To my parents and Richard

'Does the road wind uphill all the way?
Yes, to the very end.
Will the journey take the whole long day?
From morn' to night my friend.....'

Christina Rosetti

TYROSINE AMINOTRANSFERASE IN ISOLATED RAT LIVER CELLS

A dissertation submitted to the University of Kent in candidature for the Degree of Doctor of Philosophy

by

Fiona A. O. Marston B.Sc.

April, 1979

PREFACE

The studies presented in this thesis were performed in the Biological Laboratory of the University of Kent at Canterbury, from October, 1975 to September, 1978. The investigations are the original work of the author. No part of this thesis has been submitted to any other University.

I would especially like to thank Dr. C.I. Pogson for his interest, guidance and encouragement throughout the course of this work. In addition I would like to thank:

Felicity Carr, Pete Lloyd and Drs. Alan Dickson and Steve Smith for their useful criticisms, discussions and excellent 'technical' assistance.

Professor K.A. Stacey for the provision of laboratory space for the duration of this work.

Dr. D. Wildon for advice on statistical analysis.

Mr. R. Newsam for the preparation of material for electron microscopy.

Beverley Marston for preparation of the typescript.

Finally, the author is indebted to the Medical Research Council for their generous financial support. This research was funded by a grant from the Medical Research Council to Dr. C.I. Pogson.

FMarston

Fiona A. O. Marston

Conventions and Abbreviations

The conventions of The Biochemical Journal with regard to units and abbreviations have been used throughout. Additional abbreviations are indicated in the text. The words "induced" and "induction" have been used to describe an increase in enzyme activity without regard to the mechanism.

ABSTRACT

Tyrosine aminotransferase (TAT; L-tyrosine: 2-oxoglutarate transaminase) catalyses the first reaction of tyrosine catabolism in the liver. Glucagon, hydrocortisone, triamcinolone and dexamethasone each induced a significant increase in TAT activity, in liver cells isolated from fed, adrenalectomized rats. Glucagon and glucocorticoid hormones appear to act by independent mechanisms. In cells isolated from fed, normal rats, glucagon and each glucocorticoid hormone together increased TAT significantly, but alone had no effect on the enzyme activity. Glucocorticoid hormones, but not glucagon, stimulated an increase in TAT activity in cells isolated from fasted rats. Cycloheximide and cordycepin each prevented glucagon- and glucocorticoid-stimulated induction of TAT. In addition cycloheximide decreased the basal activity of TAT but lactate dehydrogenase activity remained unchanged.

At a concentration of 2.5mM tryptophan stimulated a significant increase in TAT activity in isolated liver cells from fed and adrenalectomized rats. The increase was small compared with that obtained after *in vivo* administration of tryptophan. The latter effect must be primarily an indirect one. TAT activity also increased when the level of amino acids in the medium was elevated 8-fold. The effect was not attributable to tryptophan and in combination with hydrocortisone additive increases in TAT activity were stimulated.

TAT in crude cell extracts was separated into at least 3 forms by ion-exchange chromatography. A crude particulate liver extract promoted interconversion of the forms, thus they are post-translational modifications of the same enzyme. Polyacrylamide gel electrophoresis separated TAT from crude extracts into 3 bands; these were detected using a specific activity stain. However, the forms of TAT separated by ion-exchange chromatography each migrated to the same position in acrylamide gels. One band was identified as glutamate-oxaloacetate transaminase, which reacts with tyrosine to a limited extent. When TAT activity in cells isolated from adrenalectomized rats was induced by glucagon or glucocorticoid hormones, the forms, separated by ion-exchange chromatography, increased to the same extent.

During transamination, the side-chain C-2-H of tyrosine is released. An assay for TAT was developed using L-[*side chain* 2,3-$^{3}H_2$]tyrosine; $^{3}H_2O$ formed was isolated by adsorption of the other ^{3}H-compounds onto charcoal. With this assay at least 20μU TAT can be detected. Exchange of both the 2- and 3-C-^{3}H was detected; but the latter occurred subsequent to transamination and required a factor present in crude extracts. By including L-[*side chain* 2,3-^{3}H] tyrosine in the incubation medium, TAT activity in intact isolated liver cells was measured from the $^{3}H_2O$ formed. The rate of transamination was dependent on the concentration of tyrosine and the enzyme exhibited Michaelis-Menten kinetics. A Km for tyrosine of 2.2mM-2.5mM was estimated. The time course of the changes in TAT activity *in vitro* during induction by hydrocortisone correlated with changes measured in cells . 2.5mM tryptophan decreased the rate of tyrosine transamination in cells and the results were consistent with competition between tryptophan and tyrosine for uptake or transamination. The increase in the rate of transamination in cells in the presence of elevated amino acid levels was attributed to the increased tyrosine concentration and masked the smaller, significant increase in TAT activity measured *in vitro*.

TABLE OF CONTENTS

INTRODUCTION

Mammalian organisms have apparently lost the ability, possessed by micro-organisms, to synthesise all the amino acids and certain, 'essential', amino acids must be derived from the diet. However, neither the dietary amino acid supply, nor the physiological amino acid requirement are constant. Therefore mammals must conserve, and regulate the availability of, 'essential' amino acids: this is one homeostatic role of the liver. Dietary amino acids are primarily absorbed by this organ and their subsequent fate is hormonally regulated, according to the needs of the organism. Teleologically, this necessitates co-ordinated control of hepatic amino acid transport, and metabolism, protein turnover and urea synthesis. Investigation of the mechanisms by which these hepatic processes adapt under a variety of hormonal and nutritional conditions could contribute to our understanding of mammalian metabolic regulation.

While tyrosine is not an 'essential' amino acid in mammals, it can only be synthesised by hydroxylation of the 'essential' amino acid phenylalanine; a reaction which occurs almost entirely in the liver (Kaufman, 1971). In addition, a single degradative pathway exists for tyrosine in the liver, in which the initial, irreversible reaction is catalysed by tyrosine amino-transferase ([EC 2.6.1.5] L-tyrosine : 2-oxo-glutarate transaminase; TAT). Catabolic hormones, such as glucagon and glucocorticoids, which in general stimulate rates of protein degradation, specifically increase hepatic TAT activity; similarly, a specific rise in the level of this enzyme is obtained after administration, *in vivo*, of the 'essential' amino acid tryptophan. These facts suggest that

transamination of tyrosine may be a rate-limiting step in hepatic tyrosine catabolism. Consequently, the activity of TAT may be of central importance in controlling the availability of tyrosine for both hepatic and extra-hepatic metabolism.

A liver system which is both physically and physiologically intact is required for co-ordinated investigations of the adaptation of TAT activity and rates of tyrosine catabolism. The regulatory properties of TAT have been investigated by quantitation of enzyme activity or enzyme protein *in vitro* after treatments administered *in vivo*. But the complexity of systems *in vivo* have limited the interpretation of results; also concentrations of metabolites or hormones affecting the liver could not be precisely manipulated or monitored. The use of intact, isolated liver cells could overcome these problems.

Minimum deviation hepatoma-derived cells, which respond normally to steroid hormones, have provided a simpler system for the study of the molecular mechanisms of steroid-mediated induction of TAT (Lee *et al.*, 1970; Wicks *et al.*, 1975; Granner et al., 1977). However, these cells exhibit many metabolic lesions: for example, some cultures lack phenylalanine hydroxylase, while in others, urea cycle enzymes are completely absent (Ichihara, 1976). Thus, metabolic regulation in such systems is unlikely to compare with that in liver cells *in vivo*. The development, in the last decade, of the technique for isolating viable parenchymal cells, using collagenase, has provided a simple, and physiological, model hepatic system *in vitro*. More recently, incubation conditions for maintaining cell integrity for at least 24h have been defined (Dickson and Pogson, 1977); using this system, the regulation of TAT activity and its role in tyrosine metabolism have been investigated.

To introduce the studies presented in this thesis, mammalian tyrosine metabolism and current knowledge of its regulation are described; the distribution and properties of TAT are outlined and finally, a treatise on hepatic systems *in vitro* is included, to highlight the use of isolated liver cells for investigating liver metabolism.

1) MAMMALIAN TYROSINE METABOLISM

Several observations have indicated that it is the liver which buffers plasma concentrations of ingested amino acids (Elwyn, 1970). Since the hepatic portal vein drains the major absorptive area of the gut, the liver has primary access to dietary amino acids. After a protein meal, the level of amino acids in the portal plasma increases dramatically, while systemic plasma levels are less markedly altered (Peraino and Harper, 1963; Elwyn, 1966). Using catheterized dogs, it was shown that, with the exception of branched-chain amino acids and methionine, the profile of hepatic amino acid uptake reflected gut amino acid output (Elwyn *et al.*, 1968).

A) Hepatic uptake and release of tyrosine.

Hepatic amino acid transport is an active process, and amino acids can move in the opposite direction to concentration gradients (Christensen and Handlogten, 1968). The molecules which mediate transport are proteins (Oxender, 1974); it is suggested that they extend across the plasma membrane and are sequentially, not simultaneously, accessible to amino acids on each side of the membrane (Guidotti *et al.*, 1978). The liver is the major site of tyrosine synthesis, as well as being the initial regulator of plasma levels of dietary-derived tyrosine; the rates of uptake, of ingested tyrosine, and release, of endogenous tyrosine could therefore be of importance in regulating the free, circulating, concentration of this amino acid.

The transport of an amino acid metabolised by the liver can only be studied if the relative flux of each of its metabolic pathways can be simultaneously investigated (Guidotti *et al.*, 1978). An alternative is to use non-metabolisable amino

acids, such as α-amino isobutyric acid, or valine, and assume that the transport mechanisms compare with those of metabolisable amino acids; however, there are conditions under which only non-metabolisable amino acids would accumulate in cells (Manchester, 1960). Nevertheless, using these amino acids four transport systems were characterized in Erlich Ascites tumour cells (Oxender and Christensen, 1963), and have been shown to operate in the liver *in situ* (Sanders and Riggs, 1967):

	Amino acid(s) preferentially transported
1) Na^+ dependent	Glycine
2) A system (Na^+ dependent)	Those with short, polar or linear side chains
3) L system (Na^+ dependent)	Those with branched or ring side chains
4) ASC system (Na^+ dependent)	Those with linear side chains containing 3 - 5 atoms and hydroxyl, sulphydryl or carboxamide groups

These active transport systems are retained in liver preparations *in vitro*: in perfused or sliced liver (Tews and Harper, 1969), and in enzymically isolated hepatocytes (Le Cam and Freychet, 1977b). The latter report and others published more recently (Chen and Lee, 1977; McGivan *et al.*, 1977; Donner *et al.*, 1978) clearly refute earlier observations indicating that isolated hepatocytes were freely permeable to amino acids (Schreiber and Schreiber, 1972, 1973). The mechanism by which energy for transport is provided is unknown; transport may be coupled with an ionic gradient (Christensen, 1975) or may be driven by the hydrolysis of ATP (Garcia-Sancho *et al.*, 1977; Schafer and Williams, 1977).

Rates of amino acid transport appear to adapt to changes in nutritional or hormonal conditions. Serum deprivation or inhibition of protein synthesis prevent the growth of secondary cultures of hepatoma-derived cells; simultaneously amino acid transport by the L system is increased, but transport by the A system is decreased (Oxender et al., 1976). Enhanced transport of amino acids into liver cells in situ during short-term starvation (Tews et al., 1970; Guidotti et al., 1975), is consistent with their increased utilization as substrates for gluconeogenesis (Felig et al., 1969); transport via the A system is affected.

Insulin, glucagon, catecholamines and glucocorticoids individually stimulate an increase in hepatic amino acid uptake; enhancement was observed in vivo (Noall et al., 1957; Riggs et al., 1963), in perfused liver (Chambers et al., 1965, 1968; Mallette et al., 1969; Tews et al., 1970), in hepatoma cells (Risser and Gelehrter, 1973; Grimm and Manchester, 1976) and in isolated hepatocytes in suspension (Le Cam and Freychet, 1976, 1977a, 1978a, 1978b) or in primary culture (Kletzien et al., 1975, 1976; Pariza et al., 1976, 1977). The studies of Le Cam and Freychet have revealed that these hormones each increase uptake by system A, with no effect on the rate of amino acid release; the maximum velocity (V_{max}) of transport was increased each time. In primary cultures of hepatocytes, catecholamines appear to decrease the K_m of the carrier system (Pariza et al., 1977), and V_{max} is unchanged.

Essentially all the neutral amino acids are transported by the systems A and L. In contrast to system A, system L is weakly concentrative; since tyrosine is preferentially transported by system L, only a moderate gradient of

this amino acid, across the plasma membrane, would be expected (Christensen, 1977). Such a mechanism would tend to conserve plasma tyrosine levels. It appears that transport system A is the main target of hormonal and nutritional stimuli; however it is suggested that this is a primary event, subsequent to which transport by other systems may be stimulated by exchange (Guidotti et al., 1978).

Using cultured cells, amino acid availability was shown to affect both rates of glucose transport, and cellular ATP levels (Hershko et al., 1971; Van Venrooji et al., 1972; Morhenn et al., 1974); from these observations it was suggested that rates of amino acid transport may be of direct importance in the regulation of cell metabolism (Guidotti et al., 1978). However, the intracellular availability of amino acids is determined not only by the rate of transport, but also by the rate(s) of intracellular amino acid metabolism; thus the physiological significance of an altered rate of amino acid transport can only be interpreted if the simultaneous change in the rate(s) of metabolism is known.

B) Pathways of tyrosine metabolism

In three of the four divergent pathways of its metabolism, tyrosine serves as a precursor for the synthesis of a number of biologically important molecules (Fig 1:1); these are the thyroid hormones triiodothyronine and thyroxine, the pigment melanin, and the catecholamines dopamine, noradrenaline and adrenaline. Oxidative degradation of tyrosine via the remaining pathway yields fumarate and acetoacetate. Thus tyrosine is both a glucogenic and a ketogenic amino acid. Before describing these pathways, and their regulation, in detail, the formation of tyrosine from phenylalanine will be considered.

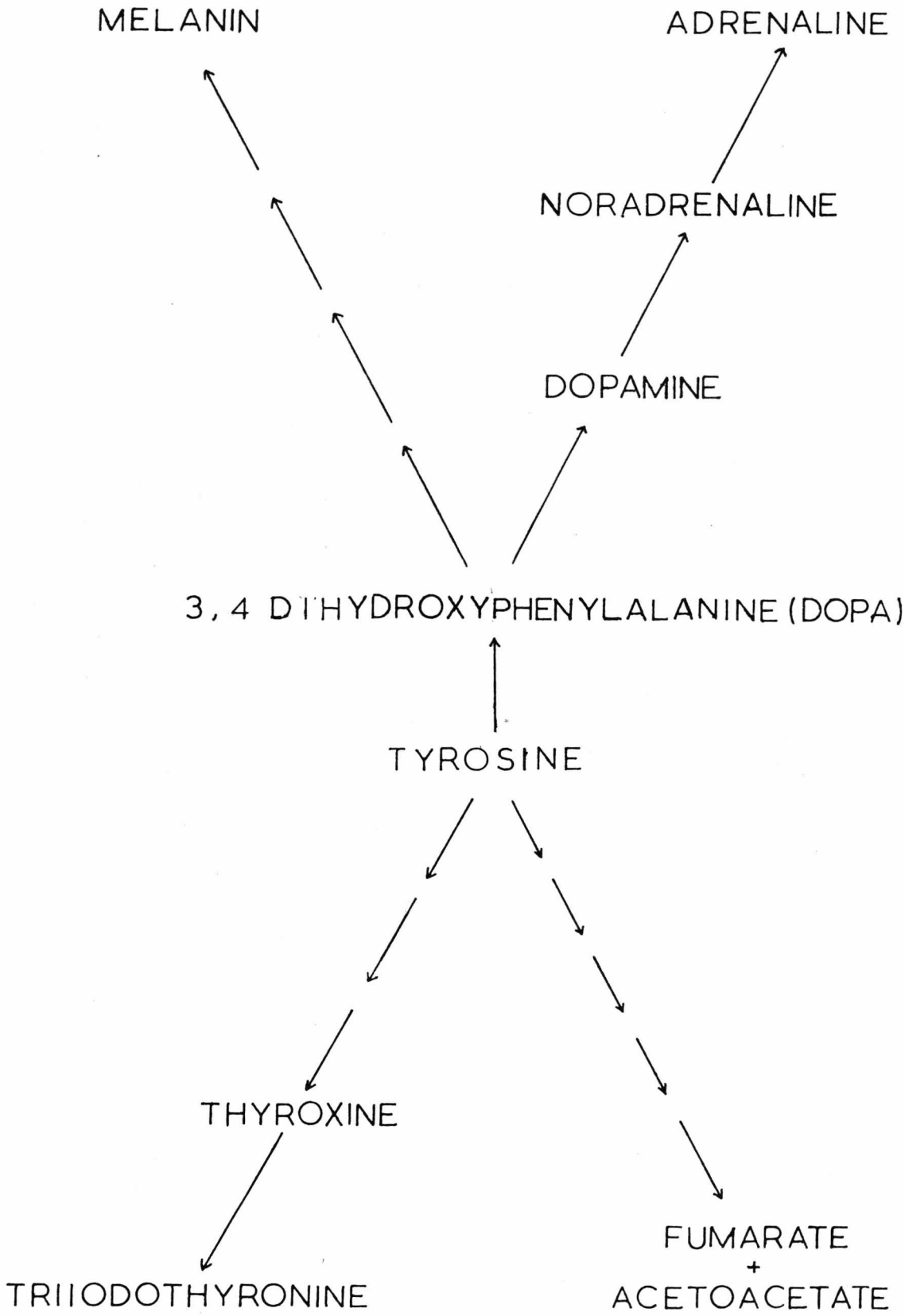


Fig 1:1 General pathways of tyrosine metabolism in mammals

i) Tyrosine synthesis

Hydroxylation of phenylalanine may be of little quantitative significance in the formation of tyrosine when an adequate dietary supply is available; certainly, individuals who lack phenylalanine hydroxylase, phenylketonuriacs, are able to survive. However, as a consequence of this metabolic lesion, phenylalanine accumulates and causes irreversible damage to the central nervous system; therefore this reaction is of importance in degrading phenylalanine as well as synthesising tyrosine.

The hepatic phenylalanine hydroxylating system requires two cofactors, tetrahydrobiopterin and NADPH (Kaufman, 1958), and consists of two activities:

I : phenylalanine hydroxylase

II : dihydrobiopterin reductase

In the reaction mechanism proposed, tetrahydrobiopterin acts to transfer reducing equivalents from NADPH to phenylalanine, and atmospheric oxygen is incorporated (Kaufman, 1971):

$$\text{phenylalanine} + O_2 + \text{tetrahydrobiopterin} \xrightarrow{\text{I}} \text{tyrosine} + \text{dihydrobiopterin}$$

$$\text{dihydrobiopterin} + \text{NADPH} + H^+ \xrightarrow{\text{II}} \text{tetrahydrobiopterin} + \text{NADP}^+$$

Hydroxylation is irreversible, as the dietary requirement for phenylalanine cannot be replaced by tyrosine (Womack and Rose, 1946).

Phenylalanine hydroxylase can catalyse the hydroxylation of tryptophan *in vitro* (Freedland *et al.*, 1961; Renson *et al.*, 1962) and phenylalanine competitively inhibits this reaction;

these observations suggest that an identical enzyme catalyses these two reactions. In nervous tissue, hydroxylation of tryptophan is the initial reaction in the synthesis of the neurotransmitter serotonin, and phenylketonuriacs display normal levels of serotonin (Kaplan and Pitot, 1970). It appears that there is a specific hydroxylase in nervous tissue which catalyses the formation of the 5-hydroxytryptophan from which serotonin is derived (Lovenberg et al., 1967).

Only a low level of phenylalanine hydroxylase was detected in foetal liver (Ryan and Orr, 1966), but there is a rapid increase in activity in the neonate. In vivo administration of glucocorticoids or glucagon stimulated an increase in phenylalanine hydroxylase activity (Freedland et al., 1963; Donlon and Kaufman, 1978); while catecholamines inactivated this enzyme in cultured hepatoma cells (Miller and Shiman, 1976).

Phenylalanine hydroxylase is a phosphoprotein and the pure enzyme contains 0.3moles of protein-bound phosphate per sub-unit (Abita et al., 1976). Chromatography of liver extracts, from untreated rats, on hydroxylapatite resolves phenylalanine activity into three forms of identical molecular weight (Barranger et al., 1972; Miller et al., 1976). A single form of the enzyme is present in extracts from hepatoma-derived cells (Miller and Shiman, 1976); but three forms of the enzyme can be resolved from cells pre-treated with hydrocortisone. The forms have been shown to differ in their extent of phosphorylation (Donlon and Kaufman, 1977). The phosphorylation state of the pure enzyme can be increased by cyclic AMP-dependent protein kinase and is accompanied by an increase in the activity of the enzyme (Abita et al., 1976). Similarly, the phosphorylation state and activity of the purified forms of phenylalanine

hydroxylase can be increased by cyclic AMP-dependent protein kinase; as a result the chromatographic behaviour of the enzyme was altered and a single, fourth form was eluted (Donlon and Kaufman, 1977). More recently, *in vivo* administration of glucagon was found to alter phosphorylation, activity and the multiple form profile of the enzyme in a comparable manner (Donlon and Kaufman, 1978).

Barranger *et al.*, (1972) described the forms of phenylalanine hydroxylase as isoenzymes and suggested that the form profiles could be features of the clinical variants of phenylketonuria. However the results of Kaufman and co-workers suggest that the forms are not true isoenzymes, but post-translation modifications of the enzyme; phosphorylation appears to be a mechanism for rapidly increasing enzyme activity. Nevertheless, the results do not eliminate the possibility that the forms differ in other, genetically-determined, structural features (Donlon and Kaufman, 1977).

ii) Thyroid hormone synthesis

The thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are synthesized by sequential iodination of free tyrosine (Fig 1:2). T_3 is the more active form of the hormone. The metabolism and functions of these hormones have been the subject of a number of reviews (Hoch, 1962; Maloof and Soodak, 1963; De Groot, 1965; Harland and Orr, 1975). The metabolic effects of the thyroid hormones are numerous: *in vivo* they have regulatory roles in growth, development, intermediary carbohydrate, fat and protein metabolism, nucleic acid and vitamin synthesis, and nerve function (Hoch, 1962). Investigations *in vitro* demonstrated that thyroid hormones alter mitochondrial

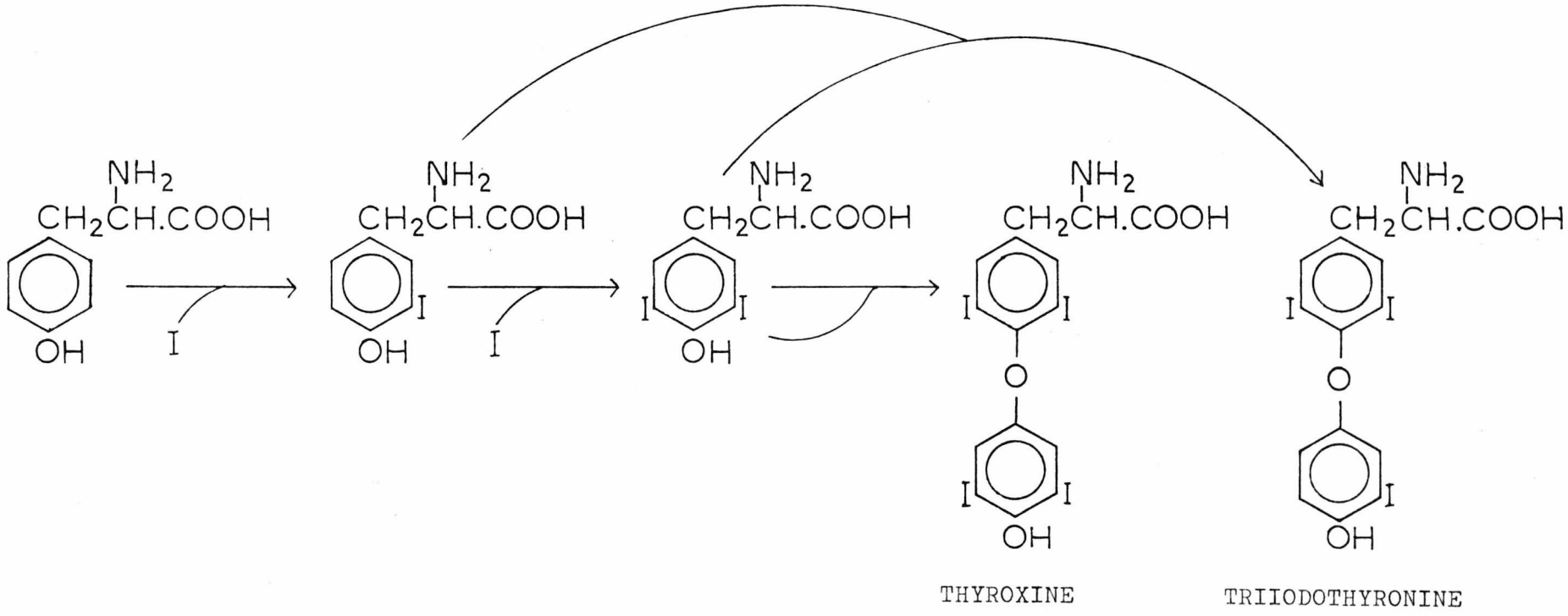


Fig 1:2 Pathway of thyroid hormone synthesis

structure and function, and the activities of certain enzymes (Pitot and Yatvin,1973). The latter effect is specific as some enzyme activities are increased, others decreased, while many remain unchanged.

In the thyroid gland, a particulate enzyme catalyses the incorporation of iodide into protein bound tyrosine (Taurog et al., 1955), forming only monoiodotyrosine. A cytoplasmic enzyme catalyses sequential iodination and the formation of T_3 and T_4 (Cunningham and Kirkwood, 1961). These enzyme systems are also present in the salivary glands and mammary glands (Taurog et al., 1956; Potter et al., 1959; Morrison and Allen, 1966). There is no evidence for hormonal control of these enzymes, rather, the rate of formation of T_3 and T_4 is related to the availability of iodide (De Groot, 1965).

Metabolism of both T_3 and T_4 can occur in tissues peripheral to their site of synthesis. Up to 80% of the circulating T_3 is derived from peripheral deiodination of T_4 (Hoffken et al., 1978). The reaction is enzyme catalysed and occurs mainly in the liver, where the activity is located in the microsomal fraction (Braverman et al., 1970; Surks and Oppenheimer, 1971). Further deiodination to form diiodothyronine has been detected (Wu et al., 1976). Hepatic enzymes also catalyse the transamination of T_3 and T_4 (Nakano, 1967; Soffer et al., 1973). The product of transamination had only 20% of the hormonal activity of T_3 which suggested that transamination was a mechanism for degrading or inactivating this hormone (Tergis et al., 1975); and there is evidence that T_3 and T_4 may regulate their own transamination.

iii) Catecholamine synthesis

Tyrosine hydroxylase catalyses the formation of 2,3-dihydroxyphenylalanine (DOPA) in brain (McGeer et al., 1965; Kindwall and Weiner, 1966), adrenal medulla (Nagatsu et al., 1964) and nervous tissue (Levitt et al., 1965). Decarboxylation of DOPA to 2,3-dihydroxyphenylethylamine (dopamine) is catalysed by aromatic amino acid decarboxylase (Udenfriend, 1963); this enzyme is pyridoxal phosphate dependent and can also decarboxylate 5-hydroxytryptophan and dihydroxy-phenylserine (Lovenberg et al., 1962). Noradrenaline is synthesized by hydroxylation of dopamine, catalysed by dopamine β hydroxylase (Levin and Kaufman, 1961); o-methylation of noradrenaline by phenylethanolamine N-methyl-transferase forms adrenaline (Wurtman, 1965; Axelrod, 1965). These reactions are illustrated in Fig 1:3.

Several reviews have been published describing both the metabolism and biochemical effects of the catecholamines (Weiner, 1970; Molinoff and Axelrod, 1971; Costa and Meek, 1974). Noradrenaline is an essential neurotransmitter, located in the ganglia of the sympathetic nervous system. Dopamine, found in high concentrations in the brain is thought to function as a synaptic neurotransmitter (Glowinski and Iverson, 1966).
The action of adrenaline on intermediary metabolism is well documented, and in general is transmitted via the 'second messenger' cyclic AMP (Robinson et al., 1971). Secretion of adrenaline is stimulated by stress, fear, or extensive exercise; the physiological and metabolic responses to this hormone provide energy for such situations. Thus an increase in the rates of respiration and heart contraction are accompanied by increased glycogenolysis and gluconeogenesis in liver, and increased lipolysis in adipose tissue.

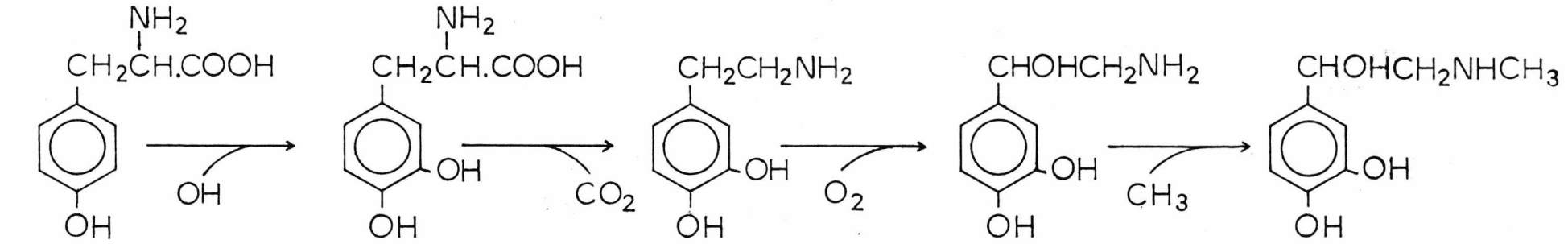


Fig 1:3 Pathway of catecholamine synthesis

Tyrosine hydroxylase is subject to feedback inhibition by both noradrenaline and dopamine (Nagatsu *et al.*,1972). The effect of dopamine is most rapid; a change is elicited in <2h. Adaptation of the level of catecholamines in response to nervous stimulation occurs too quickly to be the result of *de novo* synthesis; one possible explanation is decreased end product inhibition (Weiner, 1972). Free and membrane-bound forms of tyrosine hydroxylase are identical in amino acid sequence, but differ in conformation and catalytic activity (Udenfriend and Dairman, 1970). The K_m for the pterin cofactor and the K_i for dopamine of the bound enzyme are significantly less than those of the free enzyme (Kuczenski and Mandell, 1972); therefore short-term regulation could be achieved by changes in the relative amounts of free and bound tyrosine hydroxylase.

The methylation of noradrenaline to synthesise adrenaline, catalysed by phenylethanolamine N-methyltransferase, is inhibited by both noradrenaline and adrenaline (Fuller and Hunt, 1967); adrenaline was effective at physiological concentrations, suggesting that this may be a regulatory mechanism *in vivo*. This enzyme is located almost entirely in the adrenal medulla (Axelrod, 1962) although small amounts of activity are found in the heart and brian (Pohorecky *et al.*, 1969; Ciarenello *et al.*, 1969). Hypophysectomy results in decreased phenylethanolamine N-methyltransferase activity as well as decreased tyrosine hydroxylase and dopamine β-hydroxylase (Wurtman and Axelrod, 1966). These observations were thought to indicate a role for the pituitary gland in the long-term regulation of catecholamine biosynthesis.

Because it catalyses the first reaction of the catecholamine pathway, and is subject to feedback regulation, it was suggested that tyrosine hydroxylase is the rate-limiting step in this pathway (Levitt et al., 1965). However, this is thought to be an oversimplification (Molinoff and Axelrod, 1971). The reactions in this pathway do not all occur in the same intracellular compartment: extracellular tyrosine is transported into the adrenergic neuron where hydroxylation occurs, although the precise site of this reaction is unknown (Weiner, 1970). Decarboxylation of DOPA occurs in the axoplasm, while dopamine hydroxylation occurs in the storage vesicles at the nerve endings; therefore dopamine uptake must occur, and this is a potential regulatory site (Kopin and Weise, 1966). As a result of prolonged nervous activity or stress, the activities of a number of the enzymes in this pathway are increased (Kvetnanskey et al., 1970). Thus, there may be several sites of regulation in the catecholamine pathway.

iv) Melanin synthesis

Melanin is the pigment characteristic of skin, hair and the iris in mammals. In common with the catecholamine pathway, the first reaction in the formation of melanin is hydroxylation of tyrosine (Fig 1:4). This reaction is catalysed by Tyrosinase, a copper containing enzyme, which is dependent on DOPA for activity (Lerner, 1953). In organisms with "complete" albinism, no tyrosinase activity is detectable (Fitzpatrick et al., 1960); however noradrenaline and adrenaline are present at normal levels. Thus tyrosinase and tyrosine hydroxylase are either distinct enzymes or are regulated by different mechanisms.

