) Nature, 171, 45.

Liess, K., Varricchio, F., Mecke, D. and Holzer, H. (1968) Europ. J. Biochem. 4, 193.

Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 55, 658.

Lipmann, F. and Tuttle, L.C. (1945) J. Biol. Chem. 159, 21.

Long, C. (1961) Biochemists Handbook, 1st Edition, C & F.N. Spon Ltd., London, pages 670, 672, 687.

Manchester, K.L. and Young, F.G. (1959) J. Endocrin. 18, 381.

Martonosi, A. (1968) J. Biol. Chem. 243(1), 71.

Martonosi, A., Donley, J. and Halpin, R.A. (1968) J. Biol. Chem. 243(1), 61.

McDermott, W.V. Jr., and Adams, R.D. (1954) J. Clin. Investigation, 33, 1.

Mecke, D., Wulff, K., Liess, K. and Holzer, H. (1966) Biochem. Biophys. Res. Commun. 24, 452.

Meister, A. (1956) Physiol. Revs. 36, 103.

Meister, A. (1962) in "The Enzymes" by Boyer, Lardy & Myrbäck, 2nd Ed., Academic Press, N.Y., London, Vol. 6, pp. 443-448.

Meister, A. (1965) Biochemistry of Amino Acids, Vol. I, 2nd ed., Academic Press, New York, pp. 446-452.

Meister, A. (1968) Adv. Enzymol. 31, 183.

Meister, A., Krishnaswamy, P.R. and Pamiljans, V. (1962) Federation Proc. 21, 1013.

Meister, A., Levintow, L., Greenfield, R.E. and Abendschein, P.A. (1955) J. Biol. Chem. 215, 441.

Monder, C. (1965) Biochemistry, 4, 2677.

Monder, C. and Jacobson, B. (1964) Biochem. Biophys. Res. Commun. 17, 136.

Moscona, A.A. and Kirk, D.L. (1965) Science, 148, 519.

Moscona, A.A. and Piddington, R. (1966) Biochim. Biophys. Acta, 121, 409.

Ottaway, J.H. (1969) Q.J. Expt. Physiol. 54, 49.

Ottaway, J.H. and Khalid-Iqbal (1969) in press.

Pamiljans, V., Krishnaswamy, P.R., Dumville, G. and Meister, A. (1962) Biochemistry, 1, 153.

Panda, N.C., Goel, B.K., Mansoor, M. and Talwar, G.P. (1962) Biochem. J. 82, 176.

Peckham, W.D. and Knobil, E. (1962) Proc. Endocrine Soc., 44th Meeting, 31.

Pette, D., Klengenberg, M. and Bucher, T.H. (1962) Biochem. Biophys. Res. Commun. 7, 425.

Piddington, R. (1967) Develop. Biol. 16, 168.

Piddington, R. and Moscona, A.A. (1965) J. Cell. Biol. 27, 247.

Piddington, R. and Moscona, A.A. (1967) Biochim. Biophys. Acta, 141, 429.

Ravel, J.M., Humphreys, J.S. and Shive, W. (1965) Arch. Biochem. Biophys. 111, 720.

Recknagel, R.O. and Potter, V.R. (1951) J. Biol. Chem. 191, 263.

Reid, E. and Stevens, B.M. (1958) Biochem. J. 68, 367.

Reiner, J.M. (1964) in 'Comprehensive Biochemistry' Vol. 12, edited by Florkin, M. and Stotz, E.H., Elsevier Publishing Company, New York. pp.126-164.

Reiner, J.M. and Hudson, P.B. (1953) J. Urol. 70, 627.

Richterich, R.W. and Goldstein, L. (1958) Am. J. Physiol. 195, 316.

Riggs, T.R. and Walker, L.M. (1960) J. Biol. Chem. 235, 3603.

Russell, J.A. (1951) Endocrinology, 49, 99.

Russell, J.A. (1955) in "Hypophyseal Growth Hormone, Nature and Actions". p. 213, Ed. by Smith, R.W., Gaebler, O.H. and Long, C.N.H., New York: McGraw-Hill.

Schwerin, P., Bessman, S.P. and Waelsch, H. (1950) J. Biol. Chem. 184, 37.

Seegmiller, J.W., Schwartz, R. and Davidson, C.S. (1954) J. Clin. Investigation, 33, 984.

Sellinger, O.Z. and Verster, F. de B. (1962) J. Biol. Chem. 237, 2836.

Speck, J.F. (1947) J. Biol. Chem. 168, 403.

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

Speck, J.F. (1949b) J. Biol. Chem. 179, 1405.

Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675.

Tigerman, H. and MacVicar, R. (1951) J. Biol. Chem. 189, 793.

Trush, G.P. (1963) Ukr. Biokhim. Zh. 35, 713.

Ulrich, F., Tarner, H. and Li, C.H. (1954) J. Biol. Chem. 209, 117.

Ungar, F. and Pilsum, J.F.V. (1966) Endocrinology, 78, 1238.

Vigers, G.A. and Ziegler, F.D. (1968) Biochem. Biophys. Res. Commun. 30(1), 83.

Weinbach, E.C. (1954) J. Biol. Chem. 210, 545.

Weinbach, E.C. (1956) J. Biol. Chem. 221, 609.

Weinbach, E.C. (1957) Proc. Natl. Acad. Sci. 43, 393.

Weinbach, E.C. and Bowen, W.J. (1958) Biochim. Biophys. Acta, 30, 476.

Wellner, V.P. and Meister, A. (1966) Biochemistry, 5, 872.

Wellner, V.P., Zoukis, M. and Meister, A. (1966) Biochemistry, 5, 3509.

Wergedal, J.E. and Harper, A.E. (1964) Proc. Soc. Expt. Biol. Med. 116, 600.

White,A., Handler, P. and Smith, E.L. (1964) Principles of Biochemistry, 3rd edition, McGraw-Hill Book Company, New York, pp. 749-752.

Woolfolk, C.A. and Stadtman, E.R. (1967) Arch. Biochem. Biophys. 118, 736.

Wu, Chung (1961) Federation Proc. 20, 218.

Wu, Chung (1963a) Biochim. Biophys. Acta, 77, 482.

Wu, Chung (1963b) Comp. Biochem. Physiol. 8, 335.

Wu, Chung (1964a) Arch. Biochem. Biophys. 106, 394.

Wu, Chung (1964b) Arch. Biochem. Biophys. 106, 402.

Wu, Chung (1964c) Biochim. Biophys. Acta, 89, 137.

Wu, Chung (1965) Biochim. Biophys. Acta, 96, 134.

Wu, Chung and Bauer, J.M. (1960) Cancer Res. 20, 848.

I N D I C E S

(a) Index to Tables

(b) Index to Figures

INDEX TO TABLES

INDEX TO FIGURES