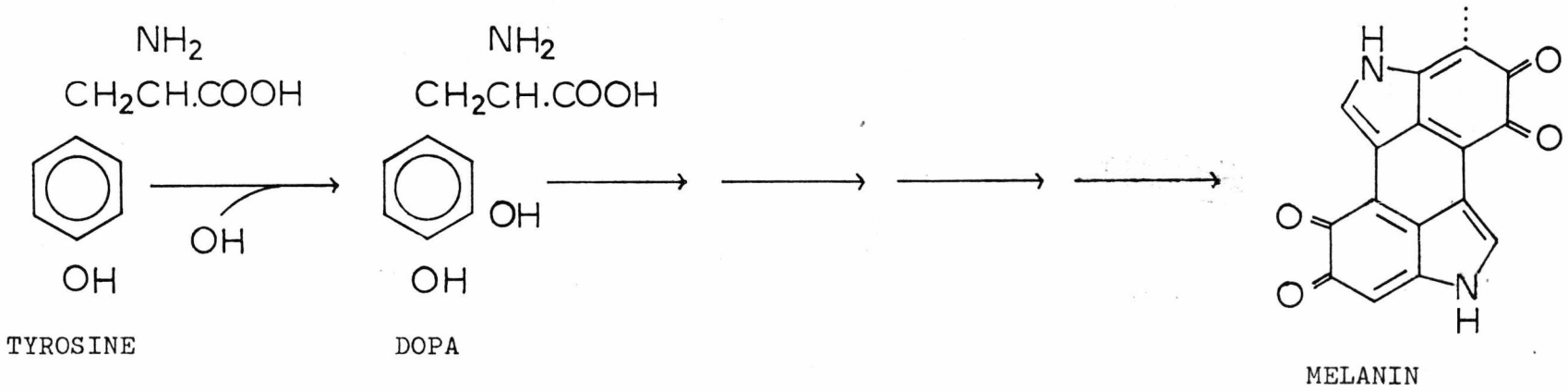


Fig 1:4 Pathway of melanin synthesis

Tyrosinase is located in the mitochondria of the melanocytes (Lerner, 1949). In a series of polymerizing reactions, DOPA is converted into melanin (Bu'Lock and Harley-Mason, 1951; Lerner, 1953; Fig 1:4); these polymerizing reactions can proceed spontaneously, but occur at an enhanced rate in the presence of enzyme.

The formation of melanin is regulated by two pituitary factors, the melanocyte-stimulating hormones, α-MSH and β-MSH (Pitot and Yatvin, 1973). Administration of β-MSH, *in vivo*, increases tyrosinase activity in the skin of mice and hamsters in 3d and 7d respectively (Pomerantz and Chuang, 1970). Catechol-*o*-methyltransferase, one of the enzymes responsible for catabolism of catecholamines, can catalyse the methylation of 5,6-dihydroxyindole and 5,6-dihydroxydihydroindole; this may be of significance as a mechanism for regulating melanin formation (Axelrod and Lerner, 1963).

v) Oxidative degradation of tyrosine

In this pathway, tyrosine is initially transaminated by TAT to form *p*-hydroxyphenylpyruvate (Fig 1:5). Oxidation of this metabolite, is catalysed by *p*-hydroxyphenylpyruvate oxidase; oxygen and ascorbate are essential for the reaction (Knox and Le-May Knox, 1951). The product of the reaction, homogentisic acid is itself oxidised. Homogentisic acid oxidase requires ferrous ions for activity (Tokuyama, 1959), and isotopic studies have demonstrated that rearrangement of the amino acid side is inherent in the reaction mechanism (Schepartz and Gurin, 1949). The requirement for atmospheric oxygen in these oxidation reactions has recently been supported by studies with isolated rat liver cells: both *p*-hydroxyphenylpyruvate and

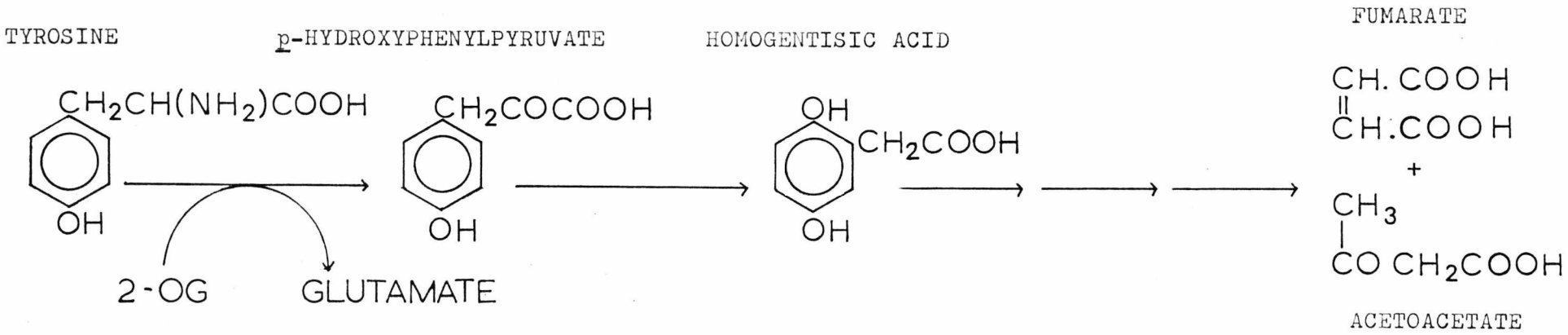


Fig 1:5 Pathway of oxidative degradation for tyrosine

homogentisic acid accumulated in cells incubated under an atmosphere of low oxygen concentration (Jones and Mason, 1978). Maleylacetoacetate (Knox and Edwards, 1955), and not fumarylacetoacetate (Ravdin and Crandall, 1951) is the product of homogentisate oxidation. The latter compound is formed subsequently by enzymic isomerization. Finally, fumarase catalyses the hydrolysis of fumarylacetoacetate to form fumarate and acetoacetate.

TAT is typically a pyridoxal phosphate-dependent enzyme (Braunstein, 1973). The activity of the hepatic enzyme is increased by administration of glucagon (Holten and Kenney, 1967; Wicks et al., 1969; McNamara and Webb, 1973, 1974), glucocorticoids (Lin and Knox, 1957; Hager and Kenney, 1968; Thompson et al., 1970; Michalopoulos and Pitot, 1975), insulin (Holten and Kenney, 1967; Iwasaki et al., 1973) and tryptophan (Kenney and Flora, 1961; Rosen and Milholland, 1963; Yatvin and Pitot, 1970; Cihak et al., 1971, 1973). Administration in vivo of growth hormone, (Kenney, 1967) and thyroxine (Litwack, 1957) result in decreased TAT activity; however the growth hormone effect was not apparent in perfused liver (Hager and Kenney, 1968) and this hormone therefore acts indirectly on the liver. The molecular mechanism of hormonal induction of TAT have been investigated extensively; a detailed discussion of these observations is presented in section 3 . While TAT activity is apparently subject to complex regulation, there is little evidence to suggest that other enzymes in this pathway have a role in controlling tyrosine degradation. In contrast to TAT, p-hydroxyphenylpyruvate oxidase activity is increased by thyroxine in vivo (Litwack et al., 1964).

2) THE PHYSICAL PROPERTIES AND METABOLIC REGULATION OF MAMMALIAN TYROSINE AMINOTRANSFERASE

TAT activity is not located solely in the liver; however, the specific activity of the hepatic enzyme is much greater than that in any other tissue. The liver is thus the major site of transamination. Also, it is the hepatic enzyme which is subject to hormonal and nutritional control. Therefore investigators have focused their attention on the molecular and regulatory properties of hepatic TAT.

A) Distribution of TAT in mammals

TAT activity was first demonstrated in liver homogenates (Knox and LeMay-Knox, 1951; La Du and Greenberg, 1951; Schepartz, 1951). Tyrosine can also be transaminated in vitro by extracts from brain, heart and kidney (Mandel and Aunis, 1974; Zigmond and Wilson, 1973; Iwasaki and Pitot, 1971). In brain TAT is located on the inner mitochondrial membrane (Mandel and Aunis, 1974). It has been suggested that this enzyme is identical to mitochondrial aspartate aminotransferase (Noguchi, 1975); however 60μM aspartate caused only 10% inhibition of tyrosine transamination, and a concentration of almost 4mM was necessary for complete inhibition (Lees, 1977). One regulatory mechanism proposed was that competition for tyrosine by TAT in brain could limit the availability of this amino acid for dopamine and noradrenaline synthesis (Lees, 1977). The concentration of tyrosine in brain is 80μM (Benkert and Matussek, 1970), while the K_m of brain TAT for tyrosine is 20mM (Mandel and Aunis, 1974). It is therefore unlikely that this enzyme has a significant physiological role. Reported effects of corticosteroids on

brain TAT are contradictory; hydrocortisone was found to have no effect on enzyme activity (Mandel and Aunis, 1974), while Laborit and Thuret (1977) obtained a significant increase. Since the latter group found hepatic TAT activity simultaneously unchanged by hydrocortisone, their observation is open to criticism.

The enzyme which transaminates tyrosine in heart and kidney elutes from hydroxylapatite before hepatic TAT (Iwasaki et al., 1973). A peak of hepatic aspartate aminotransferase elutes in a similar position (Smith et al., 1976), again suggesting the identity of these enzymes. There have also been reports of enzymes which transaminate tyrosine in thyroid glands (Igo et al., 1968), adrenal glands (Wurtman and Larin, 1968) and the placenta (Wade and Gusseck, 1976).

Hepatic TAT is found both in the cytosol and mitochondria; however, an antibody preparation directed against mitochondrial aspartate aminotransferase removed >70% of the mitochondrial activity (Miller and Litwack, 1971). It is therefore generally accepted that mitochondrial TAT is identical to mitochondrial aspartate aminotransferase.

B) Molecular properties of hepatic TAT

A purified enzyme preparation is required for investigations of the kinetic and physical properties of an enzyme. The earliest purification procedures achieved a specific activity of between 300 and 500 Units.(mg protein)$^{-1}$ (Valeriote et al., 1969; Granner et al., 1968). The molecular weight of TAT was reported to be 115,000, consisting of 4 sub-units of molecular weight 32,000 each (Aurrichio et al., 1970). Procedures published more recently attain specific activities

of at least 1,000Units TAT.(mg protein)$^{-1}$ (Belarbi et al., 1977 ; Roewekamp and Sekeris, 1977; Voight and Sekeris, 1978), and the molecular weight of the enzyme is estimated to be 90,000 - 100,000. In contrast to the previous reports of Tomkins' group, it is apparent that native TAT consists of 2, not 4, identical sub-units (Roewekamp and Sekeris, 1977; Voight and Sekeris, 1978); 4moles of pyridoxal phosphate are bound per mole of enzyme (Aurrichio et al., 1970), 2moles per sub-unit (Voight and Sekeris, 1978).

From kinetic studies, TAT was found to have average K_m values of 1.5mM for tyrosine, 1.0mM for 2-oxoglutarate, 1.0μM for pyridoxal phosphate and 5.0mM for pyridoxamine (Hayashi et al., 1967; Rosenberg and Litwack, 1970; Iwasaki et al., 1973). The reaction mechanism is ping-pong (Rosenberg and Litwack, 1970) which is consistent with the general mechanism proposed for transaminases, that substrate binding is sequential, not simultaneous (Jenkins and Sizer, 1960). Pure TAT can also catalyse the transamination of tryptophan and phenylalanine (Jacoby and La Du, 1963); however the K_m for these amino acids is $3x\ 10^{-2}$M and $8x\ 10^{-2}$M respectively. These concentrations greatly exceed the plasma levels (Wurtman et al., 1968) and hepatic levels (Wurtman, 1970) of the amino acids indicating that transamination is unlikely to occur in vivo.

TAT activity can be separated into more than one molecular form in vitro. Ion exchange chromatography (Iwasaki and Pitot, 1971; Johnson et al., 1973) and electrophoresis (Holt and Oliver, 1969; Spencer and Gelehrter, 1974) will effect a separation, but gel filtration will not (Johnson et al., 1973), which indicates a difference in charge, rather than molecular weight. There is no evidence that the forms which

separate on ion-exchange column are identical to those on polyacrylamide gels. A form of TAT, distinct from the hepatic subforms, is found in kidney, heart and brain (Iwasaki et al., 1973).

The forms in liver can be interconverted, with no change in total TAT activity (Johnson et al., 1973) and are therefore not separate gene products but modifications of the same protein. The purified forms have identical physical, chemical and kinetic properties (Iwasaki et al., 1973) which differ significantly from those of the form found in kidney, heart and brain. The nature of the difference between the forms is unknown. Free sulphydryl group content is one suggestion (Rodriguez and Pitot, 1976) and phosphorylation another (Smith et al., 1976). TAT can be phosphorylated in vivo and in vitro (Lee and Nickol, 1974; Hamm and Seubert, 1977), but it has not been demonstrated that phosphorylation or dephosphorylation accompany interconversion. The physiological role, if any, of these hepatic forms remains to be determined. Evidence that they allow distinct mechanisms of regulation by hormones is inconsistent (Holt and Oliver, 1969; Iwasaki et al., 1973; Johnson et al., 1973). Recent reports on the inactivation of TAT in vitro indicate that the forms could regulate the susceptibility of TAT to degradation (Beneking et al., 1977; Reynolds, 1978).

C) Hormonal regulation of hepatic TAT activity

Enzymes are functional macromolecules which each have a specific physiological role (Ballard, 1977). The requirement of an organism for an individual enzyme can vary considerably, both during development, and in response to

fluctuations in the environment; the ability to specifically alter the level of enzyme activity is therefore essential for survival.

There are two distinct regulatory mechanisms by which mammals can effect a change in enzyme activity: one involves alterations in the concentrations of enzyme protein, and the other, alterations in the catalytic activity of existing enzyme molecules. Mammalian proteins are continually synthesised and degraded (Swick, 1957; Buchanan, 1961; Schimke, 1964), their steady state levels being determined by the relative rates of these two processes. Simultaneous or independent adaptation of the synthetic and degradative rates results in a new steady state level of enzyme. However, the average half-life of hepatic proteins is 2 - 3 days (Goldberg and St. John, 1976), and several days may be required for a new steady state to be established. In contrast, modification of existing enzyme molecules can result in immediate activation, or inactivation. Thus, phosphorylation increases the activity of glycogen phosphorylase (Cohen, 1973; Hayakawa et al., 1973), and phenylalanine hydroxylase (Abita et al., 1976; Donlon and Kaufman, 1978), and decreases the activity of glycogen synthetase (Cohen, 1976) and acetyl CoA carboxylase (Carlson and Kim, 1974). An alternative modification mechanism is one in which the kinetic properties of the enzyme are altered. Covalent binding of the effector molecule to an allosteric site produces a conformational change in the active site. As a result, either the affinity of the enzyme for its substrate, or the maximum velocity of the reaction catalysed is altered.

The complexity of multicellular organisms, in which differentiated tissues have common requirements for certain

metabolites, necessitates a mechanism for co-ordinating the metabolic regulation of available nutrients (Walker, 1977c); this role is fulfilled by hormones. Pitot and Yatvin(1973) describe hormones as 'the "inducers and repressors" of the internal environment', and suggest that hormonal regulation is almost entirely concerned with the activity of enzymes and other proteins.

i) Glucagon-stimulated induction of hepatic TAT activity

To understand the metabolic significance of this increase of TAT activity, the effect of glucagon on metabolic processes in general must be considered. Glucagon is secreted by the pancreas, and interacts with receptors, located extracellularly, in the plasma membrane of target cells (Lawrence, 1969). The glucagon-receptor complex stimulates a rapid activation of membrane-bound adenylate cyclase and consequently cyclic adenosine 3':5'-monophosphate (cyclic AMP) accumulates (Pilkis et al., 1975; Wincek et al., 1975). There is considerable evidence that this molecule acts as the mediator through which the metabolic response to glucagon is elicited (Exton et al., 1971). The secretion of glucagon is regulated by a number of metabolic, neural, hormonal and ionic factors; a subject which has recently been reviewed (Gerich et al., 1976). Plasma glucagon levels are high during short-term starvation (Gerich, 1976), and in stressed (Bloom, 1973) or diseased (Unger, 1971) organisms. The principle actions of glucagon (cyclic AMP) are to stimulate gluconeogenesis (Exton and Park, 1968, 1969; Cornell et al., 1973) and glycogenolysis (Exton et al., 1971; Garrison and Haynes, 1973; Wagle and Ingebretsen, 1973) and to inhibit fatty acid synthesis (Capuzzi et al., 1974;

Harris, 1975) and protein synthesis (Bloxham and Akhtar, 1972; Exton et al., 1972; Ayusso-Parilla et al., 1976). Thus processes which compete for gluconeogenic substrates are inhibited. The inhibition of protein synthesis can be regarded as a mechanism for increasing the availability of amino acids for glucose synthesis (Bloxham and Klaipongpan, 1979). From these facts, glucagon-mediated induction of specific enzymes may appear to be anomalous. However, the enzymes affected include Phosphoenolpyruvate carboxykinase (Lardy et al., 1965; Eisenstein and Strack, 1968), Serine dehydratase (Jost et al., 1968), and TAT (Holten and Kenney, 1967; Labrie and Korner, 1969). These enzymes are all thought to catalyse rate-limiting reactions in metabolic pathways: Phosphoenolpyruvate carboxykinase in gluconeogenesis and Serine dehydratase, and TAT in the oxidative degradation of serine and tyrosine respectively. Thus glucagon-mediated induction of specific enxymes is consistent with its role in promoting glucose synthesis.

In vivo administration of glucagon and isotopically-labelled amino acids, coupled with immunotitration in vitro, demonstrated that the rate of TAT synthesis was increased (Holten and Kenney, 1967; Wicks et al., 1969) and the rate of degradation decreased (McNamara and Webb, 1974) during induction. The effect of glucagon (cyclic AMP) on hepatic TAT is a direct one, as induction occurs in systems in vitro (Hager and Kenney, 1968; Michalopoulos and Pitot, 1975). More recently there have been contradictory reports that glucocorticoids exert a 'permissive' effect on the glucagon (cyclic AMP) effect in vitro (Ernest et al., 1977; Granner et al., 1977). There is some evidence, obtained using inhibitors of transcription and translation, that glucagon (cyclic AMP) acts post-transcriptionally (Wicks et al., 1972a;

McNamara and Webb, 1974). Dibutyryl cyclic AMP induces both TAT and Phosphoenolpyruvate carboxykinase activity in <40min *in vitro*, which also supports a post-transcriptional site of action (Wicks *et al.*, 1974). Furthermore, in a liver-derived post-mitochondrial supernatant, dibutyryl cyclic AMP specifically decreased the ribosomal transit time for TAT (Roper and Wicks, 1978); the transit time for total soluble protein remained unchanged. Using in vitro cell-free protein synthesising systems to translate TAT-mRNA, however, glucagon was found to increase the level of TAT-mRNA (Ernest *et al.*, 1977; Noguchi *et al.*, 1977); and this observation is consistent with a transcriptional site of action. It is possible that this results from a stabilization of pre-existing mRNA, rather than stimulated *de novo* synthesis. A similar effect of glucagon on the level of Phosphoenolpyruvate carboxykinase-mRNA has been observed (Iynedjian and Hanson, 1977; Garcia-Ruiz *et al.*, 1978). Protein synthesis inhibitors, paradoxically, also increase the level of TAT-mRNA, but no change in cyclic AMP levels occurred. (Ernest *et al.*, 1978). It was suggested that a short-lived regulator protein existed for TAT-mRNA, or that translation was required for TAT-mRNA degradation.

There are many examples of glucagon-mediated induction of cyclic AMP-dependent protein kinase activity, and the role of this enzyme in activating or inactivating other enzymes is well characterized (Robinson *et al.*, 1971). As mentioned earlier, TAT can be phosphorylated *in vivo* and *in vitro*; however pure TAT is not a substrate for cyclic AMP-dependent protein kinase, *in vitro* (Wicks *et al.*, 1972b). It was suggested that glucagon-mediated induction of TAT could be related to the phosphorylation of histones or ribosomal proteins (Wicks *et al.*, 1974). In hepatoma cells only analogues

of cyclic AMP which induced TAT, stimulated phosphorylation of a serine residue in a histone protein (Wicks et al., 1975). Nevertheless, there is no direct evidence that protein phosphorylation is a pre-requisite for glucagon-stimulated induction of hepatic TAT.

iii) Glucocorticoid-mediated induction of hepatic TAT activity

The first report of glucocorticoid-mediated induction of an hepatic enzyme was made by Knox (1951), and the enzyme induced was tryptophan 2,3-dioxygenase. Subsequently induction of TAT by glucocorticoids was also demonstrated (Lin and Knox, 1957).

Glucocorticoids are a class of steroid hormone secreted by the adrenal cortex in mammals. Minor differences in the structure of adrenocortical steroid hormones alter their physiological function dramatically (Pitot and Yatvin,1973); thus, 11-oxygenated and 11-hydroxylated corticosteroids are glucocorticoid hormones, while 11-deoxygenated and 18-keto-corticosteroids are mineralocorticoid hormones. This reflects the specificity of the corticosteroid hormone receptors. These receptors are glycoproteins (Beato et al., 1971) and are located in the cytosol of target cells. The interaction between the steroid and its receptor is thought to be a two-step process (King, 1976):

1) a non-specific interaction between the steroid hydrocarbon skeleton and the steroid binding site;

2) using the energy produced by step 1), a conformational change in the binding site, which allows specific interactions between the steroids' substituent groups and the receptor protein.

The steroid-receptor complex formed becomes 'activated', enters the nucleus and binds to the chromatin (Jensen and Desombre, 1973; O'Malley and Means, 1974). 'Activation' appears to increase the acidity of the complex and hence the affinity between the complex and the nuclear histone proteins (Milgrom et al., 1973).

Glucocorticoid hormones are secreted in stress situations such as injury, infection and starvation (Litwack, 1970). They are classified as catabolic hormones since their general effect is to decrease protein synthesis and increase protein degradation (Long et al., 1960). Thus the glucocorticoid-stimulated increase of certain hepatic enzymes has been described as paradoxical (Kenney, 1970). In addition to TAT and Tryptophan 2,3-dioxygenase, glucocorticoid administration induces the activity of glutamate-alanine transaminase (Rosen et al., 1958), histidase (La Martiniere and Feigelson, 1977), serine dehydratase (Pitot and Peraino, 1964; Peraino, 1967), and the gluconeogenic enzymes, glucose-6-phosphatase (Weber et al., 1965; Weber, 1967), Fructose 1,6-diphosphatase (Weber et al., 1962; Weber and Singhal, 1964), Phosphoenolpyruvate carboxykinase (Schrago et al., 1963) and Pyruvate carboxylase (Weber et al., 1965). Therefore, as with glucagon, glucocorticoid hormones induce enzymes with important regulatory roles directly or indirectly related to the gluconeogenic pathway.

The induction of TAT by glucocorticoids has been demonstrated in vitro in perfused liver (Seglen, 1971; Ohtsuka, 1974), cultured hepatoma cells (Tomkins et al., 1966; Reel and Kenney, 1968) and cultured parenchymal cells (Bonney et al., 1974; Michalopoulos and Pitot, 1975). It is of interest that

hydrocortisone induced TAT in parenchymal cells isolated with tetraphenylboron (Haung and Ebner, 1969), since such cells are now known to be universally damaged (Dickson, 1970). However, the increase in activity obtained was much lower than that subsequently reported in cultured cells. Glucocorticoids stimulate the synthesis of TAT (Kenney, 1962a, b; Reel et al., 1970) and inhibition of degradation has also been reported (Levitan and Webb, 1970). These investigations were performed using specific antibodies directed against hepatic TAT. The effects of hydrocortisone and glucagon (cyclic AMP), in vivo, were additive indicating that the hormones act by independent mechanisms (Wicks et al., 1969, 1974).

Glucocorticoid administration in vivo stimulates an increase in hepatic DNA-dependent RNA synthesis (Feigelson et al., 1962) and RNA polymerase activity is induced (Lang and Sekeris, 1964). However this magnitude of increased RNA synthesis is not essential for the induction of TAT: no detectable change in total RNA accompanies the dexamethasone-stimulated TAT increase in hepatoma cells (Tomkins et al., 1966; Reel et al., 1970). The inhibitors of transcription, Actinomycin D, and cordycepin prevent glucocorticoid-mediated induction if administered at the same time as the hormone (Lee et al., 1970; Butcher et al., 1972). These observations, together with the fact that pre-treatment with glucocorticoid enhances glucagon (cyclic AMP)-mediated induction (Stellwagen et al., 1977), suggests a transcriptional site of action for glucocorticoids. Consistent with this, the level of TAT-mRNA is increased by glucocorticoid treatment, in vivo (Roewekamp and Sekeris, 1976) and in hepatoma cells (Roper and Wicks, 1978); also the RNA polymerase inhibitor α-amanitin prevents glucocorticoid-dependent increases in both TAT-mRNA (Ernest and

Feigelson, 1978) and TAT activity (Boctor and Grossman, 1973). In addition to increasing TAT-mRNA, glucocorticoids increase mRNA for Tryptophan 2,3-dioxygenase (Schutz et al., 1973), Glutamine synthetase (Sarkar and Griffiths, 1976) and Phosphoenolpyruvate carboxykinase (Iynedjian and Hanson, 1977); in all these investigations the level of mRNA was assayed in cell-free protein synthesising systems. These results suggest that glucocorticoid-receptor complexes interact with specific regions of the chromatin and enhance transcription.

During the course of their studies on glucocorticoid-mediated induction of TAT, Tomkins and co-workers (1966) found that administration of Actinomycin D, after induction, stimulated a further increase in TAT activity; this phenomenon was described as 'superinduction', and has been observed with tryptophan 2,3-dioxygenase as well (Garren et al., 1964). On the basis of these results the existence of a repressor protein was proposed, which interacted with mRNA, prevented its translation and stimulated its degradation (Tomkins et al., 1972). It was further suggested that glucocorticoids inactivated the repressor and stabilised the mRNA; while actinomycin D would inhibit transcription of all mRNA, it was proposed that repressor-mRNA was more labile than TAT-mRNA and thus 'superinduction' occurred. This explanation is not completely acceptable. The level of Tryptophan 2,3-dioxygenase-mRNA has been measured during 'superinduction', and while enzyme activity is increased by Actinomycin D, the mRNA was degraded at the same rate as in controls (Schutz et al., 1975). Kenney and co-workers have presented evidence that 'superinduction' results, because the rate of inhibition of TAT degradation, exceeds the rate at which TAT synthesis is inhibited. This interpretation, as with

that of Tomkins, is based on the supposition that protein synthesis is required for protein degradation, but there is a recent report which demonstrates that these processes are independent (Gunn, 1978).

D) Tryptophan: its role in the induction of specific hepatic enzymes

Tryptophan is an 'essential' amino acid in mammals, and is unique in that its structure contains an indole nucleus. It is the least abundant amino acid, both in proteins and in free amino acid pools (Munro, 1970). Tryptophan appears to have a significant role in the regulation of hepatic protein turnover, including its action in inducing the activity of several regulatory enzymes (Smith et al., 1979).

Adrenalectomy and starvation are two conditions in which rates of protein synthesis decrease, and polyribosomes disaggregate (Wittman and Miller, 1971). Only tryptophan administration stimulated protein synthesis and promoted ribosomal aggregation (Sidransky et al., 1968). Tryptophan also stimulated the incorporation of [^{3}H] leucine into soluble protein in brain and kidney (Jørgensen Majumdar, 1976).

It is possible that these effects result because tryptophan or tryptophanyl-RNA become rate-limiting for protein synthesis. Tryptophan-stimulated polyribosomal aggregation and protein synthesis are observed even in mice pre-treated with Actinomycin D (Murty and Sidransky, 1972) which indicates a post-transcriptional site of action. In rats pre-treated with both Actinomycin D and cordycepin, in which RNA is labelled with ^{14}C-orotate, tryptophan administration decreased the level of nuclear poly (A) (polyadenylic acid)-containing RNA and increase the level of cytoplasmic polyribosomal poly(A)-

containing RNA (Murty et al., 1977). Poly (A)-containing RNA is the precursor of mRNA, which suggests a role for tryptophan in promoting the translocation of mRNA from the nucleus to the cytoplasm.

In vivo administration of tryptophan was found specifically to induce Tryptophan 2,3-dioxygenase (Greengard et al., 1963), TAT (Kenney and Flora, 1961; Rosen and Milholland, 1963), Serine dehydratase (Peraino et al., 1965; Inoue and Pitot, 1970), Ornithine aminotransferase (Volpe et al., 1969; Chee and Swick, 1976), Histidase (Kaplan and Pitot, 1970; Cihak et al., 1971) and Phosphoenolpyruvate carboxykinase (Foster et al., 1966; Ballard and Hopgood, 1973). TAT activity was induced by tryptophan administration in vivo, in both normal and adrenalectomized rats (Cihak et al., 1973) which indicated that this effect of tryptophan was not mediated by corticosteroids. Using immunotitration techniques, it was found that tryptophan does not increase the rate of TAT synthesis; measuring the decay of prelabelled proteins, it was evident that tryptophan inhibited degradation (Cihak et al., 1973). Tryptophan was also shown to inhibit the degradation of Tryptophan 2,3-dioxygenase (Schimke et al., 1964), a mechanism which was distinct from that of glucocorticoid-mediated induction. In contrast, induction of serine dehydratase by tryptophan resulted from an increased rate of synthesis (Inoue and Pitot, 1970; Cihak et al., 1975). The effect of tryptophan on Phosphoenolpyruvate carboxykinase is biphasic: a rapid increase in activity occurs in <1h, followed by a slower rate of accumulation (Foster et al., 1966). The initial response must result from activation of existing enzyme, while the

later response is produced by alterations in the synthetic and degradative rates (Ballard and Hopgood, 1973).

There is some evidence that it is metabolites of tryptophan which are responsible for induction. Indole amines (decarboxylated-derivatives) induce TAT *in vivo*, but adrenalectomy diminishes the response (Deguchi and Barchas, 1971); there is therefore some mediation by adrenocortical hormones. Quinolinate, a metabolite from the "kyneurenine" pathway of tryptophan metabolism (Smith, 1977), inhibits Phosphoenolpyruvate carboxykinase activity, *in vivo*, but induces TAT activity (Hardeland, 1970; Frank *et al.*, 1976). The tryptophan effect was additive with glucocorticoid-stimulated induction.

In an attempt to elucidate the principle of tryptophan-mediated induction, six enzymes which are sensitive to this amino acid, including TAT, were studied (Smith *et al.*, 1979). In contrast with previous reports neither quinolinate nor indole amines increased these enzyme activities. No evidence was found to suggest that formation or metabolism of indole amines was required to obtain induction. Cycloheximide prevented induction of all these enzymes by tryptophan (Smith, 1977). It could only be concluded that there is no single mechanism by which tryptophan induces specific hepatic enzymes.

3) SYSTEMS IN VITRO FOR THE STUDY OF LIVER METABOLISM

An obvious advantage of investigating liver metabolism in intact animals is that the system under study is a physiological one; however, the results obtained are not easy to interpret. The metabolism of an organ is likely to be influenced by peripheral tissues with which it interacts; such effects may even result from the treatment being examined. Systems *in vitro* have therefore been developed to allow the study of the liver in isolation from the rest of the organism; those most commonly used are perfused liver, liver slices, and isolated liver cells, in culture or in suspension.

Once isolated from the organism, perfusion or incubation conditions, which maintain the structural and biochemical integrity of the liver preparation, must be defined. It is also important to assess the system for its ability to perform functions associated with the differentiated tissue from which it was derived. Only if the system is comparable, can inferences be drawn from results obtained *in vitro*, and related to the situation *in vivo*.

A) Perfused liver and liver slice preparations

The integral structure of the liver is retained in the isolated, perfused liver, and in this respect it is a good model system. Oxygen and nutrients are provided in the medium perfused, via the blood vessels, into the liver; in theory, therefore, an adequate supply to all the cells should be achieved.

Liver cells in this system *in vitro* are capable of performing several physiological functions. Gluconeogenesis occurs, from a variety of precursors (Ross *et al.*, 1967; Krebs

et al., 1969; Exton and Park, 1967, 1968, 1969) and glycogen is synthesized at rates comparable with those observed in vivo (Hems et al., 1972). However, unless glucose and gluconeogenic substrates are present in the perfusion medium, the rate of glycogen synthesis is negligible. Serum albumin and urea are synthesized in a linear fashion for at least 6h by perfused liver (Tavill, 1973), although at approximately 50% of the rate in vivo. But, supplementation of the perfusion medium with a physiological amino acid mixture increased the rate of overall protein synthesis as well as those of serum albumin and urea (Ekren et al., 1971; Tavill, 1973). The rate of general protein degradation is increased from a rate in vivo of $1\%.h^{-1}$ to $3 - 4\%.h^{-1}$ in perfused liver (Gan and Jeffay, 1971; Mortimore and Mondon, 1970). Again supplementation of the perfusion medium with either amino acids, or insulin, reversed this effect (Woodside and Mortimore, 1972). There is evidence to suggest that these changes are associated with the activity of the lysosomes (Neely et al., 1977; Ward et al., 1977; Ward and Mortimore, 1978).

In common with investigations in vivo, liver perfusion allows the investigation of only one treatment with a single animal; since natural variation between animals can be considerable, this is a disadvantage of the technique. The liver slice preparation offers a plausible alternative; slices must be very thin to enable efficient diffusion of oxygen and nutrients to all the cells. The use of liver slices to measure carbohydrate synthesis preceded that of perfused liver (Cori and Shine, 1936; Bach and Holmes, 1937; Buchanan et al., 1942). More recently, the gluconeogenic capacity of these preparations has been directly compared (Krebs et al., 1966; Wagle et al., 1966; Ross et al., 1967); with the

exception of glycerol, gluconeogenesis, from a number of substrates, in liver slices proceeded at less than 50% of the rate achieved by perfused liver. In addition, glucagon stimulated gluconeogenesis from lactate in perfused liver, but had no effect with liver slices (Ross et al., 1967). Gluconeogenesis has been described as a 'stringent test of cell integrity' as it involves the integrated work of different cell compartments (Hems et al., 1966); the results obtained suggest that the cells in liver slices are less than 50% viable. In cutting slices, cell damage is inevitable and may extend to a large proportion of the cells.

From histological studies it is apparent that the liver's cell population is not entirely homogeneous. A detailed study by Daoust (1958) showed that the cell types in adult rat liver are distributed as follows:

	% total cell number (corrected for relative nuclear sizes)
Parenchymal cells	60.6
Littoral cells	33.4
Bile duct cells	2.0
Connective tissue cells	2.2
Blood vessel wall cells	1.8

Parenchymal cells therefore constitute the bulk of the liver lobes. These are penetrated by sinusoids radiating from the central hepatic vein and the sinusoids are lined by littoral or Kupffer cells. Parenchymal cells are characteristically multinucleate and, in adult liver, divide rarely, if at all (MacDonald, 1961; Post and Hoffman, 1964). Compared with parenchymal cells, Kupffer cells are small, mononucleate and contain fewer mitochondria, less rough endoplasmic reticulum and a greater proportion of lysosomes in the cytosol (Lentz

and Di Luzio, 1971). Because of this heterogeneity, the differentiated roles of individual cell types cannot be distinguished *in vivo* or in perfused or sliced liver preparations; in addition, slices from a single liver may not be identical replicates. One further criticism of these systems *in vitro* is that the viability of the cells is very limited compared with liver cells *in situ*; on the basis of the capacity to synthesise glucose, up to 3h, compared with 200 days *in vivo* (Exton, 1975).

B) Isolated liver cell preparations

Once isolated from the collagen matrix, the major liver cell types can be easily separated. On the basis of size, parenchymal cells and Kupffer cells can be separated by differential centrifugation (Seglen, 1976b) or by rate zonal sedimentation in a stabilizing gradient (Munthe-Kas and Seglen, 1974). Alternatively, the susceptibility of parenchymal cells to trypsin or pronase can be exploited to obtain pure Kupffer cell preparations (Pisano *et al.*, 1968; Roser, 1968; Lentz and Di Luzio, 1971). When an homogeneous preparation is available, several identical samples can be obtained from a single liver; as a result, the amount of tissue available for analyses may be restricted, necessitating sensitive enzyme or substrate assays.

i) Assessment of isolated cell preparations

Measurement of the physical and metabolic integrity of the cells provides an assessment of the isolation procedure used, as well as allowing a comparison of the system *in vitro* with the physiological situation. The detailed physical structure of the cells *in vitro* can be compared with that of

liver cells *in situ* by examination using an electron microscope (Chapman *et al.*, 1973; Sattler *et al.*, 1978). A measure of the integrity of the plasma membrane, itself, is provided by the ability of the cells to exclude large dye molecules, such as trypan blue (Paul, 1970) or retain cytoplasmic enzymes, such as lactate dehydrogenase (Berg *et al.*, 1972). However, these are not definitive tests of cell viability as limited structural damage or metabolic lesions are not revealed (Berry, 1974; Seglen, 1976b). For certain metabolic processes to occur, the integrity of sub-cellular structures, as well as that of the plasma membrane, is required; thus rates of gluconeogenesis (Hems *et al.*, 1966), fatty acid synthesis (Elliott *et al.*, 1976) or cellular ATP concentrations (Krebs *et al.*, 1974) can each be used as an index of cell viability.

Another parameter of importance in assessing isolated cell preparations is the yield; that is, the percentage of the total liver cells isolated. One reason for this is that parenchymal cells constitute only 60% of the liver cell number. Also, parenchymal cells appear to be heterogeneous, with respect to biochemical function, according to their location within the liver (Jeejeebhoy and Phillips, 1976). The ability of cells to synthesise glycogen is an example of this. Investigations of glycogen distribution and synthesis in the liver indicate that the lobes themselves, and the hepatocytes within each lobe, differ in their capacity to synthesise glycogen (Corrin and Aterman, 1968; Hems *et al.*, 1972; Otter and Tait, 1972). This feature appears to be retained on isolation, as parenchymal cells in culture differ in their ability to accumulate glycogen (Walker, 1977a and b).

A good cell yield is therefore essential to obtain a representative sample of the liver cell population. In addition, the yield is important in determining the number of treatments which can be investigated with a preparation from a single liver.

ii) Methods of cell isolation

Any procedure for isolating liver cells requires the use of some degree of mechanical force (Seglen, 1976b). Mechanical techniques, alone, or in combination with the use of chemicals, successfully disrupt the intercellular matrix of the liver. Purely mechanical methods were the earliest used and included homogenization (Harrison, 1953; Tsai et al., 1966), forcing liver through filters (Kaltenbach, 1952; Schneider and Potter, 1943), shaking liver with glass beads (Aubin and Bücher, 1952), and repeated pipetting of minced liver (Prdyz and Jonsen, 1964; Howard and Green, 1965).

From the late 19th century (Roux, 1894), the importance of Ca^{2+} in maintaining cell-cell interaction, in a variety of tissues, became evident. The use of Ca^{2+}-chelating agents, such as EDTA or citrate, in addition to mechanical techniques was found to facilitate liver dispersion (Anderson, 1953; Coman, 1954; Branster and Morton, 1957; Jacob and Bhargava, 1962). The chelation of K^{+} by tetraphenylboron was also reported to decrease the amount of mechanical force required to achieve dispersion (Rappaport and Howze, 1966). These largely mechanical methods produced good cell yields and the cells were used for biochemical investigations; however, it has subsequently been shown that such cells are uniformly damaged and unable to exclude dye molecules (Klowen and Appelman, 1967; Dickson, 1970).

Efficient dispersal of liver cells was also achieved by employing enzymes to degrade the intercellular matrix. Proteolytic enzymes, such as trypsin, chymotrypsin, papain and pepsin, were used initially and good cell yields were obtained (Laws and Stickland, 1961; Günther and Goecke, 1966; Ap Gwynn et al., 1970; Poste, 1971). Collagenase was first employed to isolate cells by Rodbell, in 1967; suspensions of isolated adipocytes were prepared from epididymal fat pads. The principle of this technique was applied by Howard's group to isolate parenchymal cells from liver slices (Howard et al., 1967; Howard and Pesch, 1968; Howard et al., 1973); this technique was subsequently improved by perfusing the liver with collagenase (Berry and Friend, 1969). Comparisons of the use of mechanical methods with the use of proteolytic enzymes have shown that only collagenase techniques provide a significant proportion of viable cells (Haung and Ebner, 1969; Lipson et al., 1972; Muller et al., 1972; Fry et al., 1976). A minimal amount of mechanical force is required to disperse the cells after collagenase treatment and cell viability of 95 - 99% can routinely be achieved. Proteolytic enzymes such as trypsin and papain appear to digest the plasma membrane of parenchymal cells, as well as the intercellular matrix; therefore these enzymes can be used in the preparation of pure Kupffer cell suspensions. Despite the knowledge of these facts, reports have recently been published of ultrastructural and biochemical investigations using parenchymal cells isolated with either papain, or trypsin (Alwen and Lawn, 1974; Odashima et al., 1976; Perissel et al., 1976).

iii) Features of the collagenase technique for isolating parenchymal cells

This section is not intended as a comprehensive review of the procedures used to isolate hepatocytes which have recently been the subject of a number of excellent reviews (Schreiber and Schreiber, 1973; Berry, 1974; Krebs et al., 1974; Seglen, 1976b) and of a symposium (Proceedings of the FEBS Advanced Course No. 38, 1975). Details of the method used for the experiments presented in this thesis are described in Chapter 2.

While the techniques used to isolate hepatocytes are similar in principle, certain stages of the procedure are not common to all the methods published, and some are highly controversial. Perfusion of the liver with collagenase was the first modification of the original technique for isolating liver cells (Berry, 1969) and was reported to dramatically increase the yield of viable cells obtained. This fact is generally accepted, but, perfusion is not practically possible with large mammals and human tissue and alternative techniques must be applied (Ash and Pogson, 1977; Fry et al., 1976). The procedures published by the groups of both Howard and Berry included hyaluronidase with collagenase. While hyaluronidase has no effect on the viability of the cells isolated, it was found to increase the total cell yield (Fry et al., 1976). However there are several contradictory reports where hyaluronidase was found to be ineffective (Seglen, 1973b; Zahlten and Stradtman, 1974; Wagle and Ingebretsen, 1975; Elliott et al., 1976).

In general, exposure of the liver to 0.05%(w/v) collagenase for 15 - 20min produces a maximum yield of viable parenchymal cells (Schreiber and Schreiber, 1973; Berry, 1974; Elliott et al., 1976). It has been suggested that a lower

concentration of collagenase, 0.02%(w/v), increases the yield of metabolically active cells obtained (Ingebretsen and Wagle, 1972; Wagle and Ingebretsen, 1975). There is evidence which indicates that this is not a property of the collagenase itself, but of contaminants in the crude collagenase preparation used. The surface microvilli of parenchymal cells are thought to be the site of glucagon and insulin receptors (Wagle, 1976); after prolonged exposure of cells to collagenase, these structures are sparse or absent (Berry and Friend, 1969; Capuzzi et al., 1974; Schreiber et al., 1974). Proteolytic enzymes, such as trypsin contaminate commercially available collagenase and tryptic digestion abolishes the effect of glucagon on isolated hepatocytes (Johnson et al., 1972). Inclusion of soybean trypsin inhibitor in the perfusion medium was reported to prolong the viability of the isolated cells; albeit on the basis of a single experiment (Crane and Miller, 1977). In contrast, in this laboratory, soybean trypsin inhibitor prolonged the perfusion time required to disperse the cells and had no effect on cell viability (Dickson and Pogson, 1977); supplementation of the perfusion medium with the serine-protease inhibitor, phenylmethyl-sulphonylfluoride also had no effect on cell viability (Dickson, A.J., unpublished observation).

The perfusion buffer used in the isolation of liver cells is in general, a simple salts medium such as Krebs-Henseleit bicarbonate buffer, or Hanks buffer (Elliott et al., 1976; Wagle, 1976) or a salts-supplemented synthetic buffer such as HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid) (Seglen, 1972). Studies where chelating agents alone were used to isolate cells revealed that interactions between parenchymal cells were typical in their requirement for Ca^{2+} (Anderson, 1953; Coman, 1954); therefore perfusion buffers are initially Ca^{2+}-free

and supplemented with EDTA. It is now known that Ca^{2+} is essential to maintain the integrity of the desmosomes between cells (Amsterdam and Jamieson, 1974; Gilula and Epstein, 1976). The exact nature of this role is not known; Ca^{2+} acts either through the direct formation of ionic bridges, or by neutralising the electrostatic repulsion of the negatively charged plasma membrane (Gingell et al., 1970). Thus, chelation of Ca^{2+} facilitates cell separation; however, collagenase is a Ca^{2+}-dependent enzyme (Gallop et al., 1957; Seifter and Harper, 1970), so complete removal of EDTA before enzyme addition, is essential. Some investigators find that addition of Ca^{2+} at this stage enhances cell dispersion and the eventual cell yield (Seglen, 1973b; Edwards and Elliott, 1974; Williams and Gunn, 1974); the inclusion of Ca^{2+} with collagenase is also favoured in the isolation of cells from liver slices (Howard et al., 1973; Ash et al., 1976; Fry et al., 1976). However re-addition of Ca^{2+} to perfusion medium has been found unnecessary by others (Berry, 1974; Wagle and Ingebretsen, 1975; Elliott et al., 1976) and may, on occasions, be detrimental (Zahlten and Stratman, 1974). Seglen (1976) suggested that the latter observation could be the result of premature addition of Ca^{2+}. It is possible that these results reflect batch variation in the amount of Ca^{2+} bound by collagenase itself. Paradoxically, Ca^{2+}-supplemented incubation medium prevents the aggregation of enzymically isolated cells (Lipson et al., 1972.

As mentioned earlier, the metabolism of cells in perfused liver is affected by the composition of the perfusion medium used; it is therefore conceivable that the metabolism, and possibly the viability, of parenchymal cells could be

affected by the composition of the isolation medium used. Isolated liver cell preparations have been criticised for their low cellular K^+ and ATP concentrations compared with levels *in vivo* (Bissell *et al.*, 1973; Barnabei *et al.*, 1974). Also isolated cells exhibit increased rates of protein degradation and decreased rates of serum protein synthesis compared with rates observed *in vivo* (Seglen, 1975; Crane and Miller, 1974; Jeejeebhoy *et al.*, 1975). If the cells isolated are viable, K^+ and ATP concentrations rise rapidly during subsequent incubation, to levels comparable with those *in vivo* (Dickson and Pogson, 1977); alternatively, inclusion of either Ca^{2+} or K^+ in the isolation medium maintains cellular K^+ in the parenchymal cells isolated (Howard *et al.*, 1973; Barnabei *et al.*, 1974). The composition of the incubation medium rather than that of the perfusion medium appears to influence protein metabolism in isolated cells. Rates of general protein degradation are decreased in cells incubated in complex nutrient media (Seglen, 1977); while rates of serum protein synthesis similar to those in perfused liver and *in vivo* are obtained by hormonal supplementation of the cell incubation medium (Jeejeebhoy *et al.*, 1976; Crane and Miller, 1977). Since it appears that many alterations in metabolism initiated during the isolation procedure can be rapidly reversed subsequently, a period of preincubation is advocated by some workers (Berry and Friend, 1969; Seglen, 1973b). It is suggested that a period of 30min at 37°C also allows the completion of damage incurred by cells during the isolation procedure; thus the % viability of the final cell preparation is increased (Seglen, 1976b). In the original procedure of Berry and Friend (1969), collagenase was

included in the medium at this stage; from facts presented earlier, it appears likely that contaminants in the collagenase would decrease rather than increase the viability of the cell preparation. Seglen (1976b) reports that inclusion of collagenase at this stage has no significant effect, while Williams et al., report a decrease in viability of >30%. Again, these results may reflect variations in the crude collagenase preparations used.

iv) Maintenance of isolated liver cells

Liver cells, when isolated, may be retained in suspension or allowed to attach to a solid support and cultured. The components of the incubation medium determine whether or not hepatocytes in primary culture divide (Bissell et al., 1973); those which do proliferate enable secondary cell cultures to be established. Non-proliferating hepatocytes form confluent cell layers or monolayers.

The cells have an absolute requirement for a complex incubation medium, if their integrity is to be sustained beyond 3 - 4h (Bonney, 1974; Crane and Miller, 1974; Jeejeebhoy et al., 1975; Dickson and Pogson,1977). It is obvious that the medium should be isotonic with the cell cytoplasm, buffered and contain essential nutrients such as amino acids and vitamins (see General Materials and Methods for details), however, supplementation with serum is also necessary. As yet, the essential components in serum have not been defined, but appear to be non-dialysable (Dickson and Pogson, 1977); one, or more, factors may be hormonal in nature, as hormonal supplementation prolongs the viability of cells in culture (Laishes and Williams, 1976b). In addition, serum and insulin supplementation of the

incubation medium are essential to achieve a significant level of cell attachment and thus establish hepatocytes in culture (Bonney et al., 1974; Michalopoulos et al., 1978; Oliver et al., 1978; Savage and Bonney, 1978). Where cells are to be maintained for periods of more than 3 - 4h, antibiotics are routinely included in the medium to prevent bacterial growth.

The use of undefined media is at variance with a major principle of investigations in vitro; nevertheless, it enables cell viability to be maintained for at least 24h in suspension (Crane and Miller, 1974; Dickson and Pogson, 1977) and up to 8d, in primary culture (Michalopoulos et al., 1978; Sattler et al., 1978). This greatly exceeds the periods for which perfused or sliced liver preparations remain viable.

An efficient supply of oxygen and nutrients to all the cells in suspension or monolayer culture is ensured, as the incubation medium is in direct contact with each cell; this contrasts with both perfused liver and liver slices, where an even exposure of medium to every cell cannot be assured. This is also a problem with proliferating cultures, which are generally more than one cell layer thick. Cells in suspension must be shaken to obtain homogeneity of both cells and metabolites throughout the medium. A disadvantage of these isolated cell systems is that nutrients in the medium may be depleted, and waste products may accumulate to toxic levels during long incubation periods; the use of low cell concentrations minimizes this problem. As an alternative, cell culture medium can be decanted and replaced periodically. It appears, however, that with each medium change considerable cell loss is incurred (Bonney et al., 1974; Michalopoulos et al., 1975; Laishes and Williams, 1976a and b). The perfused liver system

is a more physiological one as a non-circulating perfusion medium can be used.

The reports of several groups indicate that cells in monolayer culture retain their integrity for longer periods than other systems *in vitro* (Williams *et al.*, 1977; Michalopoulos *et al.*, 1978; Savage and Bonney, 1978). It has been suggested that this is because efficient exchange of nutrients and gases and removal of toxic waste products is allowed (Savage and Bonney, 1978). Isolated liver cells in suspension share this advantage, but maintenance beyond 48h has not been reported (Jeejeebhoy *et al.*, 1975); the cells may be damaged by the shaking required. In the formation of monolayer cultures, parenchymal cells regain their physiological polygonal shape. Cell-cell contact is established and junctional complexes reform (Chapman *et al.*, 1973; Gebhardt *et al.*, 1978; Sattler *et al.*, 1978). If the cells are plated on plastic dishes, or in flasks, they flatten in forming monolayers. However, if floating collagen or nitrocellulose membranes are used as supports, the physiological shape is retained as the membranes shrink with the formation of cell-cell contacts (Oliver *et al.*, 1978; Sattler *et al.*, 1978; Savage and Bonney, 1978). It appears, therefore, that a structural organization reminiscent of the liver *in vivo* is regained and this may be an important factor in prolonging viability.

When primary cultures of isolated cells are established, only 40 - 50% of the viable cells isolated become attached. At least 30% of these cells detach after 48h and are lost in medium changes (Bonney, 1974; Laishes and Williams, 1976a); during the next 3 - 5d, half the remaining cells detach.

Supplementation of the medium with dexamethasone decreases the loss of attachment during the first 3d, but by 8d, the loss is comparable with that in unsupplemented medium (Laishes and Williams, 1976b). Since these investigators , themselves, propose that only viable cells attach to form monolayer cultures, this detachment of cells must reflect a loss of cell viability. Tests of cell integrity were performed only on the cells which remained attached. These observations suggest that the superiority of monolayer cultures in maintaining cell integrity is apparent rather than real.

Cells isolated from foetal liver and from normal, or regenerating, adult liver may subsequently proliferate in culture and secondary cultures or cell 'lines' can be established (Potter, 1972). The establishment of cell 'lines' from normal adult liver has been criticised, as these cells do not normally divide in vivo (Bissell et al., 1973). After extended periods of time in culture, the cells display few of the differentiated metabolic functions of adult hepatocytes (Perske et al., 1957; Davidson, 1964; Leffert and Paul, 1972; Chessebeuf et al., 1974); there is evidence to suggest that this is related to the mitotic activity of the cells (Malamud, 1971). Characteristically, the level of differentiation in foetal liver cells is lower than that of adult hepatocytes and regenerating hepatocytes resemble foetal rather than adult cells in structure and metabolic function (Church and McCarthy, 1967; Stanislawski-Birencwajg et al., 1967). Cultured liver cell 'lines' are therefore of limited use in the study of adult liver function. Nevertheless, such systems may provide information about liver cell proliferation, development and carcinogenesis (Ichihara, 1976).

v) Properties of enzymically-isolated liver cells

As mentioned earlier, to be of use for metabolic studies, a system *in vitro* must exhibit physiological functions, characteristic of the differentiated tissue from which it was derived. The properties of parenchymal cells isolated with collagenase have recently been extensively reviewed (Seglen, 1976b), therefore only a brief discussion of this subject will be included, to highlight the problems encountered with this preparation.

Isolated liver cells can synthesise ketone bodies (Ontko, 1972; Seglen, 1973c), fatty acid esters (Clark *et al.*, 1974; Nilsson *et al.*, 1974), glucose, from a variety of precursors (Krebs *et al.*, 1974; Smith *et al.*, 1978), and proteins (East *et al.*, 1973; Edwards and Elliott, 1974; Jeejeebhoy *et al.*, 1976). One major criticism of this system, however, is that glycogen synthesis can proceed only in the presence of unphysiological concentrations of potassium and glucagon (Hue *et al.*, 1975; Walli and Schimassek, 1976). More recently, synthesis of glycogen under less extreme conditions, has been reported, using isolated cells maintained in monolayer culture (Walker, 1977c; Walker and Grindle, 1977).

Although isolated liver cells exhibit normal responses to adrenocortical hormones, and pancreatic hormones (Seglen, 1976b) the extent of the response, particularly to insulin and glucagon, is less than that observed in perfused liver. A concentration of 10μM glucagon was required to stimulate glycogen breakdown (Garrison and Haynes, 1973); however, more recently concentrations of 10^{-14}M - 10^{-10}M glucagon increased glycogenolysis in isolated hepatocytes (Wagle, 1976). These values are within the range of physiological plasma concentrations (Ohneda *et al.*, 1968). It is

possible that earlier findings of hormone insensitivity resulted from the isolation procedure used; Wagle and co-workers suggest using lower concentrations of collagenase to prevent hormone receptor digestion by trypsin or other contaminants (Wagle, 1976). Their results support this hypothesis. Nevertheless, unphysiological concentrations of hormones must be used for long-term studies, such as enzyme-induction, to maintain the presence of hormone throughout the time course. Continuous infusion can overcome this problem, but, itself, presents technical problems. Similarly unphysiological concentrations of substrates must be provided, to avoid rate-limiting effects. A development which could overcome these problems is the superfusion system (Van der Meer et al., 1976) in which the cells are contained in a closed system through which the medium is perfused; thus providing a system analogous to the isolated perfused liver and more comparable with the physiological situation.

The studies presented in this thesis include:

1) the development of a sensitive assay for TAT, using L-[side chain 2,3-^{3}H] tyrosine

2) an investigation of the response of hepatic TAT activity to hormonal and nutritional imbalance

3) an investigation of the multiple forms of TAT

4) direct measurement of tyrosine transamination in intact cells using L-[side chain 2,3-^{3}H] tyrosine and a comparison of TAT activity in cells and in vitro.

C H A P T E R 2

GENERAL MATERIALS AND METHODS

Introduction

This chapter contains details of experimental procedures used routinely throughout the studies presented in this thesis. Additional techniques utilised are described subsequently in appropriate chapters. The sources of all chemicals and enzymes are given in an appendix following the list of references.

1) Animals

Male Sprague-Dawley rats (CSE/ASH strain), weighing 200 - 250g were used throughout. All animals were allowed access to diet (No. 1 maintenance diet, Cooper Nutrition Products Limited, Witham, Essex.) and water *ad libitum*, unless otherwise stated.

Adrenalectomy: Adrenal glands were removed by means of a midline dorsal incision under diethyl ether anaesthesia. Adrenalectomized animals were given 1%(w/v) NaCl in place of water and were left for 6 days before use. During the preparation of isolated liver cells from adrenalectomized rats, collagenase was added only after it was ascertained that no adrenal tissue could be observed in the region of the kidneys.

2) Glassware

To prevent cell adhesion, all glassware used in isolated cell studies was treated with 'Repelcote', a solution of dimethylchlorosilane in carbon tetrachloride (2%v/v).

3) Solutions

All solutions were prepared in deionised, double-distilled water. pH values were measured using a Radiometer Model 29 (Radiometer, Copenhagen, Denmark) pH meter equipped with a glass electrode. The adjustment of pH was made by the addition of NaOH or HCl unless otherwise specified.

A) Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932)

The composition of the buffer was as follows:

	PARTS
0.9%(w/v) NaCl (0.154M)	100
1.15%(w/v) KCl (0.154M)	4
2.11%(w/v) KH_2PO_4 (0.154M)	1
3.8%(w/v) $MgSO_4.7H_2O$ (0.154M)	1
1.3%(w/v) $NaHCO_3$ (0.154M)	21
1.22%(w/v) $CaCl_2$ (0.11M)	3

The calcium chloride was added after thorough gassing with O_2:CO_2 (95%:5%); the final pH being 7.4.

B) Minimal Essential (Eagle's) Medium

Single strength, or 10x concentrated Minimal Essential Medium (MEM) was supplied by Flow Laboratories. For certain experiments, where components were either omitted or increased in concentration, the medium was prepared in the laboratory and had the following composition:

7.25mM L-arginine	0.002mM D-Ca pantothenate
0.20mM L-cysteine	0.007mM choline chloride
0.27mM L-histidine	0.002mM folic acid
0.40mM L-isoleucine	0.011mM i-inositol
0.40mM L-leucine	0.008mM nicotinamide
0.50mM L-lysine-HCl	0.005mM pyridoxal-HCl
0.10mM L-methionine	0.30μM riboflavin
0.20mM L-phenylalanine	0.003mM thiamin-HCl
0.40mM L-threonine	0.024M $NaHCO_3$
0.05mM L-tryptophan	5.5mM D-glucose
0.20mM L-tyrosine	0.4mM $MgSO_4.7H_2O$
0.40mM L-valine	5.4mM KCl
	0.12M NaCl
	0.01M NaH_2PO_4
	0.001%(w/v) Phenol Red

The medium was stored as a 5x concentrated solution in sterile containers at 4°C. Glutamine was added, to a final concentration of 2.0mM, just before use.

C) Physiological Amino Acid Mixture (East et al., 1973).

	CONCENTRATION (mM)
Asparagine	0.027
Threonine	0.252
Serine	0.532
Glutamine	0.368
Proline	0.325
Alanine	0.109
Glycine	0.294
Valine	0.188
Methionine	0.045
Isoleucine	0.131
Leucine	0.152
Tyrosine	0.089
Phenylalanine	0.076
Lysine-HCl	0.346
Histidine-HCl	0.073
Arginine-HCl	0.175
Tryptophan	0.069
Cysteine-HCl	0.061

A 25x concentrated stock solution of the amino acid mixture in Krebs-Henseleit bicarbonate buffer (minus $CaCl_2$) was stored at -20°C. Glutamine and cysteine-HCl were omitted from the stock solution and were added immediately before use.

D) Hormone solutions

Hydrocortisone, triamcinolone and dexamethasone were prepared as concentrated solutions in methanol (A.R.); glucagon as a concentrated solution in 0.003 N-HCl. All hormones were diluted in the cell incubation medium 1:1000(v/v); equivalent volumes of methanol (A.R.) or 0.003N-HCl were added to control flasks.

E) Tryptophan

Tryptophan, as a solution in water, was added to either 10x concentrated complete MEM or MEM prepared with tryptophan omitted.

4) Preparation of isolated parenchymal cells

A) Isolation procedure

In recent years, enzymic techniques for isolating parenchymal cells have superseded all other methods. The structural organization of the liver is such that mechanical disruption, alone, or together with the use of chelating agents, inevitably produces damaged cell preparations. Parenchymal cells prepared by the use of collagenase in particular, are both morphologically and metabolically intact. The original procedure of incubating liver slices with collagenase and hyaluronidase (Howard et al., 1967; Howard and Pesch, 1968) produces a low yield of viable cells in comparison to methods involving extensive perfusion of the liver (Berry and Friend, 1969; Elliott et al., 1976; Seglen, 1972, 1973a and b). Nevertheless, the former method is in current use because it can be applied to the isolation of liver cells from large mammals (Ash et al., 1976; Fry et al., 1976). Parenchymal cells used in these studies were isolated by collagenase perfusion of the liver in the physiological direction (Elliott et al., 1976). This is essentially a modification of the method of Berry and Friend (1969).

Rats were anaesthetised by the i.p. administration of Nembutal (60mg.(kg body wt.)$^{-1}$) containing heparin (6000U.ml^{-1}). When the hind leg flexor reflex ceased, the abdomen was opened to just below the level of the diaphragm; the hepatic portal vein and inferior vena cava were exposed. The portal vein was cannulated with a 19 gauge serum needle and the inferior vena cava with a 16 gauge needle; cannulae were secured in position with ligatures. Once perfusion had been established, the thoracic cavity was opened and the blood vessels immediately above the diaphragm were clamped with a

haemostat.

All perfusion media were maintained at 37°C and equilibrated with O_2:CO_2 (95%:5%), throughout the procedure. A flow rate of 25ml.min^{-1} was provided by a peristaltic pump (Watson-Marlow MHRE 22; Watson-Marlow Ltd., Falmouth, Cornwall). A 'bubble-trap' incorporated into the perfusion apparatus served also to eliminate the pulsation produced by the peristaltic pump. The reservoirs containing the perfusion media were 20cm below the level of the perfusion table, to minimize swelling of the liver.

The basic perfusion medium was Krebs-Henseleit bicarbonate buffer (Calcium omitted). After cannulation, the liver was perfused with 40ml buffer, containing 1.0mM EDTA, then with 160ml unsupplemented buffer. The first 100ml of perfusate were discarded; the remaining 100ml were cyclically perfused and collagenase was added to a final concentration of 0.05%(w/v).

The initial perfusion cleared the liver of blood, leaving it a characteristic pale brown colour. After 10-15 min of enzymic perfusion, the liver was seen to swell slightly and the perfusion medium began to leak. This medium was returned to the reservoir. Within a further 5 min, the surface of the liver was gradually seen to redden and take on a blebby appearance. At this point the liver was carefully removed and transferred to a 250ml beaker containing 50ml buffer. The composition of the buffer was determined by the incubation medium to be used and was either:

1) Krebs-Henseleit bicarbonate buffer (complete) supplemented with 2%(w/v) bovine serum albumin.
2) Krebs-Henseleit bicarbonate buffer (complete) supplemented with 17.5%(v/v) horse serum.

3) MEM supplemented with 10%(v/v) foetal calf serum.

Treatment of the bovine serum albumin, horse serum and foetal calf serum, prior to use, will be described separately.

The liver cells were released and dispersed by gently tearing open the liver capsule with plastic teaspoons. The suspension was filtered through a single layer of nylon mesh, pore size 150μm (Henry Simon Ltd., Cheadle Heath, Stockport, Cheshire, U.K.). The debris remaining on the filter was returned to the beaker, dispersed in a further 25ml medium and filtered. The filtrate was centrifuged at 50g for 2 min at room temperature. Under these conditions, the parenchymal cells sediment, but the Kupffer and other cells remain in suspension; the supernatant was removed by aspiration. The cell pellet was washed by resuspension in 30ml medium and centrifugation, as before. If two or more incubation media were to be compared, aliquots of cells were suspended in different media, at this stage. The composition of the dispersion medium was determined according to the comparison being made. The washing procedure was performed twice to remove contaminating erythrocytes and the cells resuspended in buffer to an appropriate concentration. The medium used for dispersion, washing and resuspension of the cells was gassed continuously with O_2:CO_2 (95%:5%).

B) Cell Yield

A yield of 50% (approximately 3.5g wet weight) was obtained routinely from the liver of a 200 - 250g rat. With the filtration and centrifugation procedures used, the contamination of this preparation with non-parenchymal cells was <1.5% (Seglen, 1976).

C) Cell incubation conditions

Unless otherwise stated, the following procedure was used to prepare cell incubations:

0.2ml aliquots of cell suspension (1 - 4mg dry weight cells) were pipetted into 20ml glass vials each containing 1.3ml of the appropriate incubation medium. A wide-bore, open-ended plastic pipette was used to minimise cell damage as a result of shearing. The vials were stoppered with Suba-seals and gassed for 2 min with O_2:CO_2 (95%:5%). In initial experiments, where rates of gluconeogenesis were determined, vials were incubated in Dubnoff-type reciprocal shaking water baths (Mickle Engineering Ltd., Gomshall, Surrey), at 100 cycles.min^{-1} at 37°C. In all other experiments, vials were incubated in orbital shaking water baths (Infors, type WTR1; diameter of rotation 12.5mm) at 130 rev.min^{-1} at 37°C. All incubations were performed in triplicate. Any additions to the incubation medium were made by injection through the Suba-seals. Incubations from which substrates were to be assayed were terminated by the addition of 0.2ml 2M-$HClO_4$; those from which enzymes were to be assayed were either cooled (4°C) or frozen rapidly (-70°C) after the cells had been pelleted by centrifugation.

D) Dry weight determination

1.0ml aliquots of cell suspension were pipetted into pre-weighed glass vials and centrifuged at 50g for 2 min at room temperature. The supernatant was carefully removed by aspiration and the cells dried at 120°C to constant weight. In each experiment, determinations were performed in triplicate.

E) Assessment of viability

The quality of an isolated parenchymal cell preparation is determined by the percentage of intact cells; this is evaluated by assessing the structural and metabolic integrity of the cells. A number of methods can be used:

i) Microscopic examination:

a) The morphology of stained or unstained cells can be examined under a light microscope. Stains such as trypan blue (Paul, 1970) may be used; trypan blue is taken up by structurally damaged cells.

b) Detailed cell ultrastructure can be studied using scanning and transmission electron microscopes. This is not used routinely to measure cell viability, but is generally used to assess a method of cell preparation.

ii) Leakage of cytoplasmic enzyme, such as lactate dehydrogenase (Berg et al., 1972).

This is analagous to the staining procedures in providing a measure of the integrity of the plasma membrane.

iii) Potassium content (Baur et al., 1975). During the isolation procedure, a considerable amount of potassium is lost by parenchymal cells. However, this process is reversible; in viable cells, the content increases rapidly, on incubation, to levels comparable with levels in vivo (Quistorff et al., 1973; Dickson and Pogson, 1977).

iv) Total adenylate and ATP content (Krebs et al., 1974). The turnover of ATP is rapid, in vivo and damaged cells have low ATP contents and low ATP:ADP ratios. Immediately after isolation the ATP content of cells may be low, but rapidly increases to in vivo level in viable cells, during incubation.

v) Succinate oxidation (Mapes and Harris, 1975). Succinate cannot penetrate the plasma membrane, therefore only structurally damaged cells will oxidise succinate.

vi) Gluconeogenic capacity. The structural integrity of the liver is essential for the synthesis of glucose; the overall pathway involves interdependent metabolic processes in the cytoplasm and the mitochondria (Hems et al., 1966). The exception to this is pigeon liver; synthesis of glucose by homogenates has been demonstrated (Krebs, 1964; Gevers and Krebs, 1966).

The staining of cells with Trypan Blue is useful because it provides a rapid estimate of initial cell viability. However it should not be used in isolation to measure cell integrity because limited damage to the plasma membrane and intracellular disruption is not detected. Initial studies presented in this thesis were performed with parenchymal cells isolated from 48h fasted rats and rates of gluconeogenesis were measured. In other experiments, cell viability was assessed by cellular ATP content alone, or together with cellular lactate dehydrogenase (LDH) levels.

F) Morphological and metabolic characteristics of isolated liver cells

Scanning electron micrographs of whole cells isolated by the procedure outlined in this chapter are shown in Plates 2:1 and 2:2. The cells were fixed in glutaraldehyde and critical point dried. The individual cells are characteristically round with microvilli extensively covering their surface.

Transmission electron micrographs of thin cell sections (approximately 60nm) were prepared immediately after

PLATE 2:1

Scanning electron micrograph of isolated hepatocytes prepared from a 48h starved rat (x 600)

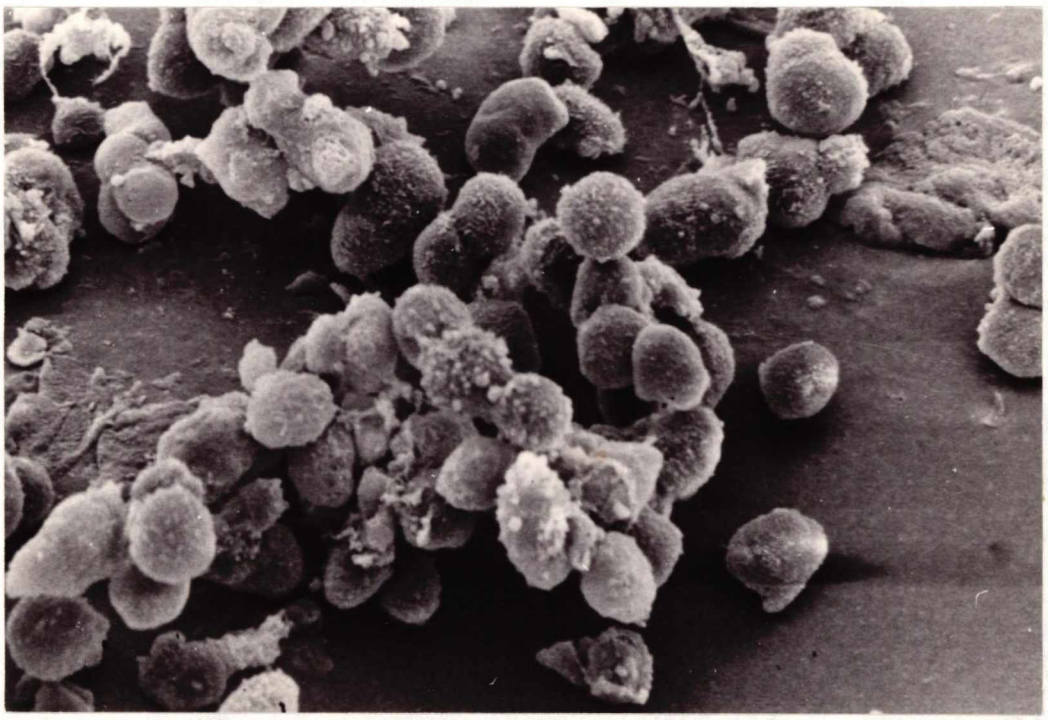


PLATE 2:2

Scanning electron micrograph of isolated hepatocytes prepared from a 48h starved rat (x 3000)

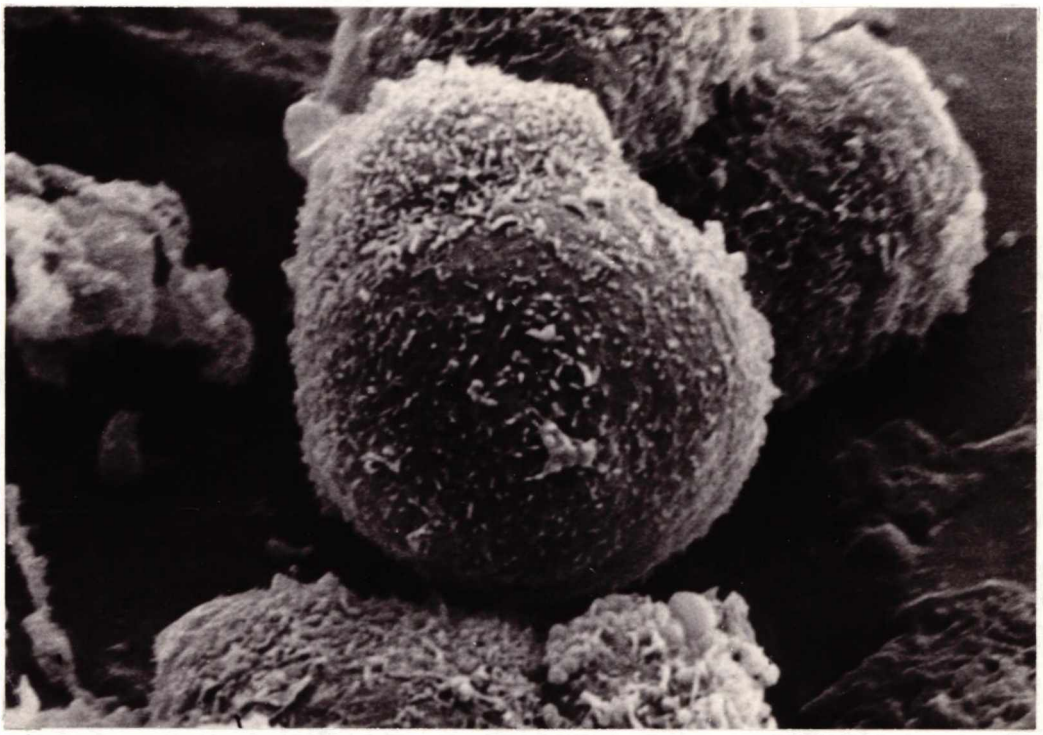


PLATE 2:3

Transmission electron micrograph of an isolated hepatocyte prepared from a 48h starved rat (x 5000)

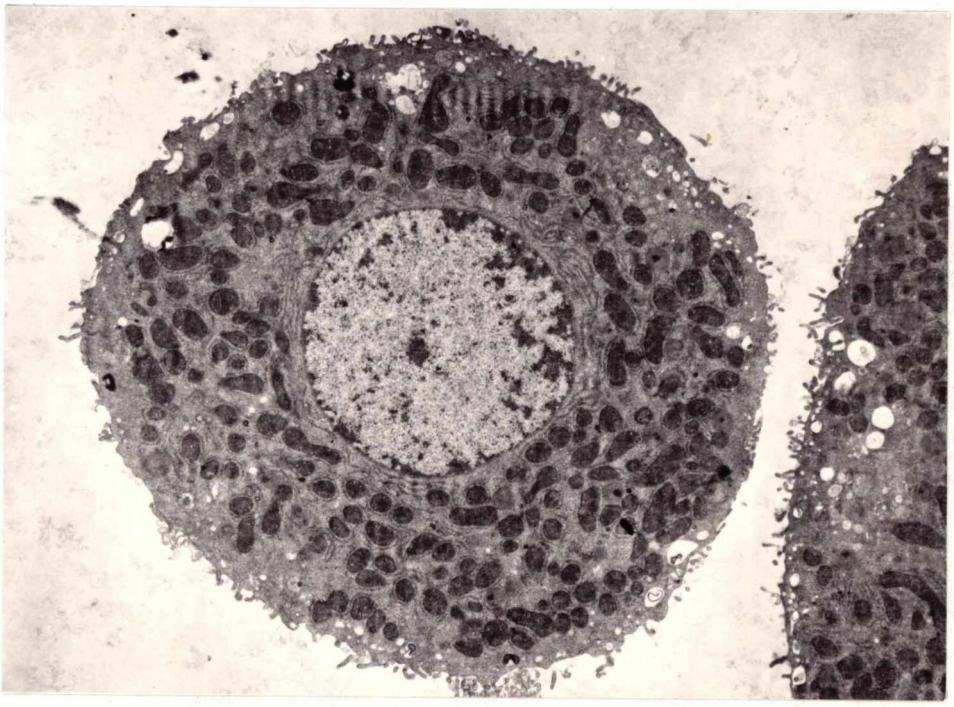
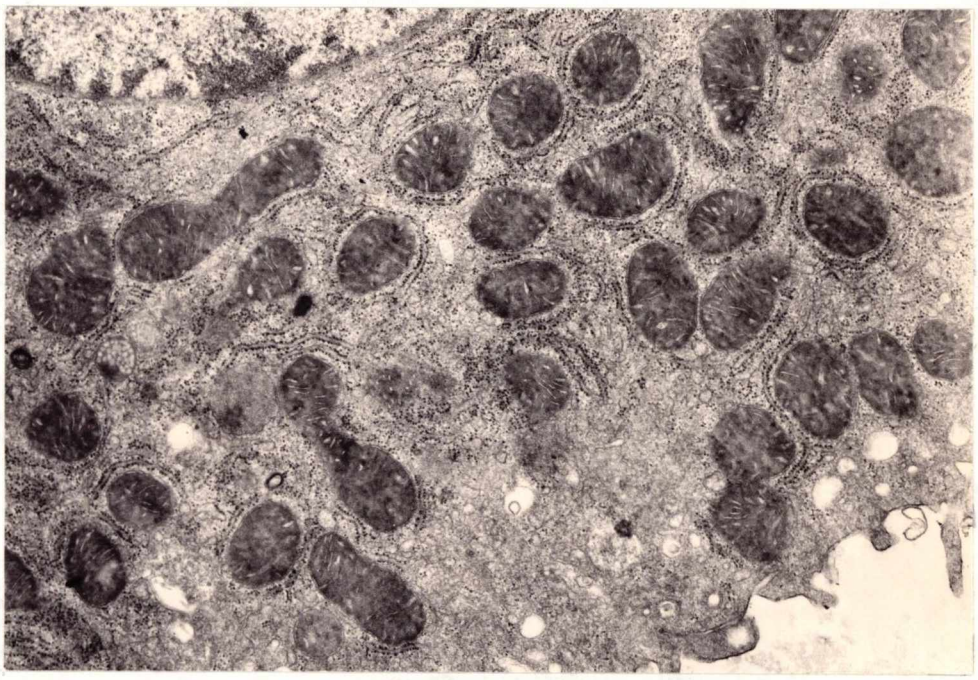


PLATE 2:4

Transmission electron micrograph of an isolated hepatocyte prepared from a 48h starved rat (x 20,000)



isolation. Cells were fixed in 0.085M - Na cacodylate pH 7.2 containing 2.5%(v/v) glutaraldehyde and post-fixed in 0.1M - Na barbitone pH 7.2 containing 1%(w/v) OsO_4. Plate 2:3 shows a whole cell (x 5,000 magnification); the microvilli on the surface of the cell are apparent and the cytoplasm contains a single nucleus and numerous mitochondria. At higher magnification (Plate 2:4; x 20,000); the mitochondria contain distinct cristae and both rough and smooth endoplasmic reticulum can be seen in the cytoplasm.

A time course of the production of glucose from 5mM pyruvate is shown in Fig 2:1; the comparative values for different methods of agitating the cell suspensions are presented. Glucose production is linear for up to 3h and rates of gluconeogenesis of 160 - 170nmoles.(mg dry weight)$^{-1}$.h^{-1} are similar to values reported previously in perfused liver and in isolated cells (Ross et al., 1967; Ingebretsen and Wagle, 1972). Glucose production from alanine is linear for $5\frac{1}{2}$h and is synthesised at a rate of 90nmoles.(mg dry weight)$^{-1}$.h^{-1} (Fig 2:2); urea was synthesised from alanine at a rate of 145nmoles.(mg dry weight)$^{-1}$.h^{-1}, which compares well with previous studies (Mendes-Murão et al., 1975; Briggs and Freedland, 1976).

5) Standard assay procedures

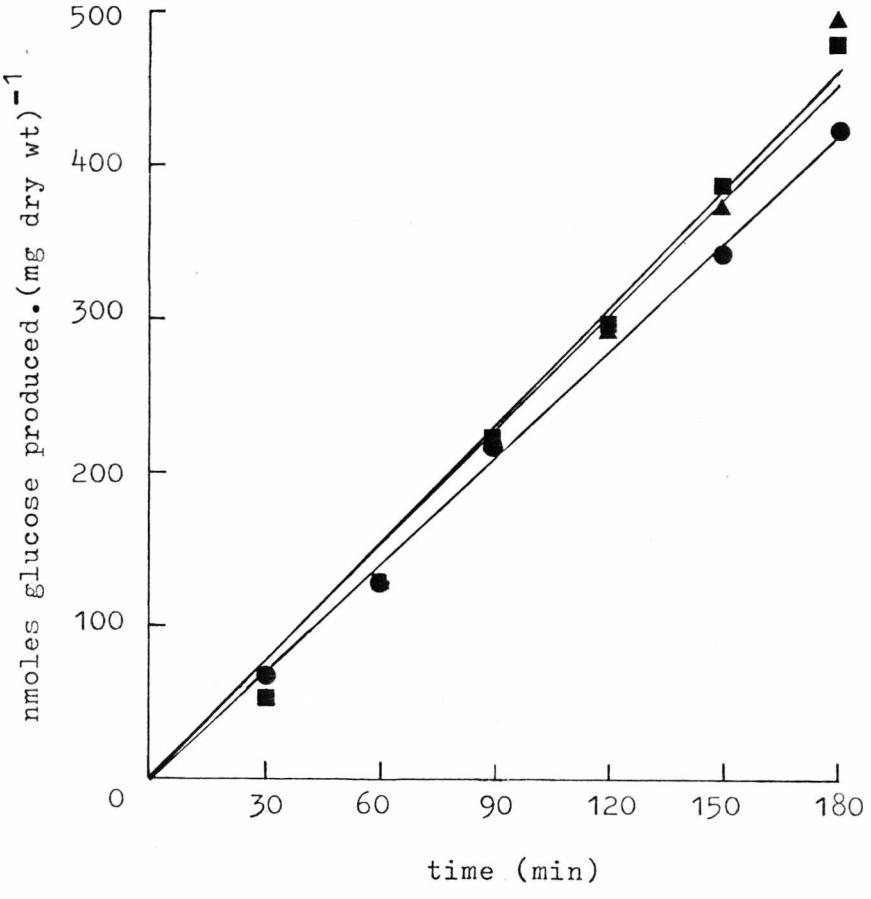
A) Spectrophotometric methods

All spectrophotometric determinations were performed using a Gilford 250 spectrophotometer.

i) Glucose

The glucose oxidase/peroxidase (GOD - POD) method of Krebs et al., (1963) was used. Incubations were terminated

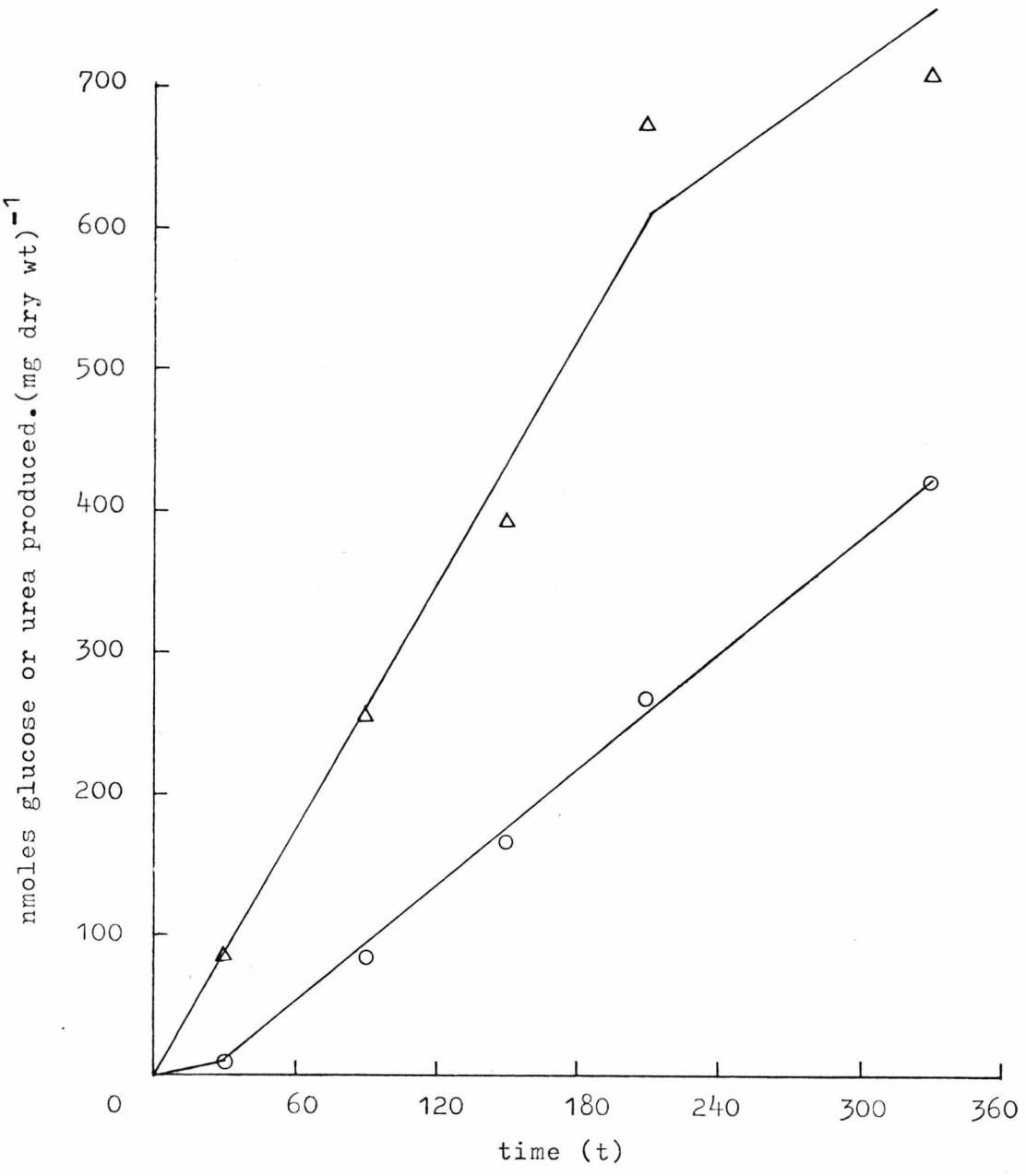
Fig 2:1 Gluconeogenesis from pyruvate: a comparison of methods of shaking



Cells isolated from 48h fasted rats were incubated in Krebs-Henseleit bicarbonate buffer supplemented with 2%(w/v) BSA. Glucose output was determined at the times indicated after the addition of pyruvate (5mM final). Points are the mean values of 3 determinations.

Method of shaking	Rate of gluconeogenesis (nmoles.(mg dry wt)$^{-1}$.h^{-1})
Reciprocal	142
Orbital	163
Magnetically stirred	170

Fig 2:2 Gluconeogenesis and urea synthesis from alanine



Cells isolated from a 48h fasted rat were incubated in Krebs-Henseleit bicarbonate buffer supplemented with 2%(w/v) BSA and a modified physiological amino acid mixture (East et al., 1973) containing 10mM alanine. Points are the mean values of three incubations.

o : glucose

Δ : urea

Rate of gluconeogenesis: 85 nmoles.(mg dry wt)$^{-1}$.h^{-1}.
Rate of urea synthesis: 176 nmoles.(mg dry wt)$^{-1}$.h^{-1}.

by the addition of $HClO_4$; after centrifugation at 2,000g for 15 min at room temperature, an aliquot of the supernatant was assayed. The assay reagent was:

0.5M Na phosphate pH 7.0)
0.005%(w/v) o-dianisidine in methanol) containing 0.1mg.ml^{-1} glucose oxidase [EC 1.1.3.4] and 0.1mg.ml^{-1} peroxidase [EC 1.11.1.7].

0.5ml of sample (containing 0 - 400 nmol glucose) was mixed with 2.5ml assay reagent and incubated at 37°C for 1h. Reagent blanks with glucose omitted and standard glucose determinations were carried out simultaneously. The absorbance at 437nm was measured.

ii) Lactate Dehydrogenase (LDH; Stinson and Gutfreund, 1971).

The preparation of cell extracts for enzyme assays is described in the appropriate chapters. The reaction medium contained:

0.1M Triethanolamine-HCl
1.0mM Na pyruvate (prepared just before use)
0.15mM NADH

Final volume 1.0ml; pH 7.4. The reaction was initiated by the addition of cell extract; blanks with pyruvate omitted were carried out simultaneously. The change in extinction at 340nm was measured, at 30°C.

E_M^{340nm} NADH = 6,220.

iii) Protein (Lowry et al., 1951)

Assay reagent:

Folin A — 0.2M Na_2CO_3
1.8mM K,Na tartrate
0.1M NaOH

Folin B — 3.5mM $CuSO_4.5H_2O$

Folin A and B were mixed 9 parts:1 part (v/v). 4ml of this reagent was mixed with 0.6ml sample (containing 0 - 300 µg protein) and left at room temperature for 10 min. 0.4ml Folin-Coicolteau reagent was added to each tube, mixed immediately and left at room temperature for 30 min. Reagent blanks (protein omitted) and standard protein determinations were carried out simultaneously. The absorbance at 750nm was measured.

iv) Urea and ammonia (Fawcett and Scott, 1960)

0.05ml urease [EC 3.5.1.5] (100 $U.ml^{-1}$ in 0.05M - Na phosphate, pH 6.5) was added to 0.1ml sample (pH 7.0). The tubes were covered and incubated for 20 min at 37°C. 1.25ml each of phenol reagent (0.106M - phenol, 0.17mM - Na nitroprusside) and hypochlorite reagent (11mM - Na hypochlorite, 0.125M - NaOH) was then added. After mixing, tubes were incubated for a further 30 min at 37°C. Reagent blanks and standard urea determinations were performed simultaneously. The optical density at 635nm was measured.

A similar procedure for the measurement of ammonia was used, but the incubation with urease was omitted.

B) Liquid scintillation counting

Samples for the determination of ^{3}H radioactivity were counted in a Packard Model 3375 liquid scintillation spectrometer using either:

i) 'Butyl PBD' scintillator:

0.6%(w/v)5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole, 8%(w/v) naphthalene, 40%(v/v) 2 methoxyethanol, 60%(v/v) toluene.

or ii) PCS scintillator (Hopkin and Williams, Chadwell Heath, Essex, U.K.).

ATP (Stanley and Williams, 1969)

Incubations were stopped by acidification with $HClO_4$ and precipitated protein removed by centrifugation. The supernatant was neutralised with 0.5M triethanolamine-HCl, pH 7.4 containing 2M- KOH.

The reagent mixture consisted of:

80mM $MgSO_4$
10mM phosphate (K^+), pH 7.4
100mM Na arsenate, pH 7.4

The solutions were mixed 1:1:1 with respect to volume. The assays were performed in 20ml glass scintillation vials which had been soaked in alkali and in acid to prevent photoemission. 5µl of neutralized cell extract (containing 20 - 80µmoles ATP) and 3ml assay reagent were mixed and left at room temperature for 10 min. Reagent blank (with ATP omitted) and ATP standard assays were prepared simultaneously. In the dark, 25µl Firefly luciferase extract (FLE-50) was added to each vial, and chemiluminescence measured immediately in a Packard 3375 liquid scintillation counter.

6) Statistics

"I can't believe that!" said Alice.
"Can't you?" the Queen said in a pitying tone.
"Try again: draw a long breath and shut your eyes".
Alice laughed. "There's no use trying", she said:
"one can't believe impossible things."
"I dare say you haven't had much practice", said
the Queen .... "Why sometimes I've believed as
many as six impossible things before breakfast".

Lewis Carroll in Alice through the Looking Glass

Statistical tests have been used throughout this thesis to provide an objective evaluation of experimental treatments. Results from replicate experiments are expressed as the arithmetic mean ($\bar{x}$) $\pm$ standard error of the mean (S.E.M.)

$$S.E.M. = \sqrt{\frac{\sum(x - \bar{x})^2}{N(N - 1)}}$$

where x = an observed value of the parameter
N = the number of observations

Students t test

This test was used when the means of two samples were to be compared. The following formula was used:

$$t_{(N_1+N_2-2)} = \frac{(\bar{x}_1-\bar{x}_2)}{\sqrt{S^2\left(\frac{1}{N_1} - \frac{1}{N_2}\right)}}$$

where 1 and 2 represent the samples being compared.
N_1+N_2-2 = number of degrees of freedom.

$$S^2 = \frac{1}{N_1+N_2-2} \left(\sum(x_1-\bar{x}_1)^2 + \sum(x_2-\bar{x}_2)^2\right)$$

If $N_1 = N_2$, then:

$$t_{(N_1+N_2-2)} = \frac{(\bar{x}_1-\bar{x}_2)}{\sqrt{(S.E.M._1)^2 + (S.E.M._2)^2}}$$

The probability (p) that the difference between two means was significant was determined by reference to the 't' values for (N_1+N_2-2) degrees of freedom (Clarke, 1969). A probability of <0.05 was considered significant.

Paired t test

This test is a derivation of Student's t test based on the differences between paired observations. The significance of proportional differences, rather than arithmetical differences were determined. The formulae used were:

$$t_{(N-1)} = \frac{\bar{d}}{s}$$

$$\bar{d} = \frac{\sum d}{N}$$

$$s = \sqrt{\frac{\Sigma(d)^2 - \frac{\Sigma(d)^2}{N}}{N(N-1)}}$$

where: d represents the difference between each paired observation
$\bar{d}$ is the arithmetic mean of the differences
N is the number of observations.

From probability tables the 't' values for (N-1) degrees of freedom were determined.

Analysis of variance

The analysis of variance allows the separation of data with respect to specific sources of variation. Therefore a two-way analysis of variance was used to assess the variation between animals as well as the variation between treatments. The test can be tabulated as follows:

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments	r-1	S_R	$S_R/r-1$
Animals	a-1	S_A	$S_A/a-1$
Error	(a-1)(r-1)	S_E	$S_E/(a-1)(r-1)$
Total	ar-1	S	

$$S = S_R + S_A + S_E$$
$$= \Sigma x^2 - \frac{(\Sigma x)^2}{N}$$

where: x represents an observed value of the parameter
N is the number of observations

$$S_R = \frac{1}{a} \cdot \Sigma(R)^2 - \frac{(\Sigma x)^2}{N}$$

where: a is the number of replicates
R is the sum of the replicates for each treatment.

$$S_A = \frac{1}{r}.\Sigma(A)^2 - \frac{(\Sigma x)^2}{N}$$

where: r is the number of treatments
A is the sum of the observed parameters for each animal

$$S_E = S - (S_R + S_A)$$

From these results, the variance ratio or F-ratio can be calculated:

$$\frac{\text{Treatments mean square}}{\text{Error mean square}} = F\left[(r-1), (a-1)(r-1)\right]$$

$$\frac{\text{Animals mean square}}{\text{Error mean square}} = F\left[(a-1), (a-1)(r-1)\right]$$

The probability that the difference between treatments or animals was significant was determined by reference to 'F' values for the degrees of freedom shown in square brackets (Clarke, 1969). The probability that the treatment means differed significantly was tested using the formula:

$$\text{Least significant difference (LSD)} = t_{(0.05,f)} \cdot \frac{2\sigma^2}{a}$$

where: $f = (a-1)(r-1)$

$t_{(0.05,f)}$ = the t value for f degrees of freedom with a probability of<5%.

σ^2 = error mean square

The treatment means were considered to differ significantly if the value of that difference was equal to or greater than the LSD.

Units

Wherever comparisons were made between parameters measured in isolated liver cells and those in intact liver, values were converted according to Seglen (1976b).

CHAPTER 3

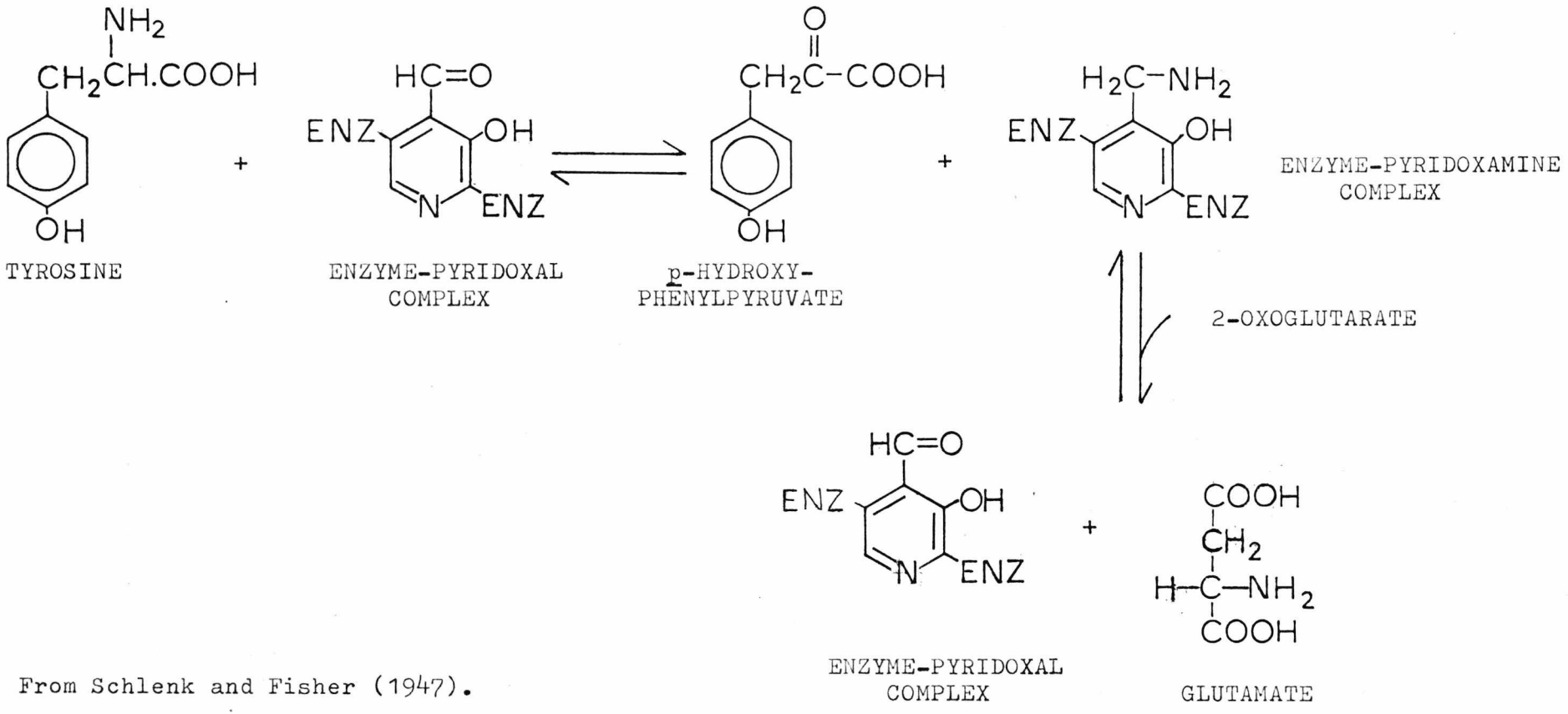
THE DEVELOPMENT OF A RADIOACTIVE ASSAY FOR TYROSINE AMINOTRANSFERASE

INTRODUCTION

Enzymic transamination of an amino acid was first reported in 1937, by Braunstein and Kritzman. Several years later it was established that all naturally-occurring amino acids could be transaminated (Braunstein, 1947; Cammarata and Cohen, 1950). It was evident that transaminases were vitamin B_6-dependent enzymes and an intrinsic role for the cofactor in a "shuttle" reaction mechanism was proposed (Schlenk and Fisher, 1947). This is illustrated, for tyrosine, in Fig 3:1; the α-amino group of tyrosine is first transferred to the enzyme-bound pyridoxal, then, in a reversal of this reaction, from the pyridoxamine to 2-oxoglutarate. There is evidence that transaminases bind their substrates sequentially, not simultaneously (Jenkins and Sizer, 1960).

The earliest assays for TAT were based on the estimate of glutamate (Cammarata and Cohen, 1950) or the absorbance of light by p-hydroxyphenylpyruvate (p-HPP) conjugated with colour reagents (Knox and Le May-Knox, 1951; Cannellakis and Cohen, 1956). p-HPP, itself, will absorb light at 300nm; the enol tautomer is responsible for this absorbance (Bücher and Kirberger, 1952), but $<4\%$ of the transamination product exists in this tautomeric form (Knox and Pitt, 1957). The keto-enol equilibrium was found to be displaced in favour of the enol-tautomer by the formation of an enol-borate complex (Fig 3:2); in consequence, one of the most sensitive spectrophotometric assays for TAT was developed (Lin et al., 1958b). This assay requires the exogenous addition of keto-enol tautomerase to ensure that tautomerisation is not rate-limiting. Critical of this need, Diamondstone (1966) developed an equally sensitive spectrophotometric assay; this was based on the

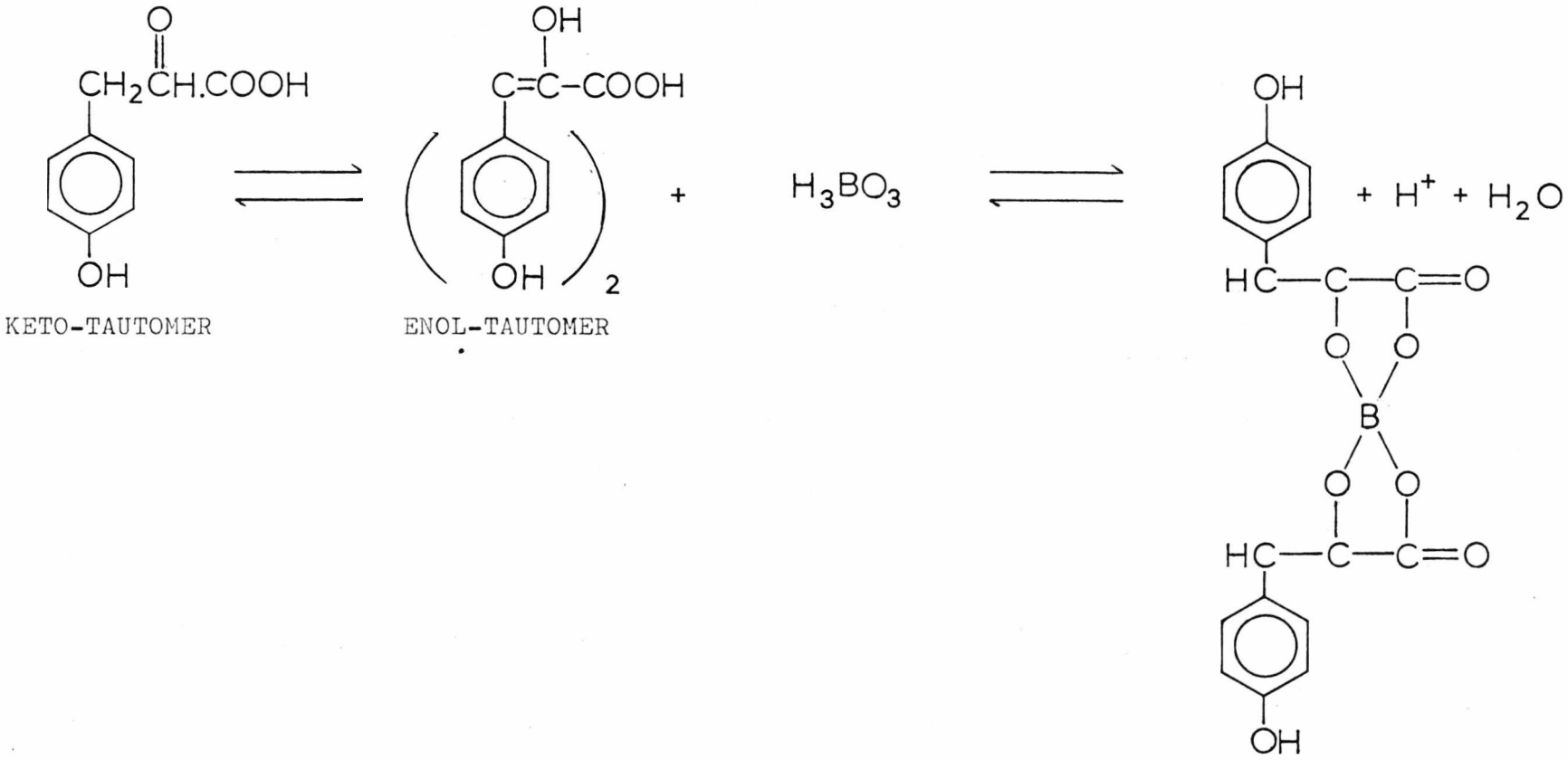
Fig 3:1 'Shuttle' Reaction Mechanism



From Schlenk and Fisher (1947).

It is suggested that pyridoxal phosphate attaches to the enzyme protein via the 2'methyl group and 5'-phosphorylated hydroxylmethyl group (Snell, 1972).

Fig 3:2 Tautomerization of p-HPP and formation of enol-borate complex



KETO-TAUTOMER

ENOL-TAUTOMER

From Knox and Pitt, 1957.

ENOL-BORATE COMPLEX

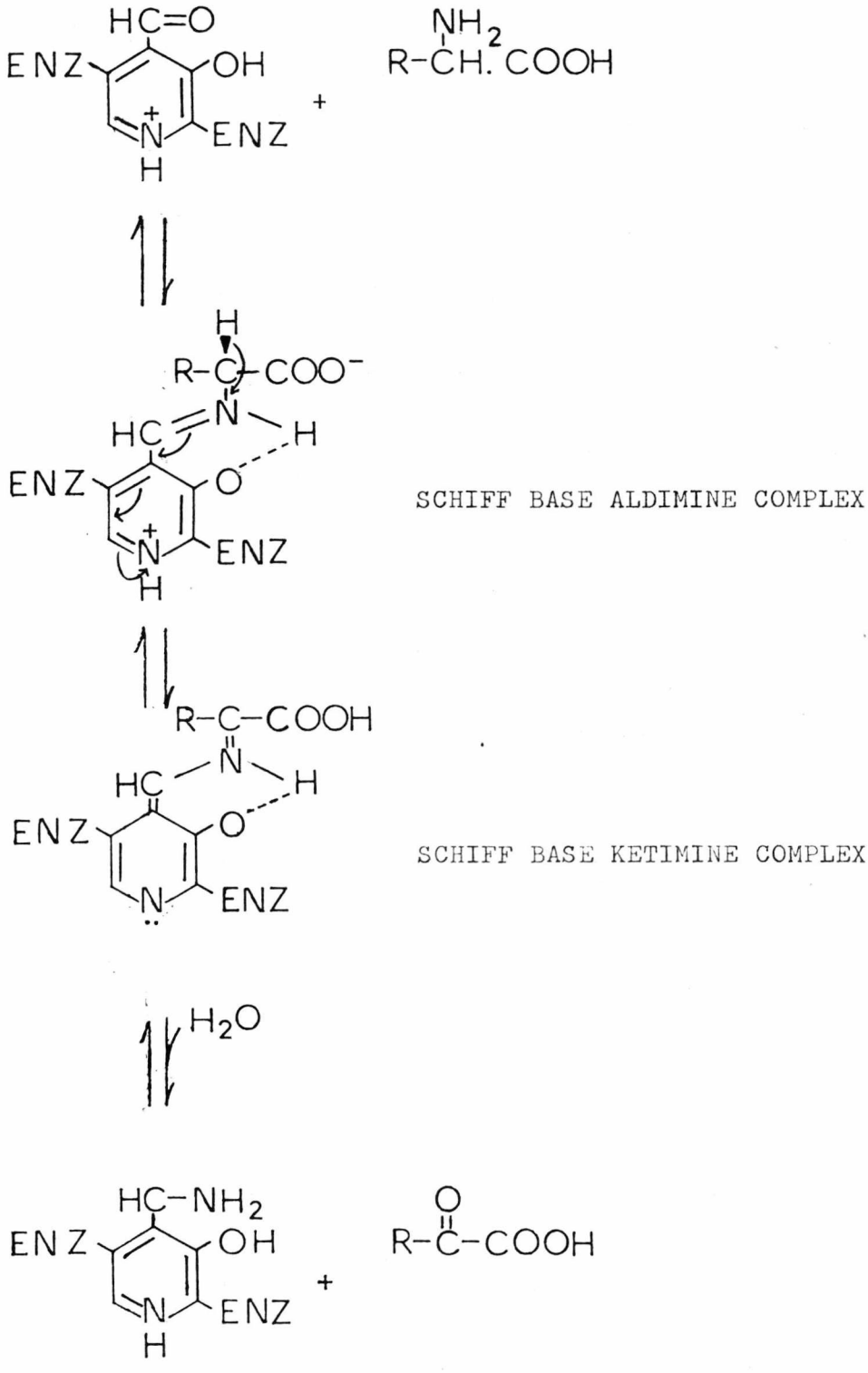
oxidation of p-HPP to p-hydroxybenzaldehyde (Hemmerle, 1917), which absorbs light at 331nm.

The sensitivity of spectrophotometric assays is limited by non-specific or background absorbance. This is a pertinent problem with TAT assays as absorbance in the region of 300nm is considerable in crude extracts. Radioisotopic assays obviate this limitation, but the radioactive metabolite to be measured must be isolated from the reaction mixture. The degree of separation is a major limitation to the sensitivity of the assay.

Radioactive assays for TAT have been developed using ^{14}C-labelled substrates (Gabay and George, 1967; Weinstein et al., 1967; Miller and Thompson, 1972) or ^{3}H-labelled substrates (Wurtman and Larin, 1968; Litwack and Squires, 1968). Reactants and products were separated by ion-exchange chromatography (Gabay and George, 1967; Weinstein et al., 1967; Litwack and Squires, 1968) or organic extraction (Wurtman and Larin, 1968; Miller and Thompson, 1972). The common intention in developing these assays was to achieve greater sensitivity than that offered by spectrophotometric assays; only that of Miller and Thompson realised this aim.

The spectrophotometric assay of Lin et al., (1958b) can be used to assay TAT in extracts from isolated rat liver cells. However, during the course of studies presented in this thesis, certain manipulations resulted in the dilution of cell extracts, necessitating the use of a more sensitive assay for TAT. Large numbers of incubations are established from a single preparation of isolated liver cells; an assay was therefore required which could be conveniently applied to the routine analysis of numerous samples.

Fig 3:3 General reaction mechanism for transaminases



R= amino acid side chain

From Braunstein, 1973.

While the requirement for sensitivity was fulfilled by the assay of Miller and Thompson, organic extraction was found to be a cumbersome procedure. In addition, great expense would be incurred by the routine use of a [^{14}C] isotope. This led to the development of the assay for TAT outlined in this chapter, using L-[side chain 2,3-^{3}H] tyrosine. By reference to the proposed mechanism for transamination (Fig 3:3) it can be seen that the C-2-^{3}H exchanges with the solvent during the reaction. Separation of the tritiated water ($^{3}H_2O$) was effected by adsorption of the [^{3}H] tyrosine and [^{3}H] p-HPP by charcoal.

MATERIALS AND METHODS

1) Animals and Dosing Procedure

Animals were maintained under conditions outlined in Chapter 2. Unless otherwise stated, animals were allowed access to food and water ad libitum. In some experiments, TAT activity was increased by i.p. injection of L-tryptophan (1g.(kg body wt)$^{-1}$) suspended in 0.9%(w/v)-NaCl, 0.1%(w/v)-Tween 80. Livers were removed three hours later and supernatant fractions were prepared as described below.

2) Preparation of Crude Extracts for Enzyme Assays

Animals were bled after decapitation. The livers were quickly removed, rinsed in 0.9%(w/v) NaCl, blotted gently and weighed. Using scissors, the tissue was minced finely in 4 volumes(w/v) of 0.1M-phosphate (Na^+) pH 6.9, containing 0.2mM-pyridoxal phosphate, and 1mM-dithiothreitol; then dispersed in a Potter-Elvehjem homogenizer. After centrifugation at 105,000g for 30 min, at 4°C, the supernatant was kept on ice and subsequently used for assays.

3) Tyrosine Aminotransferase Assays

i) Enol Borate - Tautomerase Assay (Lin et al., 1958b)

The assay mixture contained:

0.7 M Na borate
4.0mM Tyrosine
25.0mM 2-oxoglutarate
3.0mM Na diethyldithiocarbamate
40.0µM pyridoxal phosphate
10.0µl keto-enol tautomerase [EC 5.3.2.1] 93 Knox units.ml^{-1} (Constantsas and Knox, 1966).

Final volume of 1.0ml; pH 8.0. The cell extract was preincubated with pyridoxal phosphate (30μg.ml^{-1} extract) at 30°C for 5 min. before assay. The reaction was initiated by the addition of 2-oxoglutarate. Blanks (with tyrosine omitted) were run simultaneously. The increase in extinction at 310nm was measured.
E_M^{310nm} p-hydroxyphenylpyruvate-borate complex = 9850.
Unless otherwise stated, this spectrophotometric assay was used to measure TAT activity for comparative purposes, in this chapter.

ii) Conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde (Diamondstone, 1966).

Each tube (3.0ml glass test-tube) contained the following:

0.2mM phosphate (K^+)
4.0mM L-tyrosine
20.0mM 2-oxoglutarate
1.2mM pyridoxal phosphate
3.0mM EDTA
12.0mM Na diethyldithiocarbamate

Na diethyldithiocarbamate was added last. The components were preincubated for 5 min. at 30°C. The reaction was initiated by the addition of enzyme. Final volume 1.0ml; pH 7.6. Blanks, in which KOH was added prior to enzyme, were run simultaneously. The reaction was stopped by the addition of 0.1ml 10N-KOH, and mixed immediately. The tubes were left at 25°C for 30 min, after which the absorbance at 331nm was measured.
E_M^{331nm} p-hydroxybenzaldehyde = 19,900.

iii) Radioactive assay using L-[side chain 2,3-^{3}H] tyrosine

Unless otherwise stated, the standard assay medium (in 1.5ml conical 'Eppendorf' tubes) contained:

0.1M phosphate (K^+)
0.1M 2-oxoglutarate
0.8mM pyridoxal phosphate
0.05%(w/v) BSA
0.425mM L-tyrosine (containing 0.5µCi L-[side chain 2,3-^{3}H] tyrosine

Crude extract was added to a final volume of 50µl; pH 7.6. Tubes were preincubated for 5 min, at 30°C; the reaction was initiated by the addition of tyrosine, and stopped by the addition of 300µl of Norit GSX (20mg.ml^{-1} 0.06%(w/v) BSA). The tubes were mixed and centrifuged (immediately or after storage on ice) at 12,000g for 2 min at 4°C in an Eppendorf 3200 centrifuge. A portion of the supernatant (175µl) was counted with 1.0ml PCS in a minivial (Hopkin and Williams, Chadwell Heath, Essex, U.K.). Blanks, with enzyme omitted, were run simultaneously. 'Zero' time controls ($\pm$ enzyme) were performed by the addition of tyrosine subsequent to the charcoal.

4) Purification of L-[side chain 2,3-^{3}H] tyrosine

200µCi of L-[side chain 2,3-^{3}H] tyrosine, diluted to 2ml with double-distilled water, was adsorbed onto an Amberlite CG120(H^+) column. After washing, with several column volumes of water, the ^{3}H-tyrosine was eluted with 3ml of 2M - $HCOONH_4$. The eluate was lyophilized and stored at -70°C until used. When required the residue was dissolved in a tyrosine solution of appropriate concentration.

RESULTS AND DISCUSSION

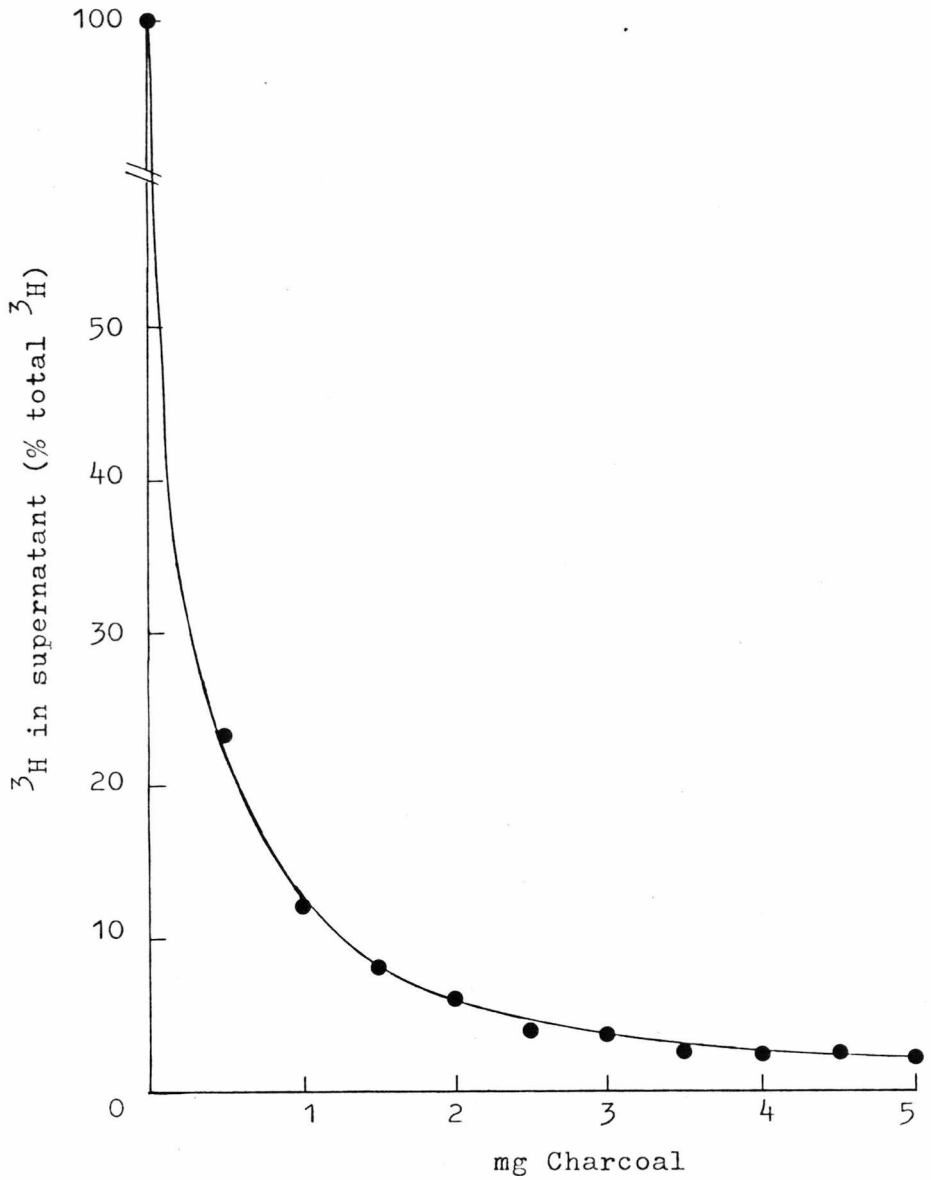
Activated charcoal will adsorb a variety of compounds, both hydrophilic and hydrophobic (Mantell, 1951; Hassler, 1963). While charcoal will readily adsorb aromatic ring structures, through non-polar interactions, some adsorption of water may also occur. However, the recovery of $^{3}H_2O$, formed during transamination was assumed to be 100%; this assumption is justified by the fact that the reaction medium contains a vast excess of unlabelled water.

Factors affecting the sensitivity of the assay

Charcoal suspended in a dilute solution of BSA was used routinely, as a more compact precipitate was obtained, on centrifugation, than with water alone. Charcoal will adsorb BSA, non-specifically, on its surface (Herbert *et al.*, 1968) effectively increasing the density of the particles. While this prevents further adsorption of large molecules, small molecules can penetrate the pores in the particle and are adsorbed on the inner surface.

The addition of 5mg charcoal to a standard assay mixture reduced the level of ^{3}H in the supernatant to approximately 2% of the total ^{3}H present (Fig 3:4). Greater amounts of charcoal did not reduce this background further. The fact that charcoal may not stop transamination completely was considered. In previous radioisotopic assays, acid conditions were effective in stopping the reaction. Using charcoal suspended in 2N - acetic acid, the background actually increased to $>10\%$ (Table 3:1). The acid may have interacted with the charcoal and as a result, its adsorptive properties may have been decreased. An alternative is that under acid conditions

Fig 3:4 Precipitation of L-[side chain 2,3-^{3}H] tyrosine with charcoal



L-[side chain 2,3-^{3}H] tyrosine was added to assay tubes after the addition of various amounts of charcoal. Each tube contained 0.2mU TAT (measured spectrophotometrically in crude extract). After centrifugation, the amount of ^{3}H remaining in the supernatant was measured. The results are expressed as a percentage of the total ^{3}H in the reaction mixture. Points represent the means of 3 determinations.

Table 3:1 The effect of acetic acid and L-glutamate on the precipitation of L-[side chain 2,3-^{3}H] tyrosine with charcoal

Time at 4°C (min)	^{3}H in supernatant (% total ^{3}H in reaction mixture)		
	0.06% BSA	0.2N-acetic acid	0.06% BSA + 1mM L-glutamate
0	0.89	14.0	0.85
5	0.75	12.3	-
10	0.75	12.0	0.78
15	0.71	11.7	0.82
30	0.77	12.1	0.68

L-[side chain 2,3-^{3}H] tyrosine was added to assay tubes after the addition of 6mg charcoal suspended in either 0.06% BSA, 0.2N-acetic acid, or 0.06% BSA containing 1mM L-glutamate. Each tube contained 0.2mU TAT (measured spectrophotometrically) in crude extract. Tubes were either centrifuged immediately or after maintenance at 4°C for the amount of time indicated and the amount of ^{3}H remaining in the supernatant measured. Values are the means of 3 determinations.

the ^{3}H-C bond is weakened and non-enzymic exchange with the solvent occurs rapidly. Inclusion of a high concentration of glutamate in the charcoal suspension had no effect on the level of the background (Table 3:1). Under all conditions, the background remained constant for at least 30 min (at 4°C) after charcoal additions (Table 3:1). These facts indicate that charcoal alone is effective in stopping the enzymic reaction.

The background is due, in part, to contaminants in the isotope solution, which can be removed by ion-exchange chromatography on Amberlite CG-120(H^+) resin as described in Materials and Methods (Table 3:2). Contaminants such as [^{3}H] alanine and [^{3}H] glycine, which would not be removed by this procedure, may contribute to the residual background.

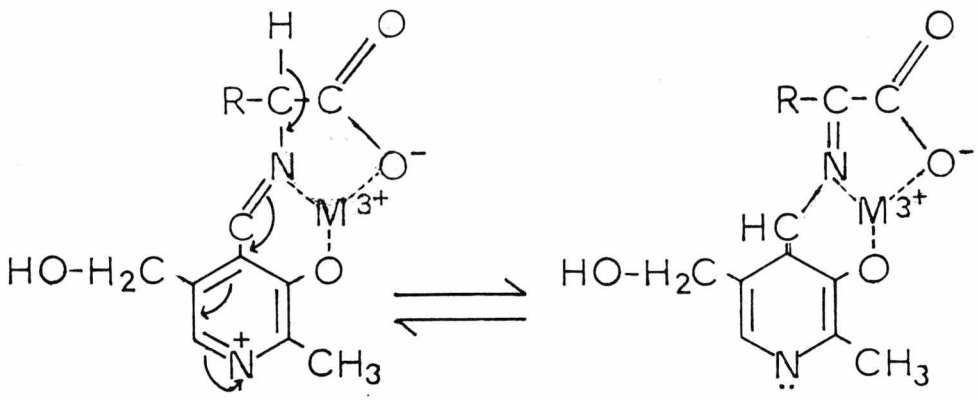
In the absence of crude extract, the background increases during the incubation of the reaction mixture at 30°C (Table 3:2). This rate of exchange of ^{3}H with the solvent is slow compared with that due to the enzyme catalysed reaction; it may be attributable to transamination catalysed by basic molecules in the medium. In model systems, imidazole, Fe^{3+} and Al^{3+} can all catalyse a low rate of transamination (Metzler et al., 1954; Bruice and Topping, 1963; Auld and Bruice, 1967); it is suggested that these molecules facilitate the formation of the Schiff base aldimine complex as shown (Fig 3:5). The background rate is reduced when purified [^{3}H] tyrosine is used (Table 3:2). Results obtained with crude extracts are identical using standard or purified [^{3}H] tyrosine; however purification reduces the background and potentiates the sensitivity of the assay. All subsequent studies presented in this chapter were performed using purified [^{3}H] tyrosine.

Table 3:2 Background rate and TAT activity measured using untreated and purified [^{3}H]tyrosine

Incubation time (min)	% total ^{3}H in assay medium	
	Untreated	Purified
0	0.43±0.01	0.36±0.02
30	1.10±0.02	0.48±0.02
60	1.23±0.06	0.55±0.03
Rate of TAT activity (μmole.min^{-1}.(g wet liver)$^{-1}$)	0.452±0.017	0.436±0.022

The background rate was measured using standard assay conditions, with crude extract omitted. All values are means ± S.D. of 6 observations.

Fig 3:5 Formation of a Schiff base aldimine complex and tautomerization in a non-enzymic system



ALDIMINE KETIMINE

R= amino acid side chain

M^{3+}= metal ion

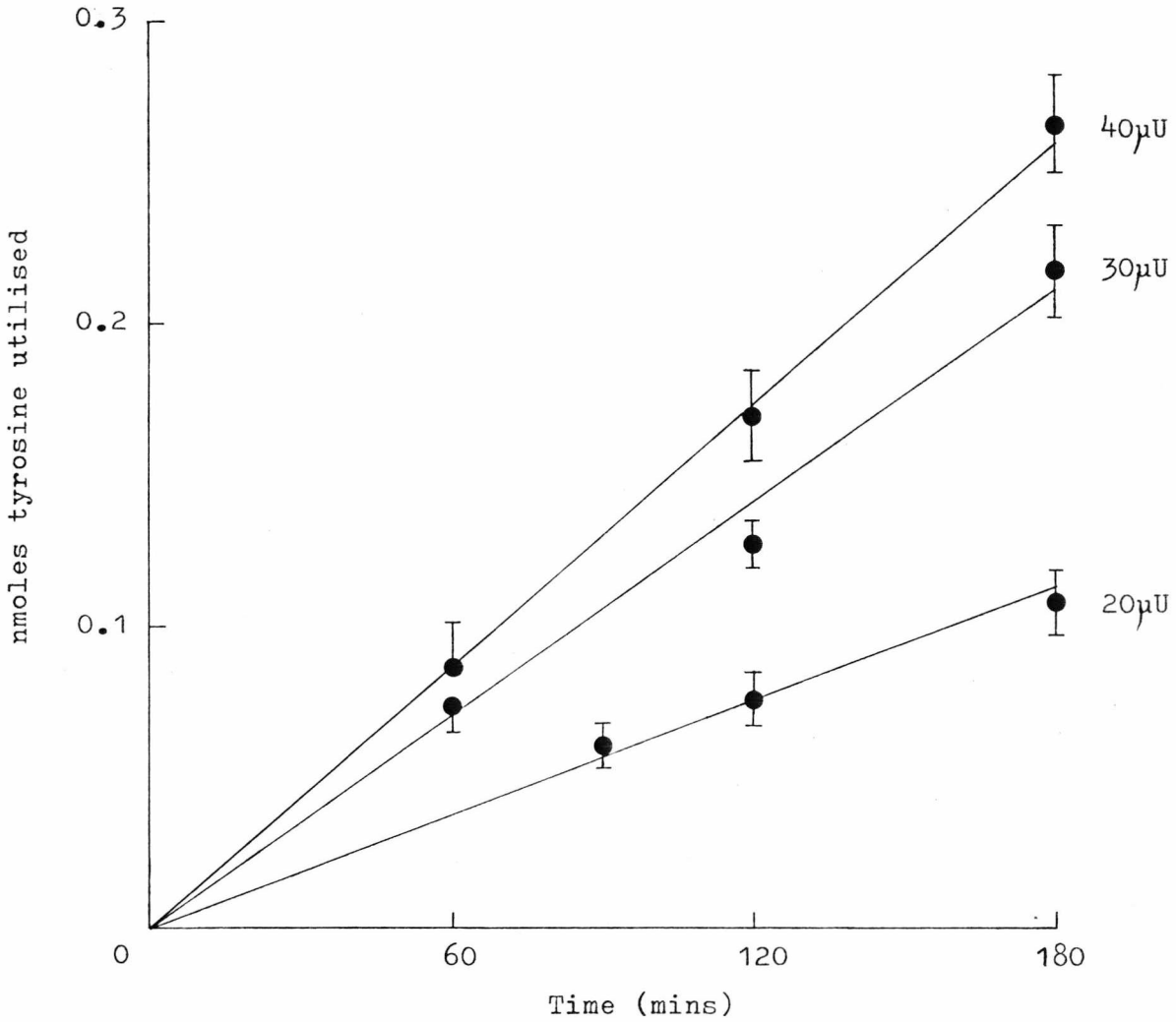
From Bender (1972).

Characterisation of the assay

The sensitivity of a radioisotopic assay is determined by both the level of the background and the specific activity of the labelled substrate. The standard assay mixture contained 0.5μCi L-[side chain 2,3-^{3}H] tyrosine. Using a concentration of 0.425mM tyrosine, the assay is linear with respect to time for a considerable range of enzyme concentrations (Figs 3:6 and 3:7). In addition, Fig 3:8 shows that the reaction rate is a linear function of the enzyme concentration in the crude extract. The lower limit of the most sensitive spectrophotometric assays for TAT is approximately 100μU (Miller and Thompson, 1972); with this assay, as little as 20μU TAT can be detected. This is comparable with the sensitivity achieved by the standard assay of Miller and Thompson (1972).

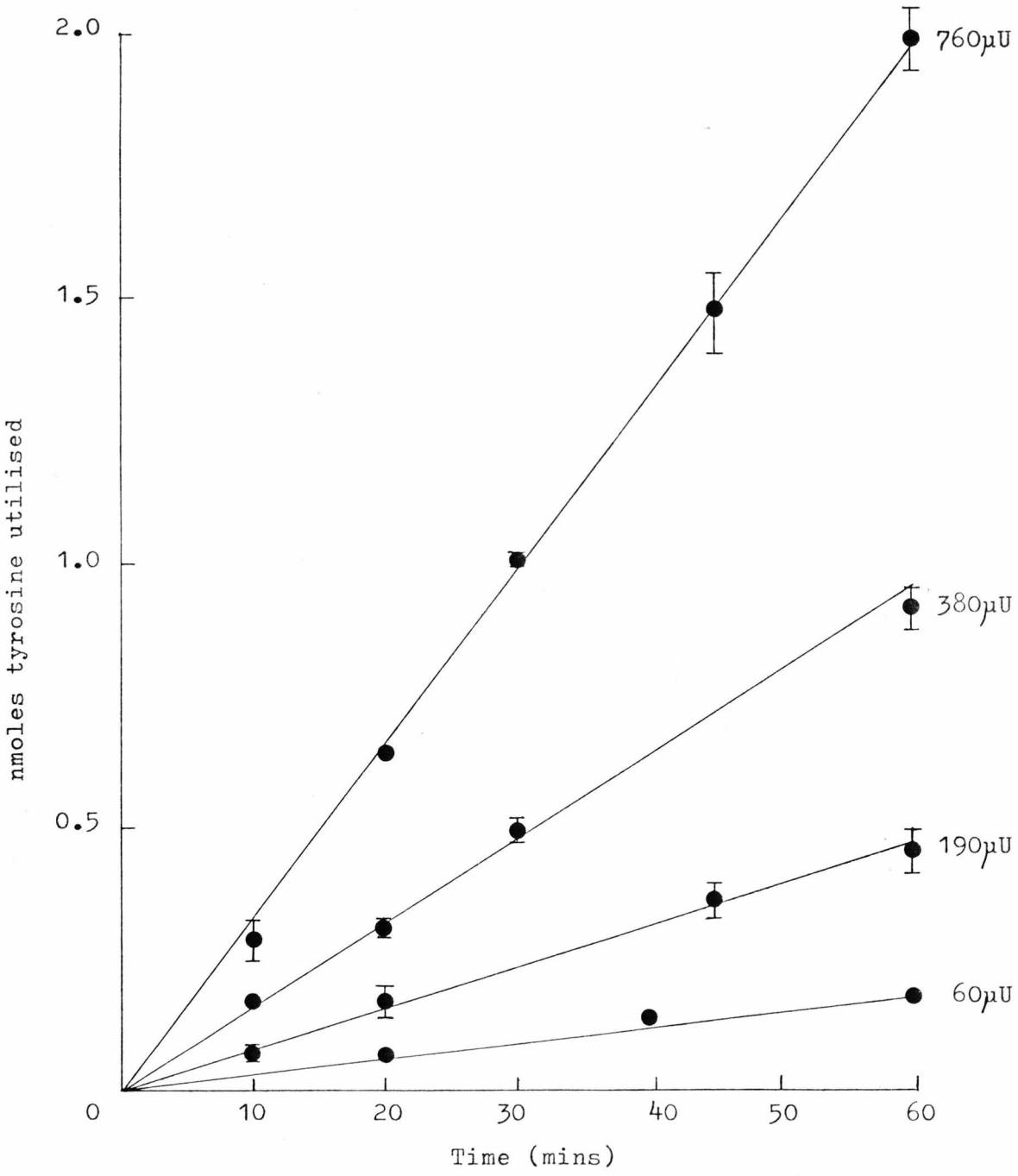
The rate of reaction was measured as a function of tyrosine concentration, maintaining a constant specific activity of tyrosine. A Lineweaver-Burk plot from these results (Fig 3:9) yields a K_m for tyrosine of 1.92mM, in good agreement with previously published values of 1.5 - 2.4mM (Jacoby and LaDu, 1964; Weinstein et al., 1968; Hayashi et al., 1967; Litwack and Squires, 1968). However, with this assay V_{max} is 40% of the value obtained by assaying the crude homogenate spectrophotometrically (Table 3:3). It has been suggested that tautomerisation, with the prototropic rearrangement it entails, is the rate-limiting step of transamination (Braunstein, 1973; Snell, 1963); there is evidence to support this from non-enzymic model systems (Bruice and Topping, 1963). It is likely that the observed discrepancy in the value of V_{max} is attributable to an isotope effect of between 2 and 3.

Fig 3:6 Time course of ^{3}H release from 0.425mM L-[side chain 2,3-^{3}H] tyrosine with various amounts of crude extract



TAT activities indicated were assayed spectrophotometrically. Points represent the means ± S.D. of 3 determinations.

Fig 3:7 Time course of ^{3}H release from 0.425mM L-[side chain 2,3-^{3}H] tyrosine with various amounts of crude extract



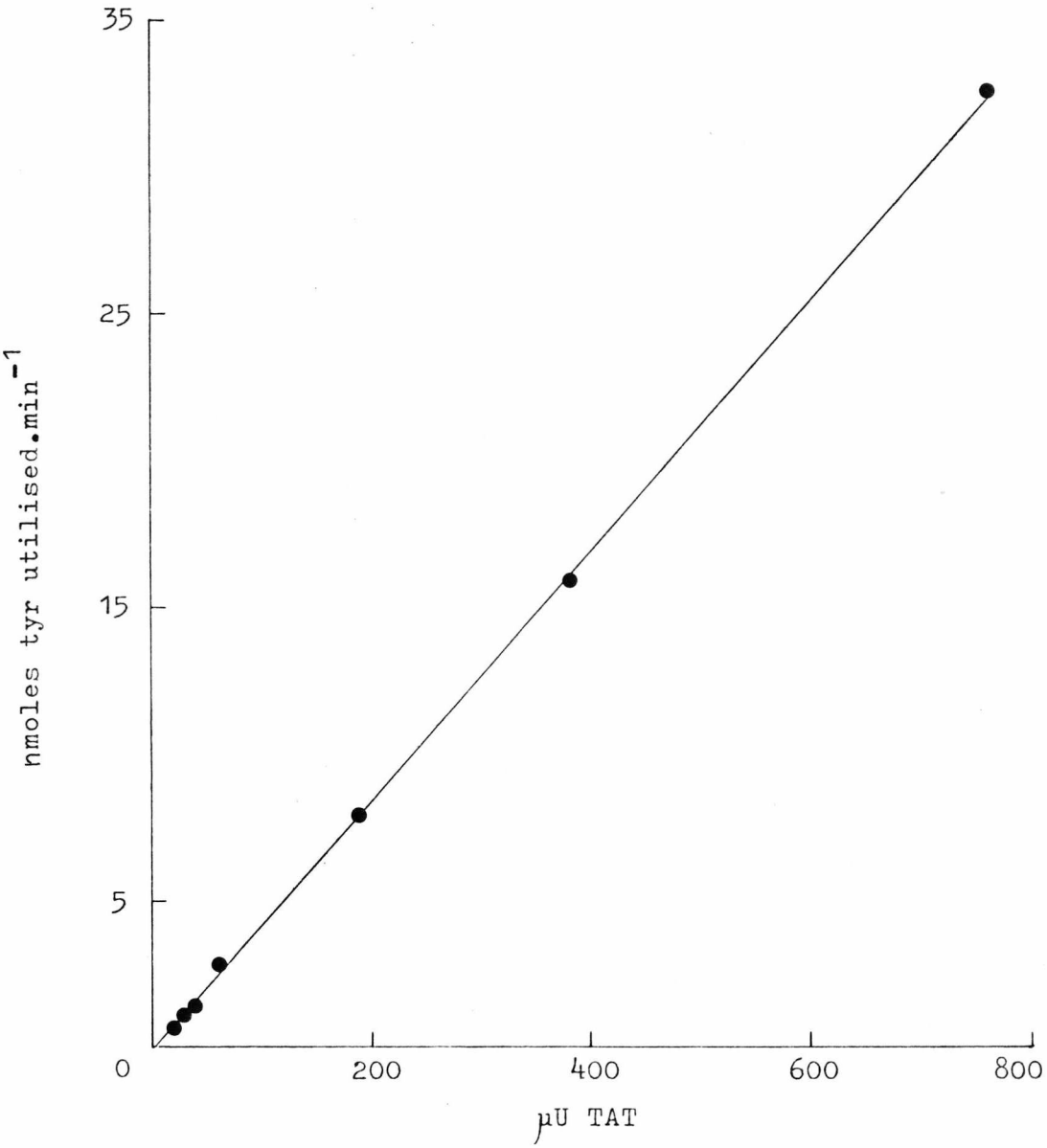
TAT activities indicated were assayed spectrophotometrically. Points represent the means ± S.D. of 3 determinations.

Table 3:3 Measurement of TAT activity in a crude extract using spectrophotometric and radioactive assays

Assay	[Tyrosine] in assay medium	Rate of activity ($nmoles.min^{-1}.(g\ wet\ liver)^{-1}$)
Spectrophotometric (Lin et al., 1958b)	4.0mM	410±18
Spectrophotometric (Diamondstone, 1966)	4.0mM	450±14
Radioactive	4.25mM	190±6
Radioactive	0.425mM	19±0.4

Details of the assay procedures were as described in Materials and Methods with the following exception: radioactive assays were terminated by the addition of 600µl Norit GSX ($100mg.ml^{-1}$). Values are means ± S.D. of 10 observations.

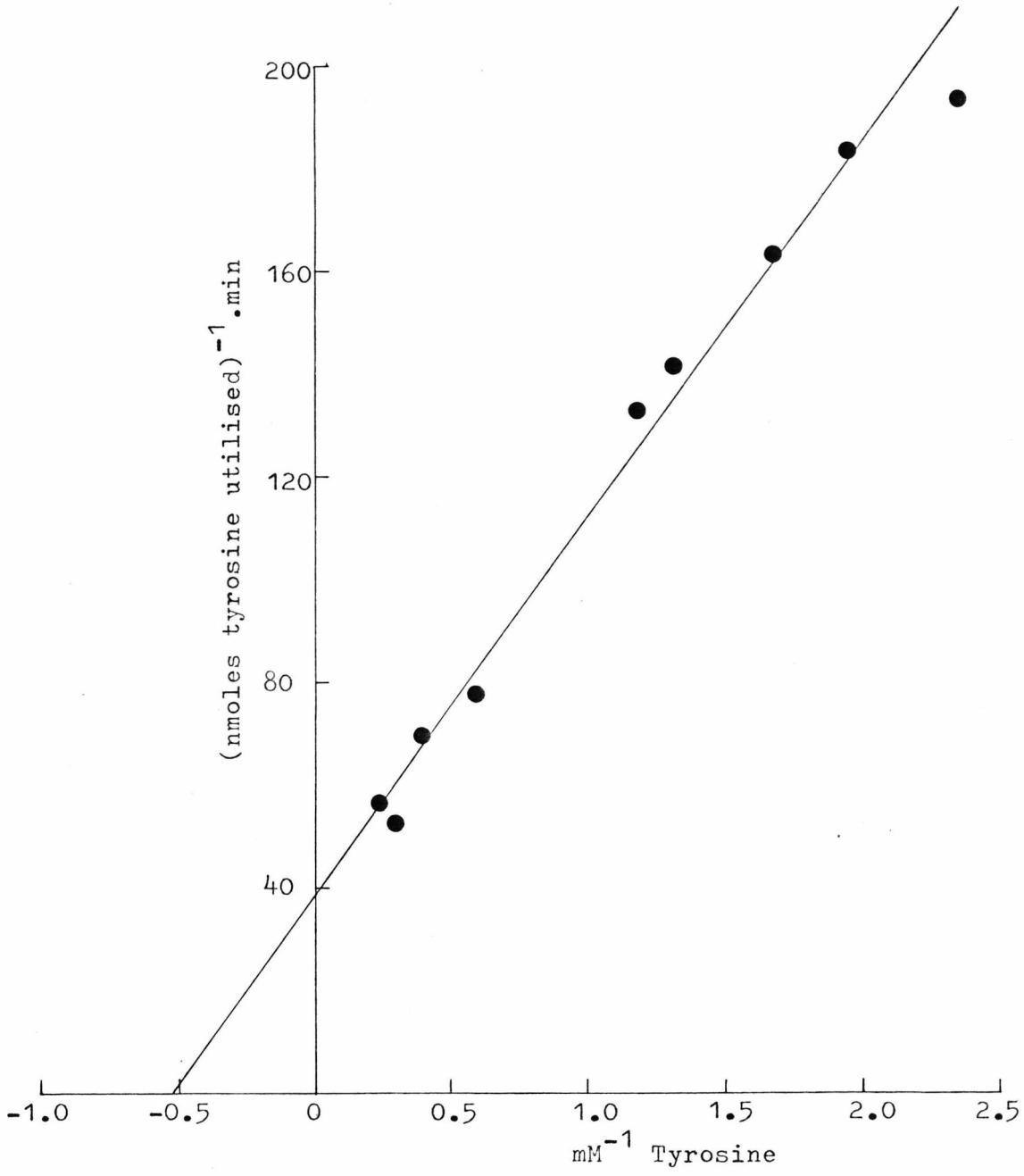
Fig 3:8 Effect of increasing enzyme concentration in crude extracts on the utilisation of 0.425mM L-[side chain 2,3-^{3}H] tyrosine



Enzyme activities indicated on the abscissa were determined spectrophotometrically.

Values are taken from Figs 3:6 and 3:7.

Fig 3:9 Lineweaver-Burk plot



^{3}H release was measured from reaction mixtures containing varying concentrations of L-[side chain 2,3-^{3}H] tyrosine of constant specific activity. Points represent the means of 3 determinations.

From the double-reciprocal plot (Lineweaver and Burk, 1934):

$$K_m \text{ (tyrosine)} = 1.92\text{mM}$$

It was proposed earlier that the ^{3}H in L-[side-chain 2,3-^{3}H] Tyrosine exchanged from both the C-2 and C-3 positions (Marston and Pogson, 1977). This has been shown to hold for glutamate-pyruvate transaminase (Oshima and Tamiyama, 1961; Babu and Johnston, 1974) and glutamate-oxaloacetate transaminase (Walter et al., 1975). It was shown that exchange of the C-3-H is an inherent part of the reaction mechanism and not, as had been suggested, a result of tautomerization of the oxo-acid product (Besmer and Anigon, 1968).

When the transamination of 0.425mM L-[side chain 2,3-^{3}H] tyrosine by TAT in a crude extract was allowed to go to completion, all the ^{3}H from the [^{3}H] tyrosine was lost (Table 3:4). The exogenous addition of keto-enol tautomerase had no effect. However, when transamination catalysed by pure TAT was allowed to go to completion, only half the ^{3}H from the [^{3}H] tyrosine exchanged with the solvent (Table 3:5). Exogenous addition of keto-enol tautomerase resulted in a further 20% of the ^{3}H exchanging with the solvent. This observation is consistent with the fact that approximately half the hydrogens at the C-3 position are replaced by tritium in L-[side chain 2,3-^{3}H] tyrosine; therefore when tautomerisation reaches equilibrium, approximately 25% of this ^{3}H should have exchanged with the solvent. The exchange of the C-3-^{3}H, in the presence of crude extract, is therefore not entirely the result of tautomerisation. Direct exchange of the tritium with the solvent may be catalysed by an enzyme present in the crude extract; this exchange may be facilitated during the translocation of the amino acid side chain in the formation of homogentisic acid from p-HPP.

Table 3:4 Measurement of the extent of exchange of ^{3}H with the solvent after the complete transamination of [^{3}H] tyrosine by TAT in a crude extract

Incubation time (min)	% total [^{3}H] in assay medium	
	TAT	TAT + KET
0	0.9	0.9
30	26	24
60	47	43
120	71	68
180	86	82
240	93	94
255	98	93
270	95	94

Where added, assay tubes contained 0.5 Knox units of keto-enol tautomerase (KET). Values are the means of 3 determinations. The experiment was performed using standard assay conditions; each assay tube therefore contained 21.25 nmoles tyrosine. The amount of TAT required to complete transamination in 5h was calculated from:

$$V = \frac{K_m + S}{t_{(0.98)}}$$

where: V = rate reaction

K_m = Michaelis constant for tyrosine

S = [tyrosine]

$t_{(0.98)}$ = time in minutes for the reaction to reach 98% completion

(from: Lowry and Passoneau, 1972)

Table 3:5 Measurement of the extent of exchange of ^{3}H with the solvent after the complete transamination of [^{3}H] tyrosine catalysed by pure TAT

Incubation time (min)	% total ^{3}H in assay medium	
	TAT	TAT + KET
420	51	70
450	51	69
480	52	70

Values are the means of 6 determinations. Where added, assay tubes each contained 0.5 Knox units KET. Pure TAT was provided by Dr. A. J. Dickson; the amount required to completely transaminate 21.25nmoles tyrosine in 6h was calculated as described in the legend for Table 3:4.

Results have therefore been calculated throughout on the basis of exchange of ^{3}H from both the C-2 and C-3 positions. The rate of reaction measured with this assay, using 0.425mM tyrosine can be related to V_{max}, measured spectrophotometrically, using a standard conversion factor; rates should be multiplied throughout by 19.6 ± 1.14 (6 observations).

That the participation of keto-enol tautomerase in this exchange reaction does not render this assay unsuitable for induction studies is shown by the results in Table 3:6. TAT activity was induced and measured either spectrophotometrically or radioactively; the assays yielded a similar value for the ratio of induced:basal activity.

Table 3:6 Measurement of increased TAT activity after L-tryptophan administration

Assay	Rate (μmole.min^{-1}.g liver^{-1})		Ratio (Tryptophan : Control)
	Control	+ Tryptophan	
Spectrophotometric	0.382	3.87	10.1 : 1
Radiometric	0.347$\pm$0.026	3.63$\pm$0.37	10.4 : 1

Activity in each extract was measured both spectrophotometrically and by release of $^{3}H_2O$ from L-[side chain 2,3-^{3}H] tyrosine. Rates (means $\pm$ S.D. for 10 observations) with the radiometric assay were calculated using the factor discussed in the text.

GENERAL DISCUSSION

Results obtained during the development of this assay have demonstrated that only the C-2-H of tyrosine is exchanged during transamination catalysed by TAT.

In the presence of enzyme protein, rates of transamination increase 6-fold (Fasella, 1967). The formation of an enzyme-substrate complex both orientates the reacting groups precisely and stabilises ionic intermediate forms during the reaction (Ivanov and Karpeisky, 1969). When alanine is transaminated it has been proposed that a more rigid enzyme-substrate intermediate is formed by the interaction of the side-chain methyl group in the active site (Babu and Johnston, 1974); subsequently a basic group in the active site removes hydrogen at the 3-position. A more extensive rate of exchange is obtained with alanine than with glutamate, during transamination; none is apparent with aspartate (Walter et al., 1975). It was suggested that the presence and proximity of the carboxyl group in the amino acid side chain affected the exchange of H at the C-3 position. It is therefore likely that the aromatic ring of tyrosine causes steric hindrance and prevents exchange of tritium at the C-3 position by TAT.

The basis of the assay presented in this chapter, the exchange of ^{3}H at the side chain C-2 position with water, has been used previously (Litwack and Squires, 1968). In essence, therefore, this assay is a refinement of the one previously described. The improvement is the use of charcoal to stop the reaction and isolate the $^{3}H_2O$; between 100 - 200 assays can be performed simultaneously by one person.

The assay described enables the detection of smaller amounts of enzyme in crude extracts than is possible with spectrophotometric assays. The sensitivity could be further increased

by a reduction of the background. This could be achieved by more extensive purification of the isotope. In addition the use of an isotope tritiated only at the C-2 position would eliminate any contribution to the blank rate of the exchange of the C-3-^{3}H with the solvent.

Subsequent to its development, this assay has been used routinely in the studies presented in this thesis. It is possible that the principle of this assay could be applied to the assay of any aromatic amino acid, provided a suitably labelled substrate could be obtained.

C H A P T E R 4

THE EFFECT OF HORMONAL AND NUTRITIONAL IMBALANCE ON TYROSINE AMINOTRANSFERASE ACTIVITY IN ISOLATED RAT LIVER CELLS

INTRODUCTION

The intracellular levels of proteins are determined by their relative rates of synthesis and degradation (Schimke, 1973; Goldberg and Dice, 1974). Proteins in the liver have diverse rates of degradation, the average half-life being 2 - 3 days (Goldberg and St. John, 1976). Hepatic TAT, with a half-life of 2 - 3h (Kenney, 1962b; Berlin and Schimke, 1965) has one of the most rapid rates of turnover. Teleologically, the advantage of this is that, in response to alterations in the synthetic rate, TAT can quickly adapt to a new intracellular level (Berlin and Schimke, 1965). A corollary is that the level of TAT can fluctuate rapidly in a changing physiological environment dictated by hormonal and nutritional stimuli.

The hypothesis that hormones could regulate cell function by altering the activities of enzymes (Green, 1941) has been substantiated by studies with a variety of mammalian tissues (Schimke and Doyle, 1970; Pitot and Yatvin, 1973). Hepatic TAT activity was first shown to be increased by the administration of hydrocortisone to intact and adrenalectomised rats (Lin and Knox, 1957). Several hormonal and nutritional agents have subsequently been shown to increase TAT activity; a summary is shown in Table 4:1.

Compounds administered *in vivo* have been found to induce TAT activity indirectly (Kenney and Flora, 1961; Labrie and Korner, 1968b), and the route of administration of a compound can greatly affect the response elicited (Yatvin and Pitot, 1970). These possibilities must always be taken into consideration when interpreting results obtained *in vivo*. The development of systems *in vitro* for studying hepatic enzyme

Table 4:1 A summary of the hormonal and nutritional agents which induce hepatic TAT activity

	IN VIVO	IN VITRO
Glucagon/*cyclic AMP	Adex (7)	Perfused liver (14) *Hepatoma tissue culture (HTC) cells (15) Hepatocytes (monolayer culture) (12)
Hydrocortisone	Intact (11) Adex (10,11)	Perfused liver (14) Reuber H35 hepatoma cells (13) Hepatocytes (monolayer culture) (1,12)
Dexamethasone	-	HTC cells (6) Reuber H35 cells (13) Hepatocytes (suspension) (5) Hepatocytes (monolayer culture) (3)
Insulin	Adex (7)	Perfused liver (4) Reuber H35 cells (9) Hepatocytes (monolayer culture) (1)
Tryptophan	Intact (2,8) Adex (2, 8)	- -
Amino acids	Intact (2) Adex (2)	- -

Adex = adrenalectomized.

Legend to Table 4:1 / Continued

References:

1. Bonney et al., 1974
2. Cihak et al., 1973
3. Davidson and Gerschenson, 1977
4. Hager and Kenney, 1968
5. Haung and Ebner, 1969
6. Hershko and Tomkins, 1971
7. Holten and Kenney, 1967.
8. Kenney and Flora, 1961
9. Lee et al., 1970
10. Levitan and Webb, 1970
11. Lin and Knox, 1957.
12. Michalopoulos et al., 1978
13. Reel et al., 1970
14. Seglen, 1970
15. Stellwagen et al., 1977
16. Wicks et al., 1972a

induction has served both to facilitate interpretation and to enable more detailed investigations of the mechanism of induction.

Cultured hepatoma cells have provided much information about the hormonal regulation of TAT activity (Table 4:1). However, these cells are intrinsically altered (Reel et al., 1970; Thompson and Gelehrter, 1971; Wicks et al., 1972a) and the results cannot be extrapolated to normal liver cells.

Suspensions of parenchymal cells, isolated by collagenase perfusion of the liver (Howard and Pesch, 1968; Berry and Friend, 1969; Elliott et al., 1976) retain the morphological and metabolic characteristics of cells in intact liver (Berry, 1974; Seglen, 1976b). Non-proliferating cultures of these hepatocytes, which have prolonged viability, have provided a system for the study of protein turnover (Edwards et al., 1977; Hopgood et al., 1977) and the induction of TAT activity (Bonney et al, 1974; Michalopoulos et al., 1978). The usefulness of this system is limited as parenchymal cells in culture have been found to lose chromosomes (Gerschenson et al., 1970), to de-differentiate (Lambiotte et al., 1972) and to become neoplastically transformed (Oshiro et al., 1972).

The integrity of isolated parenchymal cells suspended in simple salt solutions is limited to 2 - 3h, but incubation conditions have been defined in this laboratory which maintain their physical and metabolic integrity for up to 12h (Dickson and Pogson, 1977). In common with other isolated cell systems it has the technical advantages over the perfused liver system, but is unique in enabling the direct extrapolation of data to the normal liver cell in situ.

Using these conditions, a study is presented in this chapter of the regulation of TAT activity in enzymically isolated hepatocytes. The effect of the composition of the incubation medium on the turnover of TAT, as well as cell integrity has been studied. The hormonal and nutritional state of the rats from which the hepatocytes are isolated are compared with respect to hormonal response and are shown to differ markedly. Hormonal and nutritional induction of TAT activity is compared with results obtained previously *in vivo* and *in vitro*. During the course of this study isolated parenchymal cells in suspension have been used by other groups to study TAT activity (Crane and Miller, 1977; Ernest *et al.*, 1977) and a direct comparison is made with their results.

MATERIALS AND METHODS

1) Animals

Animals were maintained under conditions outlined in Chapter 2. Unless otherwise stated, access to food and water was allowed ad libitum.

2) Incubation media

The composition of the simple or complex media used was described in Chapter 2. One of the following media was used to supplement the basic nutrient medium:

i) Bovine serum albumin (BSA)

In all experiments, the BSA used had been defatted by the charcoal method of Chen (1967). To remove all traces of charcoal, the BSA was filtered first through a 5.0 μm Millipore filter and then through Celite. Finally the BSA was equilibrated against Krebs-Henseleit bicarbonate buffer ($CaCl_2$ omitted) on a sephadex G-25 column.

ii) Horse serum

Before use, the horse serum was dialysed against two changes of 15 volumes of Krebs-Henseleit bicarbonate buffer ($CaCl_2$ omitted) for 20h at 4°C.

iii) Foetal calf serum (FCS)

In some experiments, untreated foetal calf serum was used; however for the majority of experiments the serum was charcoal-treated and dialysed. 300 ml FCS and 10 g Norit GSX charcoal were stirred together for 30 min at room temperature.

The charcoal was removed by centrifugation at 2,000g for 30 min at room temperature. The FCS was filtered through Celite to remove charcoal fines, then dialysed as described earlier for horse serum. As a precaution against bacterial contamination the FCS was filtered through a 0.22 μm sterile Millipore filter and stored in sterile containers at -20°C until use.

3) Cell incubation conditions

0.2 ml portions of isolated parenchymal cell suspension were pipetted into 20 ml glass scintillation vials containing 1.3 ml incubation medium. The vials were stoppered with rubber Suba-seals, then gassed and incubated according to the procedure outlined in Chapter 2. The concentration of cells incubated was between 2 and 4 mg dry wt.ml^{-1} medium; this was reduced after initial experiments, as discussed in the results section, to approximately 1 mg dry wt.ml^{-1} medium.

Measurements of cellular ATP content and LDH leakage (see Chapter 2 for details) were used to assess the viability of each cell preparation.

4) Preparation of isolated liver cell extracts for enzyme assay

The cells suspended in incubation medium were transferred from incubation vials to Eppendorf centrifuge tubes and centrifuged at 50g for 2 min at room temperature. The medium was removed by aspiration and the cells resuspended in 0.2 ml extraction buffer. The composition of the buffers used was determined by the enzyme to be assayed:

i) Tyrosine Aminotransferase:

0.1M phosphate (Na^+) pH 6.9
0.2mM pyridoxal phosphate
1.0mM dithiothreitol

ii) Tryptophan Aminotransferase and Tryptophan 2,3-dioxygenase:

20.0mM phosphate (Na^+) pH 7.0
0.14M KCl

iii) Serine Dehydratase:

0.154M KCl
50.0mM phosphate (K^+) pH 7.4
1.0mM dithiothreitol
0.1mM 2-mercaptoethanol

iv) Phosphoenolpyruvate Carboxykinase:

0.25M sucrose
5.0mM Tris-HCl pH 7.4
1.0mM 2-mercaptoethanol

Enzyme was extracted from the cells by one of the following methods:

A) The cells were frozen rapidly at -70°C and thawed at 30°C, three times.

B) Extraction buffers containing 0.1%(w/v) digitonin or 0.2%(w/v) triton X-100 were used to resuspend the cell pellets. After 5 min at 4°C, the suspensions were centrifuged for 30 sec at 12,000g (Eppendorf Model 3200 centrifuge); the supernatants were retained for assay.

C) The cells were sonicated (MSE Sonicator, 150W) for 10s, three times, at 0°C.

Extracts prepared using methods A) and C) were centrifuged for $2\frac{1}{2}$ min at 12,000g. If enzyme assays were to be performed immediately, the supernatant extracts were maintained

on ice; alternatively, they were stored, overnight, at below -70°C and assays performed the next day.

5) Enzyme assays

All enzyme activities are expressed as μmole product formed.min^{-1}.(g wet wt cells)$^{-1}$ at 30°C (Phosphoenolpyruvate Carboxykinase, 37°C).

i) Tyrosine Aminotransferase [EC 2.6.1.5]

TAT was assayed using either the enol borate tautomerase method (Lin et al., 1958b) or radioactively with L side-chain 2,3-^{3}H tyrosine. Details of these assays were outlined in Chapter 3.

ii) Tryptophan Aminotransferase

The enol borate tautomerase assay of Lin et al., (1958b) was employed. The composition of the assay medium was:

0.7M Na borate
40.0mM tryptophan
6.7mM 2-oxoglutarate
40.0μM pyridoxal phosphate

Final volume of 1.0ml; pH 8.0. The cell extract was preincubated with pyridoxal phosphate (30μg.ml^{-1} extract) at 30°C for 5 min before assay. The reaction was initiated by the addition of 2-oxoglutarate (omitted in blank); the increase in extinction at 328nm was measured.

E_M^{328nm} indole pyruvate-borate complex = 14,000.

iii) Tryptophan 2,3-dioxygenase [EC 1.13.11.11]

Two methods were used to assay this enzyme:

a) Piras and Knox (1967)

In soluble liver extracts, tryptophan 2,3-dioxygenase exists largely as the inactive apoenzyme. In order to measure total enzyme activity the cell extract was preincubated at 37°C for 30 min in the following medium:

50.0mM phosphate (Na^+)
2.5mM tryptophan
0.5mg methaemoglobin
30mM Na ascorbate (freshly neutralized to pH 7.0)
0.5ml cell extract

Final volume of 1.0ml, pH 7.0.

In this medium, the apoenzyme is conjugated (methaemoglobin) and reduced (ascorbate) to form the active holoenzyme. The presence of tryptophan is required for both steps to occur.

An aliquot of the preincubation medium was assayed in:

45.0mM phosphate (Na^+)
3.3mM L-tryptophan
10.0mM Na ascorbate (freshly neutralized to pH 7.0)
0.2mg methaemoglobin

Final volume of 3.0ml; ph 7.0.

Blanks, with tryptophan omitted, were carried out simultaneously. The increase in extinction at 360nm was measured.

E_M^{360nm} kynurenine = 4530. Rat liver contains excess formylase which catalyses the conversion of N-formyl kynurenine to kynurenine (Schimke et al., 1965).

b) Schutz and Feigelson (1972)

The buffer used in the preparation of cell extracts for this assay additionally contained 10mM L-tryptophan.

To promote conjugation of the apoenzyme with haem, the supernatant extract was incubated for 5 min at 55°C, chilled in ice and centrifuged for 10 min at 12,000g to obtain a clarified supernatant. At this temperature, haem-binding proteins such as globin

and albumin are denatured and the availability of haem to bind with the apoenzyme is increased. Tryptophan is required to maintain the holoenzyme in the reduced state; inclusion of ascorbate produces no further increase in enzyme activity. The assay was modified by the inclusion of Kynurenine Formylase (purified as described below) to ensure that the formation of kynurenine from N-formyl kynurenine is not rate-limiting in the reaction.

The assay medium contained:

0.2M phosphate (Na^+)
4.6μM hematin
30.0mM L-tryptophan
2.0μl kynurenine formylase
(6 units.ml^{-1})

Final volume 1.0ml; pH 7.0. Blanks, with tryptophan omitted were run simultaneously. The increase in extinction at 360nm was measured. E_M^{360nm} kynurenine = 4530.

Purification of kynurenine formylase (formamidase) (Mehler and Knox, 1950).

All procedures were performed at 4°C. Rat livers were dispersed in 3 volumes(w/v) of 0.14M KCl, containing 2.5mM NaOH, and centrifuged at 11,000g for 20 min. The supernatant was acidified by the gradual addition of first 0.1 volume (v/v) 0.5M NaH_2PO_4, then 0.05 volume(v/v) 0.5M NaH_2PO_4 containing 1%(w/v) glacial acetic acid. The solution was centrifuged at 13K for 10 min, and the supernatant adjusted to pH 7.0 by the addition of 1.0N NaOH.

The neutralised supernatant was transferred to an Ehrlenmeyer flask, heated rapidly to 60°C in a water bath and maintained at this temperature for 5 min. The coagulated protein was removed by filtration through coarse grade filter

paper. The final step in the purification was the addition of solid ammonium sulphate to the filtrate, to give 45% saturation. After centrifugation at 10,000g for 10 min, ammonium sulphate was added to the supernatant to bring it to 68% saturation. The precipitate from this step was retained, dissolved in a small volume of water and dialysed extensively against water.

At each stage during the purification procedure, kynurenine formylase activity was assayed in a medium containing:

0.7mM L-formyl kynurenine (prepared by the procedure of Auerbach and Knox, 1957)
7.0mM phosphate (Na^+)

Final volume 3ml; pH 7.5. The reaction was initiated by the addition of enzyme (diluted with 0.14M KCl). Blanks, with L-formyl kynurenine omitted were run simultaneously. The increase in extinction at 360nm was measured.

E_M^{360nm} kynurenine = 4530.

Stage in purification	Specific Activity (μmoles kynurenine.(mg protein)$^{-1}$)
Homogenate	0.014
Acid precipitation	0.018
Heat precipitation	0.037
Ammonium sulphate (45% saturation)	0.034
Ammonium sulphate (precipitate after 68% saturation)	0.25

iv) Serine Dehydratase [EC 4.2.1.13]

The assay was performed according to the method of Cheung et al., (1969). The reaction medium contained:

20.0mM Tris-HCl
50.0mM KCl
0.6mM pyridoxal phosphate
0.15mM NADH
1.0mM dithiothreitol
2units lactate dehydrogenase
65mM serine

Final volume 1.0ml; pH 8.5.

Medium (serine omitted) and cell extract were preincubated together for 30 min at 30°C. The reaction was initiated by the addition of serine and the decrease in extinction at 340nm measured. Blanks, with serine omitted were run simultaneously. E_M^{340nm} NADH = 6220.

v) Phosphoenolpyruvate Carboxykinase [EC 4.1.1.32]

The enzyme was assayed by the method of Seubert and Huth (1965), as modified by Pogson and Smith (1975). The assay medium consisted of:

0.1M Tris-HCl
18mM $MgCl_2$
6mM ITP
15.0mM NaF
1.0mM reduced glutathione
4.5mM oxaloacetate

Final volume 1.0ml; pH 8.1.

The reaction medium was preincubated for 2 min at 37°C, before the assay was initiated by the addition of cell extract. After 10 min the reaction was stopped by the addition of 0.1ml potassium borohydride (50mg.ml^{-1} in 1mM NaOH); subsequently 10µl 20%(v/v) octan-2-ol, in ethanol, was added as an anti-foaming agent. Samples were chilled on ice for 2 - 3 min.

Excess borohydride was destroyed by the addition of 0.2ml 20%(w/v) $HClO_4$ and the precipitated protein removed by centrifugation. 1.0ml of supernatant was neutralized by the addition of approximately 0.2ml 0.5M-triethanolamine-HCl (ph 7.4) containing 2M-KOH; samples were mixed vigorously to prevent hydrolysis of phosphoenolpyruvate by alkali. Precipitated $KClO_4$ was removed by centrifugation; phosphoenolpyruvate in the supernatant was assayed in a medium containing:

0.1M triethanolamine-HCl
0.5mM ADP
0.15mM NADH
5.0mM $MgSO_4$
1.0unit pyruvate kinase
1.0unit lactate dehydrogenase

Final volume 1.0ml, pH 7.4.

The concentration of phosphoenolpyruvate was calculated from the decrease in extinction at 340nm, 5 min after the addition of the pyruvate kinase/lactate dehydrogenase mixture. Blanks with extract omitted were carried, simultaneously, through the entire assay procedure. The reaction was linear throughout the assay period.

6) Incorporation of L-[4,5-^{3}H] leucine into protein

A modification of the method of Mans and Novelli (1961) was employed. Standard cell incubation conditions were used. The assay medium was Krebs-Henseleit bicarbonate buffer supplemented with 10% dialysed horse serum and a physiological amino acid mixture (East et al., 1973). The medium contained 0.152mM L-leucine and 0.05μCi [^{3}H] leucine.ml^{-1}.

0.1ml cells suspended in incubation medium was pipetted on to a Whatman No.3MM filter paper disc, and dried in a stream of warm air. Each disc was placed in an ice-cold solution of 10%(w/v) trichloroacetic acid (TCA) containing 0.1M L-leucine; this stopped the reaction, diluted out the [^{3}H] leucine and precipitated the protein into the matrix of the disc. After storage for 60min at 4°C in this solution, the discs were washed three times with 25ml 5%(w/v) TCA in a Millipore filtration apparatus. After drying in air, each disc was counted for ^{3}H radioactivity in 5ml Butyl PBD scintillator.

Statistical analysis

To minimise differences which result from animal variation, some of the data in this chapter have been normalised as follows:

$$\frac{S.A._{(t)}}{S.A._{(o)}} = R.S.A.$$

where: $S.A._{(o)}$ = initial specific activity

$S.A._{(t)}$ = specific activity at time t

R.S.A. = relative specific activity

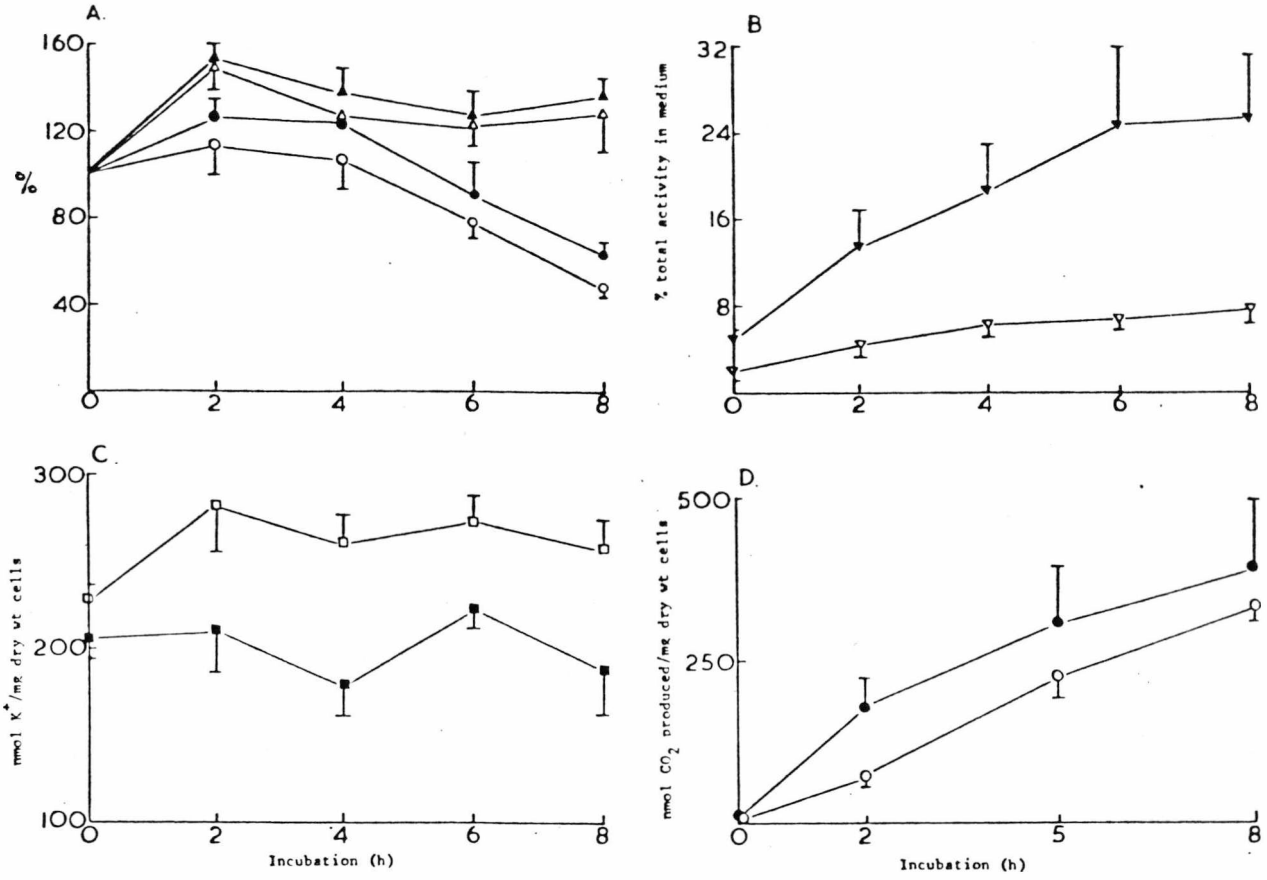
RESULTS AND DISCUSSION

1) Incubation media suitable for protein turnover studies in isolated liver cells

Incubation of isolated hepatocytes in a simple defined medium facilitates the interpretation of experimental results. Isolated hepatocytes incubated in Krebs-Henseleit bicarbonate buffer supplemented with 2%(w/v) BSA synthesise glucose at a constant rate for at least 4h (Fig 4:1). Studies in this laboratory have shown that the physical and metabolic integrity of cells incubated in this medium declines after 3 - 4h incubation, but is sustained in a more complex incubation medium (Fig 4:2).

Minimum essential medium (MEM), a bicarbonate buffer supplemented with physiological concentrations of glucose, amino acids, vitamins and cofactors, will alone not sustain the viability of isolated hepatocytes (East et al., 1973; Grant and Black, 1974). Supplementation of the medium with serum is also necessary (Crane and Miller, 1974; Jeejeebhoy et al., 1975). Uptake of serum factors by hepatoma cells has been demonstrated (Kaminskas, 1975) and serum factors have been shown to both prolong the survival of normal liver cells (Pickart and Thaler, 1973) and improve the response of perfused liver (Ohtsuka, 1970) and isolated cells (Siess and Wieland, 1975) to hormones. Supplementation of MEM with Foetal Calf Serum (FCS) or BSA is compared in Table 4:2. Initial cellular ATP levels are similar in both media. In the FCS-supplemented medium, ATP content increases between 0 - 2h and is maintained at this level until 8h; while in the BSA-supplemented medium the ATP content decreases between 4 - 8h. This is accompanied by leakage of cytoplasmic LDH between 6 - 8h which is not

Fig 4:2



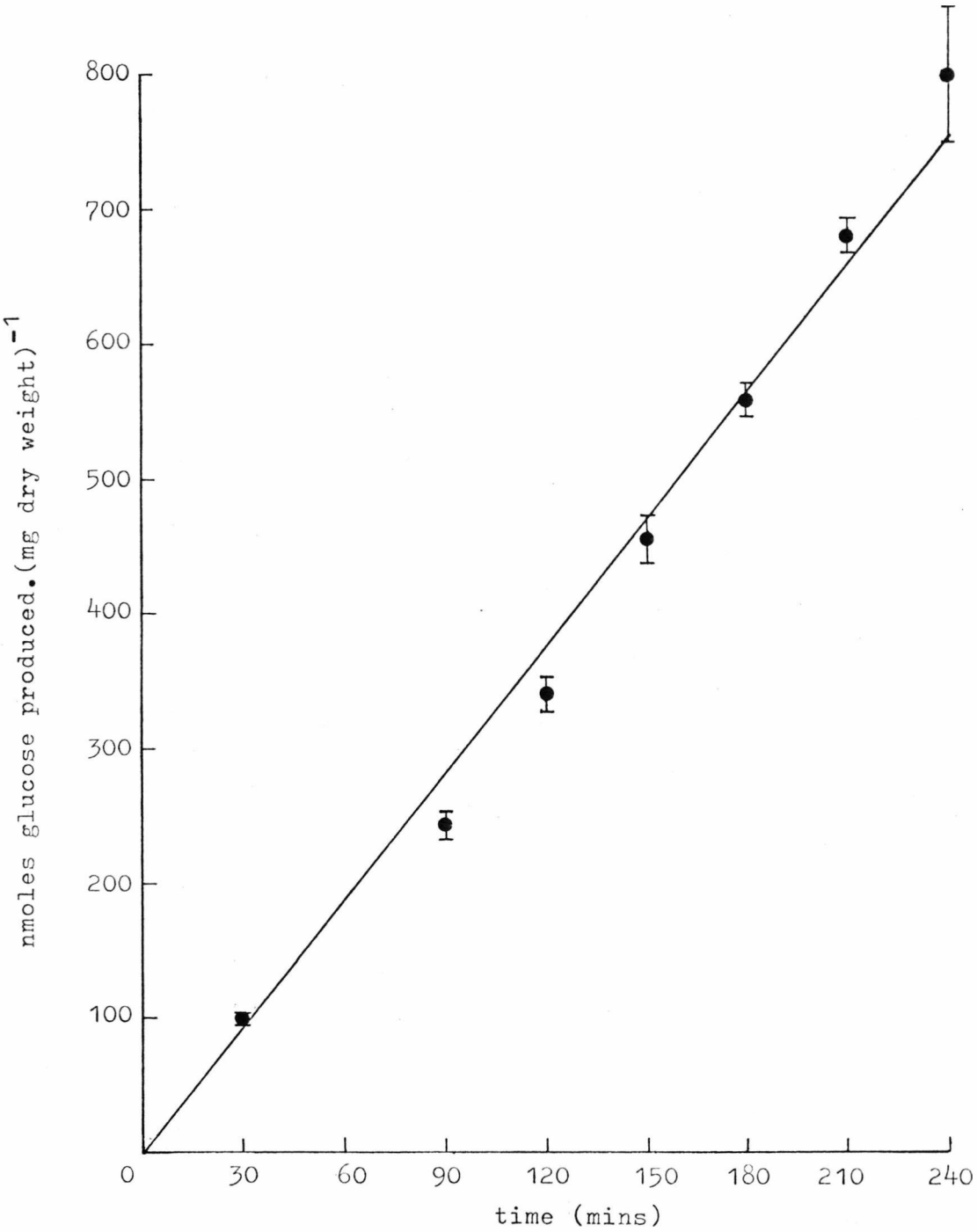
Alteration of metabolic characteristics of hepatocytes incubated in media A and B. Each point represents the mean of 3 experiments ± SEM. (A) ATP: (•) Medium A; (▲) Medium B and total adenylate; (○) Medium A; (△) Medium B contents. (B) Lactate dehydrogenase leakage: (▼) Medium A; (▽) Medium B. (C) K^+ content: (■) Medium A; (□) Medium B. (D) Conversion of [1,5-^{14}C] citrate to $^{14}CO_2$. Final spec. act. citrate 1.53 μCi/μmol. (●) Medium A; (○) Medium B.

Medium A = Krebs-Henseleit bicarbonate buffer supplemented with 2% (w/v) BSA.

Medium B = MEM supplemented with 10% (v/v) FCS.

Taken from Dickson and Pogson, (1977).

FIG 4:1 GLUCOSE PRODUCTION BY ISOLATED RAT LIVER CELLS



Cells were isolated from a 48-hour fasted rat and incubated in Krebs-Henseleit bicarbonate buffer supplemented with 2%(w/v) BSA (2.6mg dry weight cells.ml^{-1} final) under an atmosphere of O_2:CO_2 (95%:5%) at 37°C. Glucose output was measured at the times indicated after the addition of pyruvate (5mM final) and points are means $\pm$ S.D. (3 determinations).

Rate of gluconeogenesis = 189 nmoles glucose/h/mg dry weight.

Table 4:2 A comparison of MEM (Eagle's) supplemented with 2%(w/v) BSA or 10%(v/v) FCS

Incubation time (h)	ATP		Lactate dehydrogenase		TAT	
	BSA	FCS	BSA	FCS	BSA	FCS
0	6.4	7.0	247	311	0.27	0.27
2	6.5	9.1	229	309	0.27	0.43
4	6.7	8.8	247	300	0.24	0.38
6	5.7	8.8	239	290	0.19	0.32
8	4.7	9.8	196	312	0.057	0.11

Cells were isolated from a fed rat and incubated in MEM supplemented with serum, as indicated, at a final concentration of 4.3mg dry wt.cells.ml^{-1}. Values are the means from triplicate incubations. ATP levels are expressed as nmoles.(mg dry wt. cells)$^{-1}$; units of enzyme activity are μmoles.min^{-1}.(g wet wt. cells)$^{-1}$.

Table 4:3 A comparison of MEM (Eagle's) supplemented with untreated FCS or charcoal-treated, dialysed FCS

Incubation time (h)	ATP		TAT	
	Untreated	Treated	Untreated	Treated
0	15.0	14.9	0.70	0.69
1	-	-	1.10	1.02
2	16.5	19.4	1.52	1.38
3	-	-	1.43	1.05
4	15.7	14.9	1.49	0.84

Cells were isolated from a fed rat and incubated in MEM supplemented with 10%(w/v) FCS as indicated, at a final concentration of 2.0mg dry wt.ml^{-1}. Values are the means of triplicate incubations.

ATP concentrations are expressed as nmoles.(mg dry wt.)$^{-1}$; units of enzyme activity are μmoles.min^{-1}.(g wet wt. cells)$^{-1}$.

parallelled in the FCS-supplemented medium; this indicates a loss of physical and metabolic integrity of hepatocytes in the BSA-supplemented medium after 4 - 6h. Horse serum has been found as efficient as FCS in maintaining the integrity of isolated liver cells in suspension (Crane and Miller, 1977) while, in contrast, others have found horse serum more efficient than FCS (Jeejeebhoy et al., 1975). This may represent batch variation in sera.

Between 0 - 6h, TAT activity undergoes a transient increase in the FCS-supplemented medium only. Insulin or glucagon induce a transient increase in TAT activity in vivo and in vitro (Holten and Kenney, 1967; Hager and Kenney, 1968). A comparison was therefore made between untreated FCS and FCS which had been charcoal-treated (see Materials and Methods) and subsequently dialysed against Krebs-Henseleit bicarbonate buffer (Table 4:3). Dialysis alone does not affect the ability of FCS to maintain the integrity of isolated hepatocytes (Dickson and Pogson, 1977) and charcoal treatment, in addition, has no effect as can be seen from the ATP values (Table 4:3). The transient increase in TAT activity is reduced, but not abolished, by treatment of the FCS. The amount of charcoal used was in excess of that required to remove physiological concentrations of hormones. The factor responsible, in part, for this transient increase may be tightly bound to serum protein or of high molecular weight.

TAT activity decreases in BSA-supplemented and FCS-supplemented medium after 6h (Table 4:2). This decrease cannot be entirely attributed to leakage in the BSA-supplemented medium, and may be due to enhanced degradation or inactivation of TAT.

In vivo and *in vitro*, hepatic protein degradation is increased by the limitation of nutrients (Woodside *et al.*, 1974; Garlick *et al.*, 1975); the degradation of TAT, in particular, is enhanced in hepatoma cells cultured in medium devoid of amino acids or serum (Hershko and Tomkins, 1971). To reduce the possibility of the exhaustion of essential nutrients, the cell concentration in incubations was reduced in subsequent experiments.

2) The effect of hormonal imbalance on TAT activity.

To minimise the effect of the four-fold diurnal variation of TAT activity (Hardeland, 1972; Wurtman, 1974) all cell suspensions were prepared between 9 and 11 a.m. Despite this precaution, considerable variation between animals is seen in basal TAT activity and in the response to hormonal imbalance.

The activities of all enzymes are expressed relative to the wet weight of cells, using a dry:wet weight ratio of 1:3.7 (Seglen, 1976b). Measurements were made of enzyme activity in cells alone, to enable an estimate of cytoplasmic enzyme leakage. The contents of incubation vessels were transferred to tubes to permit the separation of cells and medium by centrifugation. Occasionally it was found that, after extended periods of incubation, some cells had adhered to the walls of the incubation vessels. It was necessary to resuspend them before transferring the contents of each vessel to a centrifuge tube. It is likely that some cell breakage and enzyme leakage occurred as a direct result of this procedure. This problem did not arise in the estimation of cellular ATP content; the procedure employed does not require the separation of cells and medium (see Materials and Methods). Under these conditions cellular ATP content gives a more accurate evaluation of cell viability than leakage of cytoplasmic enzyme.

In the preparation of isolated liver cells, there is a 10 - 20 min period after the administration of the anaesthetic before the blood-flow through the liver is interrupted. During this time it is possible for endogenous hormones to interact with hepatocytes in intact liver; metabolic reactions may be initiated which affect TAT activity subsequently in the isolated liver cells. To investigate this, the response of isolated parenchymal cells from rats in various hormonal and nutritional states was compared. The concentration of all hormones used approach or are within the physiological range (Gray and Bacharach, 1967).

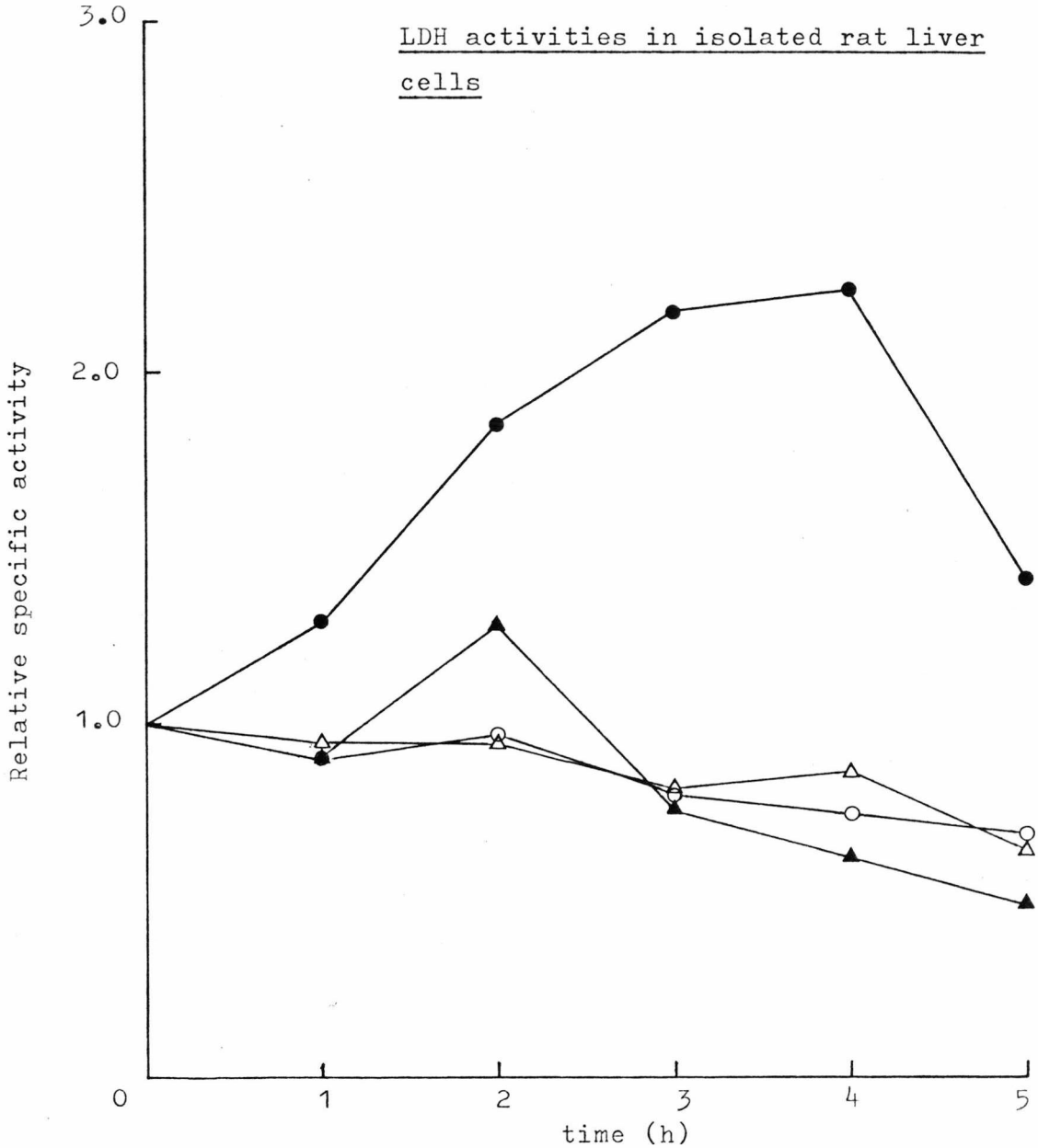
A) Isolated hepatocytes from fed, adrenalectomized rats.

Adrenal hormones produced in response to stress can complicate the interpretation of the effects of exogenously added inducers (Rosen and Milholland, 1963; Kenney and Albritton, 1965). Adrenalectomy has no effect on the activity of TAT in isolated cells (Tables 4:4 and 4:10). In vivo, adrenalectomy has either no effect on TAT activity (Rosen and Milholland, 1963) or results in a decrease in activity (Lin and Knox, 1957; Wicks et al., 1969). Adrenalectomy has previously been reported to decrease TAT activity in isolated liver cells (Crane and Miller, 1977).

i) The effect of hydrocortisone on hepatic enzyme activities.

Hydrocortisone induces a significant increase in TAT activity within 2h (Fig 4:3; Table 4:4). A transient increase in the control activity also occurs between 1 - 3h. Induction by

Fig 4:3 The effect of Hydrocortisone on TAT and LDH activities in isolated rat liver cells



Values are means from 3 separate cell batches (see Table 4:4); S.E.Ms have been omitted for clarity. All other details as given in the legend to Table 4:4.

-▲- control TAT activity

-△- control LDH activity

-●- hydrocortisone-treated TAT activity

-○- hydrocortisone-treated LDH activity

Table 4:4 The effect of Hydrocortisone on TAT and LDH activities in isolated liver cells

Time (h)	TAT				LDH			
	Control		Hydrocortisone		Control		Hydrocortisone	
	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.
0	0.38±0.08	1.0	-	-	239±6	1.0	-	-
1	0.34±0.07	0.91±0.03	0.44±0.08	1.29±0.43	228±6	0.95±0.03	216±28	0.90±0.13
2	0.47±0.07	1.28±0.09	0.67±0.10	1.85±0.15*	227±42	0.95±0.2	231±40	0.97±0.19
3	0.29±0.09	0.76±0.07	0.84±0.20	2.17±0.49*	196±19	0.82±0.09	192±15	0.80±0.08
4	0.25±0.09	0.62±0.08	0.85±0.2	2.23±0.21***	209±52	0.87±0.30	180±13	0.75±0.04
5	0.19±0.06	0.49±0.07	0.57±0.2	1.41±0.22**	155±7	0.65±0.02	165±5	0.69±0.01

Hepatocytes were isolated from fed, adrenalectomized rats and incubated in Krebs-Henseleit bicarbonate buffer supplemented with 17.5%(v/v) dialysed horse serum, and a physiological amino acid mixture (East et al., 1973). Values are the means ± S.E.M. from 3 separate cell preparations.

The specific activities (S.A.) of TAT and LDH are expressed as μmoles.min^{-1}.(g wet wt. cells)$^{-1}$. Relative specific activity (R.S.A.) is the specific activity at a given time expressed relative to the initial or

(Legend to Table 4:4 / Continued)

Oh specific activity.

The probability (p) that the hormonally induced enzyme activity was significantly different from the appropriate control activity was tested using Students t test.

* $p < 0.05$

** $p < 0.02$

*** $p < 0.01$

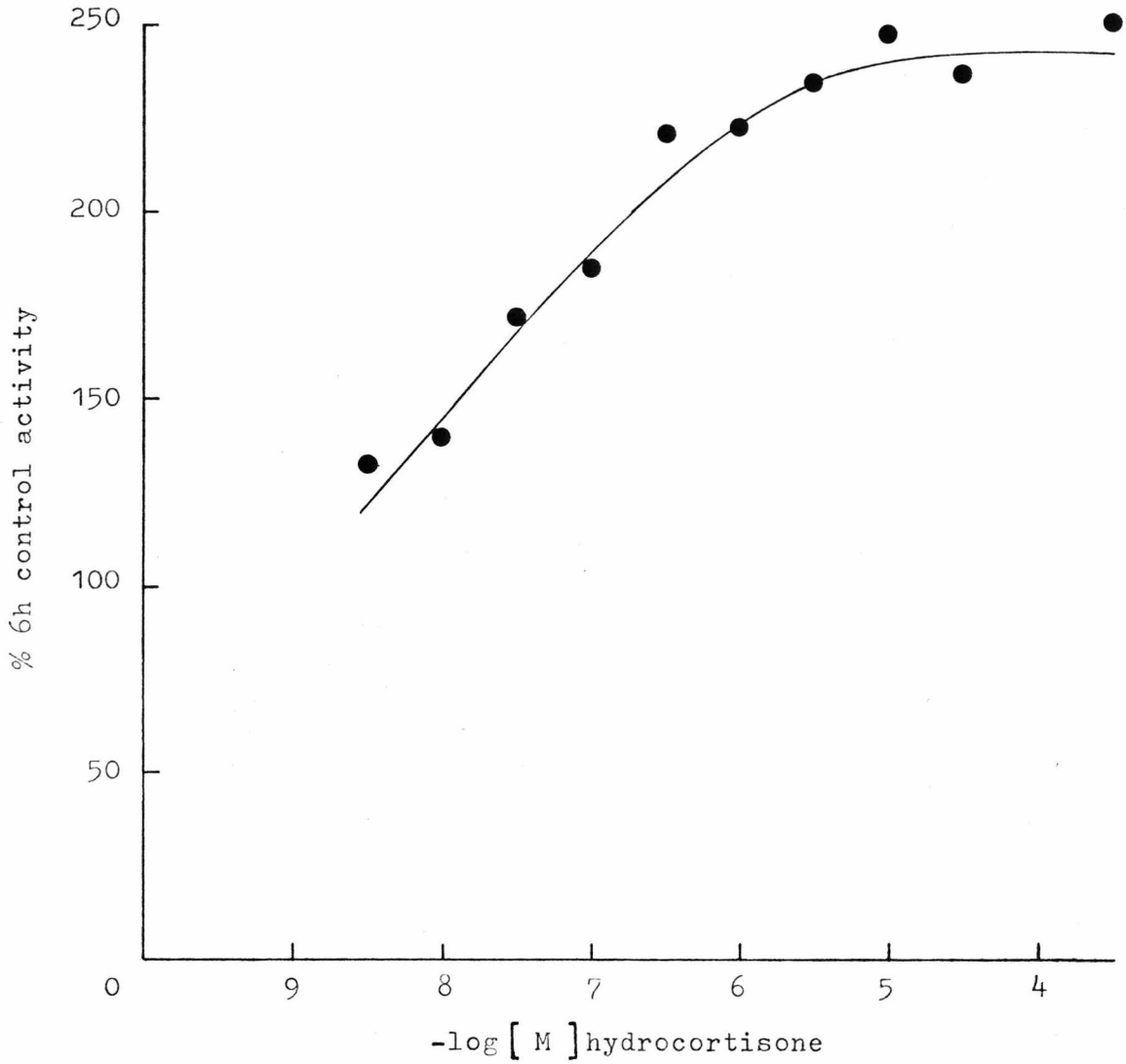
hydrocortisone increases to a maximum by 4h and decreases rapidly between 4 - 5h to a level still significantly different from the control. This pattern of induction was obtained previously with isolated cells from an adrenalectomised rat (Crane and Miller, 1977) and is similar to that seen *in vivo* after the administration of a single injection of hydrocortisone (Levitan and Webb, 1970).

Immunochemical analysis has shown that induction by hydrocortisone *in vivo* is due to increased synthesis and inhibition of degradation (Kenney, 1962a; Levitan and Webb, 1970). The decrease in the induced level of TAT is due to a decrease in the induced synthetic rate. Further administration of steroid during this phase results in the re-induction of TAT activity (Grossman and Mavrides, 1967). In perfused liver, continuous infusion of hydrocortisone will maintain the induced level of TAT activity (Hager and Kenney, 1968). These facts suggest that the continued presence of hormone will maintain induced TAT activity and that hydrocortisone is metabolised by hepatocytes *in vivo* and *in vitro*. In some hepatoma cells in culture, hydrocortisone maintains an induced level of TAT for up to 36h (Lee *et al.*, 1970; Reel *et al.*, 1970) suggesting that the steroid degradation mechanism is impaired.

A dose response of TAT activity to hydrocortisone is shown in Fig 4:4. A concentration of 3.16×10^{-9}M will significantly increase TAT activity in 6h; 10^{-5}M is the lowest concentration which results in maximum induction of TAT.

Hydrocortisone does not appear to increase the synthesis of proteins in general, as LDH activity in the same cell preparations is not increased (Table 4:4). In addition synthesis of soluble cell protein, measured by the incorporation

Fig 4:4 Dose-response curve for hydrocortisone



Hepatocytes were isolated from an adrenalectomized rat and incubated in MEM supplemented with 10%(v/v) charcoal-treated, dialysed FCS and hydrocortisone as indicated (added at the beginning of the incubation period (0h)). Values are the means of triplicate incubations; standard deviations were <5%.

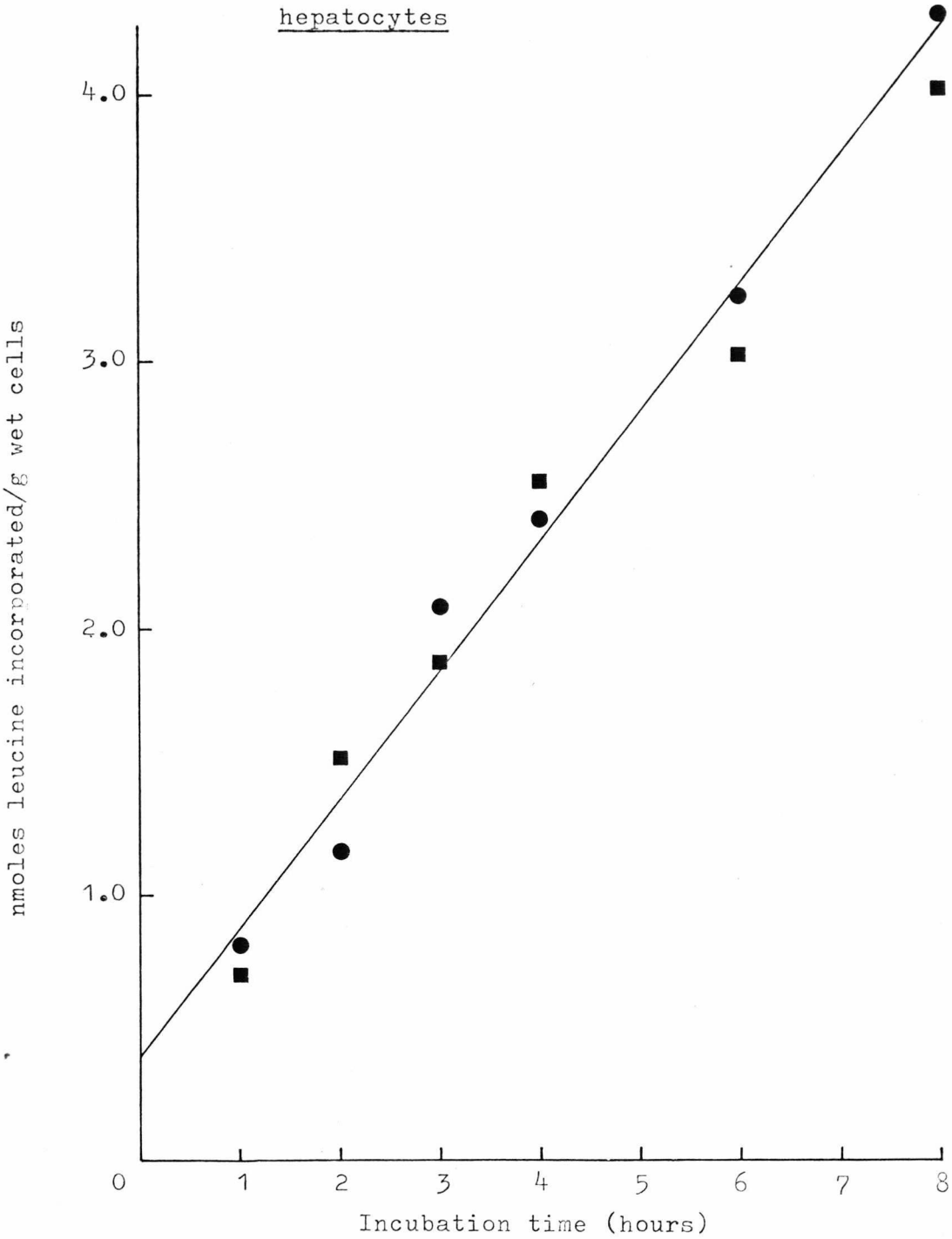
ATP content at 6h = 9.4 ± 0.2 nmoles.(mg dry wt. cells)$^{-1}$

Final cell concentration = 1.2mg dry wt.ml^{-1}

of leucine, is not altered by inducing concentrations of hydrocortisone (Fig 4:5). The rate of protein synthesis is linear for up to 8h, in agreement with previous observations (Jeejeebhoy et al., 1975). The calculation of amino acid incorporation is based on the extracellular specific activity of leucine. However, the data cannot be interpreted quantitatively as there is evidence to suggest that amino acids used for protein synthesis do not originate exclusively from the intracellular or extracellular pools (Vidrich et al., 1977; Seglen and Solheim, 1978a).

Administration of hydrocortisone in vivo specifically induces the activities of the amino acid metabolizing enzymes Tryptophan Aminotransferase (Lin et al.,1958b), Serine Dehydratase [EC 4.2.1.13] (Pitot and Peraino, 1964; Cihak et al., 1975), Tryptophan 2,3-dioxygenase [EC 1.13.1.12] (Knox and Mehler, 1951; Feigelson and Greengard, 1962), and the key gluconeogenic enzyme Phosphoenolpyruvate Carboxykinase [EC 4.1.1.32] (Wicks et al., 1974; Krone et al., 1974). In isolated hepatocytes, during 5h incubation, hydrocortisone elicited no significant increase in the activity of Tryptophan Aminotransferase (Table 4:5), Serine Dehydratase (Table 4:6), or Phosphoenolpyruvate Carboxykinase (Table 4:7). Using the assay for Tryptophan 2,3-dioxygenase of Schutz and Feigelson (1972), no activity was detectable in extracts from isolated hepatocytes. With the assay of Piras and Knox(1967), the change in optical density in the absence of exogenously added tryptophan was equal to or greater than that with tryptophan added. As a result, no data was obtained on the effect of hydrocortisone on Tryptophan 2,3-dioxygenase in isolated liver cells. The effect of dexamethasone on Tryptophan 2,3-dioxygenase in isolated cells has been reported (Berg et al., 1972); however assays were performed on large amounts of concentrated cell extracts. The development of

Fig 4:5 The effect of hydrocortisone on the incorporation of L- 4,5-^{3}H leucine into TCA-precipitable protein in isolated hepatocytes



Cells were isolated from a fed, adrenalectomized rat and incubated as described in Materials and Methods. Hydrocortisone (10^{-5}M) was added at the beginning of the incubation period (0h). Final cell concentration = 3.75mg dry wt. ml^{-1}. Values are the means of triplicate incubations.

(Legend to Fig 4:5 / Continued)

Incubation time (h)	TAT activity in the presence of hydrocortisone relative to control
0	1.00
2	1.68
4	1.57
6	2.32
8	3.33

—●— control

—■— 10^{-5}M hydrocortisone

Table 4:5 The effect of hydrocortisone on Tryptophan Aminotransferase in hepatocytes isolated from adrenalectomized rats

Incubation time (h)	Control		Hydrocortisone	
	S.A.	R.S.A.	S.A.	R.S.A.
0	0.062 ± 0.002	1.0	-	-
1	0.073 ± 0.005	1.18 ± 0.09	0.08 ± 0.02	1.29 ± 0.3
2	0.068 ± 0.008	1.09 ± 0.11	0.058 ± 0.004	0.94 ± 0.09
3	0.046 ± 0.008	0.74 ± 0.11	0.059 ± 0.013	0.95 ± 0.09
4	0.036 ± 0.003	0.59 ± 0.05	0.056 ± 0.011	0.90 ± 0.17
5	0.051 ± 0.007	0.83 ± 0.13	0.086 ± 0.027	1.37 ± 0.39

The cells isolated were incubated in Krebs-Henseleit bicarbonate buffer supplemented with 17.5%(v/v) dialysed horse serum and a physiological amino acid mixture. Hydrocortisone (10^{-5}M) was added at the beginning of the incubation period (Oh). Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$.
Values are means from 3 separate cell batches $\pm$S.E.M.;
LDH activities are as shown in Table 4:4.
Using Students t test the mean RSA for control and hydrocortisone-treated incubations were compared; the values do not differ significantly at any incubation time.

Table 4:6 The effect of Hydrocortisone on Serine Dehydratase and LDH activities in isolated hepatocytes from adrenalectomized rats

Incubation time (h)	Serine Dehydratase				LDH			
	Control		Hydrocortisone		Control		Hydrocortisone	
	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.
0	0.39 ± 0.05	1.0	-	-	283 ± 46	1.0	-	-
1	0.36 ± 0.06	0.92 ± 0.06	0.34 ± 0.08	0.87 ± 0.11	279 ± 41	0.99 ± 0.04	256 ± 45	0.9 ± 0.07
2	0.31 ± 0.08	0.80 ± 0.14	0.30 ± 0.05	0.77 ± 0.09	275 ± 53	0.97 ± 0.04	234 ± 54	0.83 ± 0.08
3	0.29 ± 0.04	0.74 ± 0.11	0.30 ± 0.04	0.77 ± 0.09	228 ± 24	0.86 ± 0.06	221 ± 56	0.78 ± 0.09
4	0.21 ± 0.04	0.54 ± 0.19	0.26 ± 0.06	0.67 ± 0.22	185 ± 30	0.65 ± 0.07	181 ± 39	0.64 ± 0.06
5	0.21 ± 0.02	0.54 ± 0.11	0.25 ± 0.04	0.64 ± 0.17	199 ± 29	0.70 ± 0.17	180 ± 49	0.64 ± 0.09

The cells isolated were incubated in Krebs-Henseleit bicarbonate buffer supplemented with 17.5%(v/v) dialysed horse serum and a physiological amino acid mixture. Hydrocortisone (10^{-5}M) was added at the beginning of the incubation period (Oh). Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. Values are means from 3 separate cell batches $\pm$S.E.M. The mean RSA for control and hydrocortisone-treated incubations were compared

(Legend to Table 4:6 / Continued)

using Students t test; these values, for either Serine Dehydratase or LDH, do not differ significantly at any incubation time.

Table 4:7 The effect of hydrocortisone on Phosphoenolpyruvate Carboxykinase (PEPCK) and LDH activities in hepatocytes isolated from adrenalectomized rats

Incubation time (h)	PEPCK				LDH			
	Control		Hydrocortisone		Control		Hydrocortisone	
	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.	S.A.	R.S.A.
0	1.28±0.53	1.0	-	-	277±49	1.0	-	-
1	1.12±0.24	0.88±0.12	1.2±0.38	0.94±0.21	233±35	0.84±0.05	216±28	0.78±0.04
2	1.03±0.31	0.80±0.10	1.41±0.67	1.1±0.52	223±54	0.81±0.08	246±38	0.89±0.11
3	0.77±0.22	0.60±0.13	1.11±0.44	0.87±0.31	180±32	0.65±0.06	183±43	0.66±0.08
4	0.65±0.25	0.51±0.18	1.19±0.52	0.93±0.40	165±	0.60±0.12	204±76	0.74±0.26
5	0.72±0.39	0.56±0.22	0.89±0.36	0.70±0.28	177±	0.64±0.10	204±63	0.74±0.19

The cells isolated were incubated in Krebs-Henseleit bicarbonate buffer supplemented with 17.5%(v/v) dialysed horse serum and a physiological amino acid mixture. Hydrocortisone (10^{-5}M) was added at the beginning of the incubation period (Oh). Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. Values are means from 3 separate cell batches ± S.E.M. The mean RSA for control and hydrocortisone-treated incubations were compared using Students t test; these values, for either PEPCK or LDH, do not differ significantly at any incubation time.

a more sensitive assay for Tryptophan 2,3-dioxygenase is necessary to enable the measurement of activity in extracts from 1 - 2 mg dry weight of cells.

ii) The effect of glucagon on hepatic TAT activity.

Glucagon alone induces a significant increase in TAT activity after 6h (Table 4:8). This indicates that the isolated hepatocytes have retained intact hormone receptors in the plasma membrane. It is likely that this increase in TAT activity is mediated by cyclic adenosine 3':5'-monophosphate (cyclic AMP). Adenylate cyclase activity in isolated cells is comparable with that in intact liver (Wincek et al., 1975). Glucagon stimulates the activity of adenylate cyclase in isolated cells; the intracellular concentration of cyclic AMP increases 30-fold in 3 - 6 min and subsequently falls rapidly (Pilkis et al., 1975). Glucagon and cyclic AMP combined induce TAT activity to the same extent as either inducer alone in isolated cells from fed rats (Ernest et al., 1977).

Glucagon or cyclic AMP will induce TAT activity in vivo and in perfused liver (McNamara and Webb, 1973; Hager and Kenney, 1968; Krone et al., 1974). Results obtained with cultured hepatoma cells are varied, reflecting the transformed nature of the liver cells. HTC cells possess no adenylate cyclase and exogenously added cyclic AMP has no effect on TAT activity (Granner et al., 1968) but stimulates induction in the presence of glucocorticoids (Granner et al., 1977). In Reuber H-35 cells, cyclic AMP but not glucagon will induce TAT due to either defective glucagon receptors (Wicks et al., 1972) or lack of adenylate cyclase (Reel et al., 1970).

fect of glucagon and glucocorticoid hormones on TAT activity in hepatocytes isolated from fed, adrenalectomized rats

on)	Hydrocortisone (6h)	Hydrocortisone + Glucagon (6h)	(Hydrocortisone + Glucagon (6h) $_{CALC}$	Triamcinolone (6h)	Triamcinolone + Glucagon (6h)	(Triamcinolone + Glucagon) $_{CALC}$ (6h)	Dexamethasone (6h)	Dexamethasone + Glucagon (6h)	(Dexamethasone + Glucagon) $_{CALC}$ (6h)
.05	0.61±0.05	0.74±0.09	0.97±0.05	0.34±0.03	0.58±0.07	0.70±0.03	0.37±0.03	0.52±0.04	0.73±0.02
.04	0.78±0.05	2.17±0.08	1.12±0.02	0.81±0.11	2.41±0.23	1.15±0.16	0.81±0.01	2.00±0.16	1.14±-0.05
.14	1.07±0.13	1.55±0.13	1.92±0.18	1.05±0.05	1.48±0.09	1.90±0.13	1.07±0.06	1.59±0.15	1.92±0.19
.07	0.61±0.04	0.78±0.04	0.95±0.08	0.68±0.09	0.78±0.08	1.02±0.10	0.53±0.08	0.69±0.08	0.88±0.10
.14	0.77±0.11	1.31±0.34	1.24±0.30	0.72±0.15	1.32±0.40	1.19±0.25	0.70±0.15	1.20±0.36	1.17±0.26
.29	1.92±0.19	3.18±0.65	2.92±0.52	1.77±0.30	3.15±0.82	2.95±0.51	1.70±0.30	2.88±0.70	2.88±0.54

(Legend to Table 4:8)

The cells isolated were incubated in MEM supplemented with 10%(v/v) dialysed, charcoal-treated FCS. Hormones were added at the start of the incubation period. Final concentrations were: 10^{-8}M glucagon; 10^{-5}M hydrocortisone; 10^{-5}M triamcinolone; 10^{-7}M dexamethasone.

Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. A, B, C, and D are the values from individual cell preparations, which are expressed as the means (3 incubations) $\pm$ S.D. The specific activities and relative specific activities, denoted *, are the mean values of the four cell batches $\pm$ S.E.M. Values in the columns headed $(\ )_{CALC}$ are the specific activities for treatment with glucagon and glucocorticoid combined calculated from the specific activities obtained after treatment with glucagon or glucocorticoid alone.

Incubation time (h)	ATP content nmoles.(mg dry wt cells)$^{-1}$	LDH activity μmoles.min^{-1}.(g wet wt cells)$^{-1}$
0	-	292 ± 47
3	8.6 ± 0.2	-
6	6.2 ± 0.4	227 ± 12

Values are the means $\pm$ S.E.M. (4 cell preparations) from unsupplemented incubations with the exception of the 6h LDH activity: this is the mean value of the LDH activity in the control and hormone-supplemented incubations $\pm$ S.E.M.

(Legend to Table 4:8 / Continued)

Statistical analysis

The significance of the treatments was tested using a 2-way analysis of variance as described in Chapter 2; the analysis was performed on the normalised (relative specific activities) data from the individual animals. A probability (p) of <0.05 was considered significant; N.S. = no significant difference.

	Variance ratio	p
Between treatments	$F_{(7,21)} = 5.06$	<0.001
Between animals	$F_{(3,21)} = 6.33$	<0.001

From Students t test:

Treatment	p (compared with no additions (6h))
Glucagon	<0.05
Hydrocortisone	<0.05
Hydrocortisone + Glucagon	<0.001
Triamcinolone	<0.05
Triamcinolone + Glucagon	<0.001
Dexamethasone	<0.05
Dexamethasone + Glucagon	<0.01

Treatment	p (compared with glucocorticoid + glucagon (6h) calculated)
Hydrocortisone + Glucagon	N.S.
Triamcinolone + Glucagon	N.S.
Dexamethasone + Glucagon	N.S.

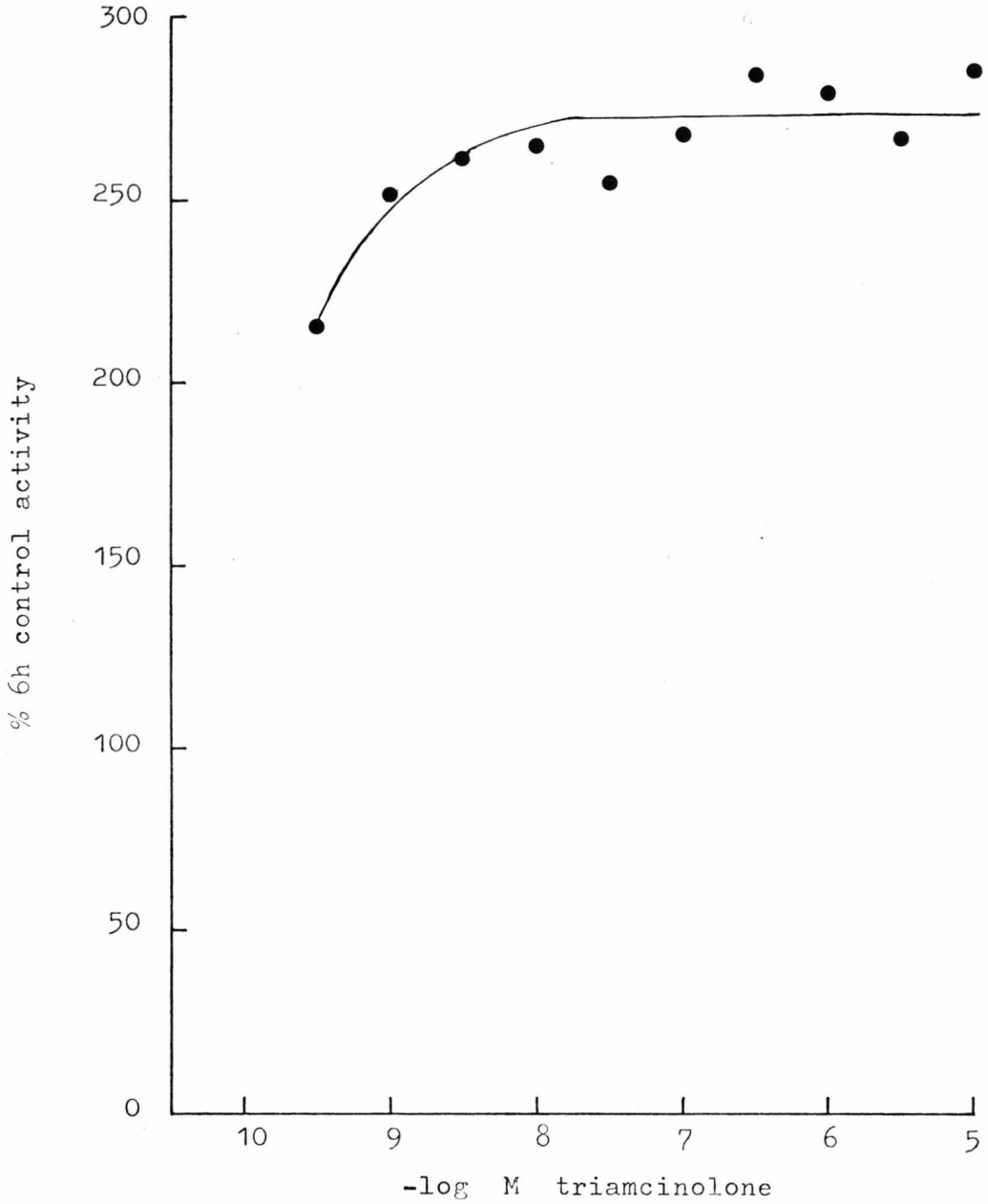
The effect of hydrocortisone and glucagon combined on TAT activity is additive (Table 4:8) and is consistent with *in vivo* observations (Holten and Kenney, 1967; Wicks *et al.*, 1969). The synthetic steroid hormones, triamcinolone and dexamethasone each induced TAT activity significantly after 6h. Either of the synthetic steroids in combination with glucagon induces an additive increase in TAT activity (Table 4:8). These results indicate that glucagon and steroid hormones induce TAT activity by different mechanisms and are not consistent with the theory that glucocorticoids have a "permissive" effect on glucagon (cyclic AMP)-mediated induction of TAT (Wicks *et al.*, 1974; Butcher *et al.*, 1971).

A dose response curve for triamcinolone in the presence of 10^{-8}M glucagon shows that a concentration of 10^{-7}M will induce a maximal increase in TAT activity (Fig 4:6). A maximum 3-fold induction is obtained with this combination of hormones in 4h (Table 4:9 ; Fig 4:7) and is maintained up to 6h. A decrease in the induced level of TAT is not observed since triamcinolone is metabolised more slowly by the liver than naturally occuring steroids (Florini *et al.*, 1961). The magnitude and extent of induction by triamcinolone was also greater than that by hydrocortisone in primary cultures of isolated hepatocytes (Bonney *et al.*, 1974; Michalopoulos and Pitot, 1975). The results are consistent with the fact that the presence of steroid hormone is required to maintain induction of TAT.

B) Isolated Hepatocytes from fed, normal rats.

In contrast to hepatocytes isolated from fed, adrenalectomized rats, the basal activity of TAT increased in 6h (Table 4:10). The initial specific activity of TAT is comparable

Fig 4:6 Dose-response curve for triamcinolone and glucagon



Hepatocytes were isolated from an adrenalectomized rat and incubated in MEM supplemented with 10%(v/v) charcoal-treated, dialysed FCS, glucagon (10^{-8}M) and triamcinolone, as indicated. The hormones were added at the start of the incubation period (0h). Values are the means of 3 incubations; standard deviations were <5%. ATP content at 6h = 9.4 ± 0.2nmoles.(mg dry wt cells)$^{-1}$. Final cell concentration = 1.2mg dry wt.ml^{-1}.

Table 4:9 The effect of triamcinolone + glucagon on TAT activity in isolated hepatocytes

Incubation time (h)	TYROSINE AMINOTRANSFERASE			LACTATE DEHYDROGENASE		
	Control	Triamcinolone + Glucagon	p	Control	Triamcinolone + Glucagon	p
0	0.34 ± 0.01	0.40 ± 0.01	N.S.	245 ± 9	-	-
1	0.45 ± 0.02	0.40 ± 0.01	N.S.	213 ± 7	202 ± 4	N.S.
2	0.58 ± 0.03	0.67 ± 0.02	N.S.	214 ± 11	196 ± 5	N.S.
3	0.50 ± 0.02	0.93 ± 0.04	<0.001	202 ± 9	202 ± 9	N.S.
4	0.38 ± 0.02	1.02 ± 0.06	<0.001	189 ± 5	162 ± 8	N.S.
6	0.25 ± 0.02	0.93 ± 0.13	<0.01	182 ± 11	161 ± 12	N.S.

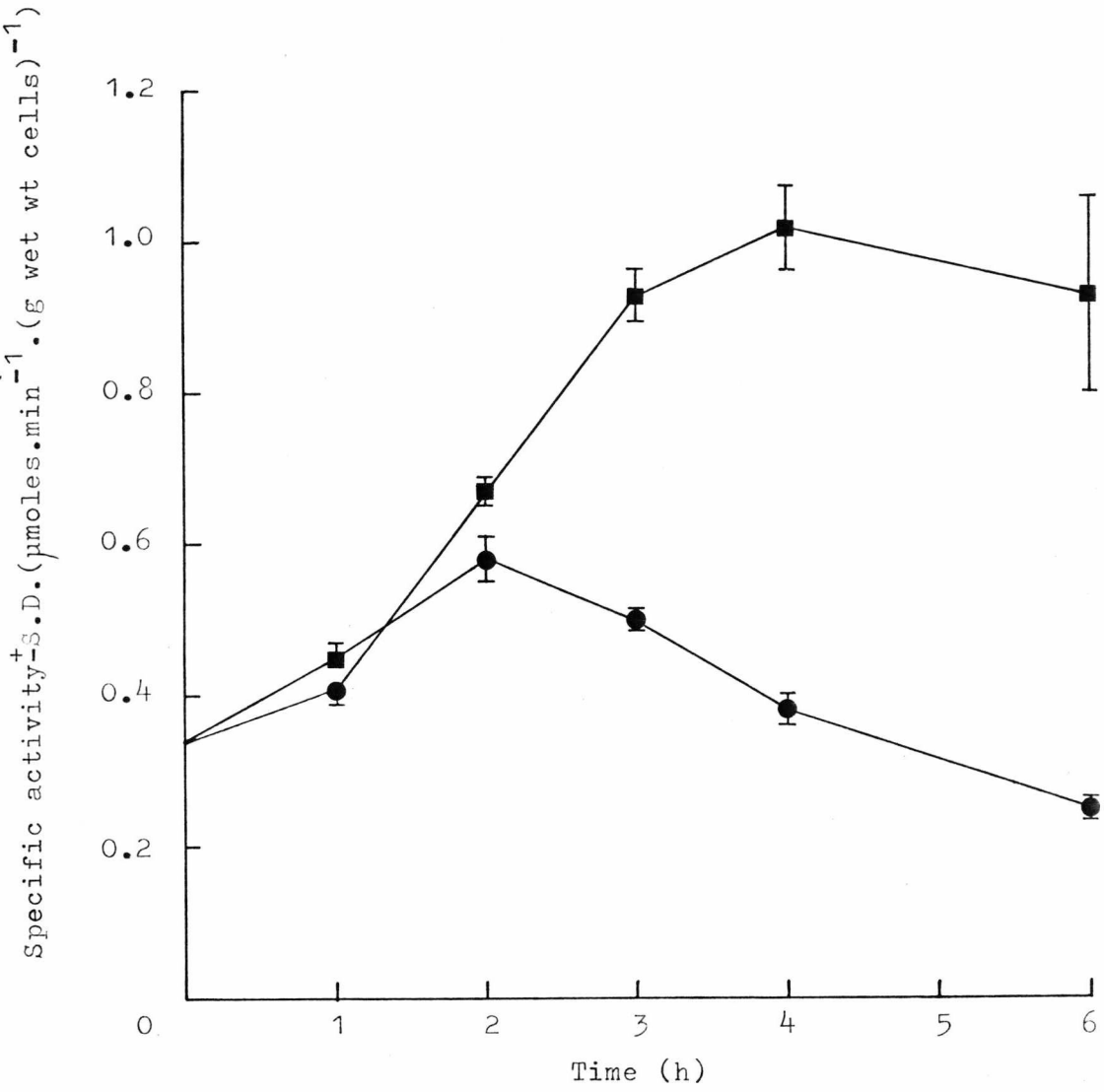
Cells were isolated from an adrenalectomized rat and incubated in MEM supplemented with 10%(v/v) charcoal-treated, dialysed FCS. Triamcinolone (10^{-5}M) and glucagon (10^{-8}M) were added at the beginning of the incubation period (0h). Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$; values are the means (3 incubations) $\pm$ S.D.

Legend to Table 4:9 / Continued

The mean specific activities for control and hormone-treated incubations were compared using Student's t test; a probability (p) of <0.05 was considered significant. N.S. = no significant difference.

ATP content (nmoles.(mg dry wt cells)$^{-1}$: 3h = 11.6; 6h = 8.7.

Fig 4:7 The effect of triamcinolone and glucagon on TAT activity in isolated hepatocytes from an adrenalectomized rat



Values are taken from Table 4:9.

The effect of glucagon and glucocorticoid hormones on TAT activity in hepatocytes isolated from fed, normal rats

Glucagon (6h)	Hydrocortisone (6h)	Hydrocortisone + Glucagon (6h)	(Hydrocortisone + Glucagon)$_{CALC}$ (6h)	Triamcinolone (6h)	Triamcinolone + Glucagon	(Triamcinolone + Glucagon)$_{CALC}$ (6h)	Dexamethasone (6h)	Dexamethasone + Glucagon (6h)	(Dexamethasone + Glucagon)$_{CALC}$ (6h)
$0.99^{+}0.11$	$1.39^{+}0.04$	$1.75^{+}0.05$	$1.33^{+}0.04$	$1.33^{+}0.05$	$1.59^{+}0.07$	$1.24^{+}0.16$	$1.40^{+}0.03$	$1.81^{+}0.13$	$1.31^{+}0.09$
$1.02^{+}0.11$	$0.68^{+}0.08$	$1.00^{+}0.01$	$1.06^{+}0.06$	$0.68^{+}0.06$	$0.88^{+}0.08$	$1.06^{+}0.10$	$0.56^{+}0.13$	$0.83^{+}0.10$	$0.94^{+}0.06$
$1.35^{+}0.07$	$0.93^{+}0.05$	$1.32^{+}0.11$	$1.36^{+}0.08$	$0.80^{+}0.10$	$1.26^{+}0.08$	$1.23^{+}0.04$	$0.85^{+}0.06$	$1.30^{+}0.03$	$1.28^{+}0.08$
$1.11^{+}0.12$	$1.0^{+}0.20$	$1.36^{+}0.05$	$1.25^{+}0.10$	$0.94^{+}0.20$	$1.24^{+}0.33$	$1.18^{+}0.08$	$0.94^{+}0.25$	$1.31^{+}0.28$	$1.18^{+}0.12$
$2.36^{+}0.70$	$2.13^{+}0.18$	$2.89^{+}0.32$	$2.87^{+}0.52$	$1.99^{+}0.08$	$2.65^{+}0.14$	$2.71^{+}0.48$	$1.99^{+}0.20$	$2.79^{+}0.35$	$2.67^{+}0.46$

Legend to Table 4:10

The cells isolated were incubated in MEM supplemented with 10%(v/v) dialysed, charcoal-treated FCS. Hormones were added at the start of the incubation period. Final concentrations were: 10^{-8}M glucagon; 10^{-5}M hydrocortisone; 10^{-5}M triamcinolone; 10^{-7}M dexamethasone.

Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. A, B and C are the values from individual cell preparations, which are expressed as the means (3 incubations) $\pm$ S.D. The specific activities and relative specific activities, denoted *, are the mean values of the three cell batches $\pm$S.E.M. Values in the columns headed $(\ )_{CALC}$ are the specific activities for treatment with glucagon and glucocorticoid combined calculated from the specific activities obtained after treatment with glucagon or glucocorticoid alone.

Incubation time (h)	ATP content nmoles.(mg dry wt cells)$^{-1}$	LDH activity μmoles.min^{-1}.(g wet wt cells)$^{-1}$
0	-	192 ± 27
3	9.3 ± 1.6	-
6	6.9 ± 1.7	139 ± 7

Values are the means $\pm$ S.E.M. (3 cell preparations) from unsupplemented incubations with the exception of the 6h LDH activity: this is the mean value of the LDH activity in the control and hormone-supplemented incubations $\pm$ S.E.M.

(Legend to Table 4:10/ Continued)

Statistical analysis

The significance of the treatments was tested using a 2-way analysis of variance as described in Chapter 2; the analysis was performed on the normalised (relative specific activities) data from the individual animals. A probability (p) of <0.05 was considered significant; N.S. = no significant difference.

	Variance ratio	p
Between treatments	$F_{(7,14)} = 4.15$	<0.025
Between animals	$F_{(7,14)} = 13.91$	<0.001

From LSD:

Treatment	p (compared with no additions (6h))
Glucagon	N.S.
Hydrocortisone	N.S.
Hydrocortisone + Glucagon	<0.01
Triamcinolone	N.S.
Triamcinolone + Glucagon	<0.02
Dexamethasone	N.S.
Dexamethasone + Glucagon	<0.01

Treatment	p (compared with (glucocorticoid + glucagon (6h) calculated)
Hydrocortisone + Glucagon	N.S.
Triamcinolone + Glucagon	N.S.
Dexamethasone + Glucagon	N.S.

with values reported *in vivo* and *in vitro* (Levitan and Webb, 1970; Seglen, 1971; Cihak *et al.*, 1972). This contrasts with previous measurements of TAT activity in isolated liver cells from fed rats (Crane and Miller, 1977; Ernest *et al.*, 1977); the values reported were between 2 and 3 fold higher than those noted previously.

The response of these cells to exogenously added hormones differs markedly from that of cells isolated from adrenalectomized rats (Table 4:10). In the presence of glucagon, the activity of TAT is increased, but does not differ significantly from the increased control value at 6h. The data shows that the response of the individual animals is varied. Similarly, no significant change in TAT activity was elicited by hydrocortisone or either of the synthetic steroid hormones. In contrast, however, glucagon together with hydrocortisone induces a statistically significant increase in TAT activity; the effect of the hormones combined appears to be additive. LDH activity is unchanged after the addition of glucocorticoids or glucagon alone or in combination.

These observations are fundamentally in agreement with those of Ernest *et al.*; Glucagon alone, or dexamethasone alone were found to have no significant effect on TAT activity, but together a greater than additive increase in TAT activity was obtained. It has been shown that glucagon alone (10^{-8}M - 10^{-6}M) will induce TAT activity in primary cultures of isolated hepatocytes (Michalopoulos *et al.*, 1978), albeit in the presence of insulin and after the 5th day of culture.

C) Isolated hepatocytes from 48h fasted rats

The initial activity of TAT is significantly higher in hepatocytes isolated from 48h fasted rats than in those isolated from fed rats (normal or adrenalectomized). This is not paralleled *in vivo* (Smith *et al.*, 1979). Isolated cells from rats fed a protein-deficient diet are smaller than those from rats fed a complete diet (Grant and Hoffenberg, 1977); this suggests that the increase in TAT activity, expressed relative to cell mass, is apparent rather than real. However, parenchymal cells constitute 66% of intact rat liver (Daoust, 1958) and therefore one might expect that an increase should also be observed *in vivo*.

The hormonal response of these cells contrasts with that of cells isolated from fed, normal or adrenalectomized rats (Table 4:11). In the presence of glucagon alone the mean specific activity of TAT is numerically greater than the control at 6h, but the values do not differ significantly; this is a direct result of the variation in response of the individual animals.

TAT activity is significantly increased by each of the steroid hormones alone, and in combination with glucagon. The magnitude of the increase observed in the presence of steroid together with glucagon again appears to be additive. In all experiments, LDH activity was unchanged in the presence of glucagon or glucocorticoids.

3) The effect of nutritional imbalance on TAT activity

A major role of the liver is to regulate plasma amino acid levels according to the need of the organism. This involves the combined mechanisms of the absorption of amino acids

The effect of glucagon and glucocorticoid hormones on TAT activity in hepatocytes isolated from 48h-fasted rats

Glucagon (6h)	Hydrocortisone (6h)	Hydrocortisone + Glucagon (6h)	(Hydrocortisone + Glucagon)$_{CALC}$ (6h)	Triamcinolone (6h)	Triamcinolone + Glucagon) (6h)	(Triamcinolone + Glucagon)$_{CALC}$ (6h)	Dexamethasone (6h)	Dexamethasone + Glucagon (6h)	(Dexamethasone + Glucagon) (6h)
3.5±0.10	5.90±0.06	6.5±0.84	6.3±0.57	5.8±0.53	6.0±0.07	6.2±0.35	5.9±0.20	5.85±0.05	6.3±0.30
2.40±0.07	2.64±0.04	5.66±0.10	4.22±0.10	2.51±0.27	5.25±0.45	4.09±0.21	2.70±0.23	5.30±0.42	4.28±0.17
1.38±0.07	2.88±0.06	2.83±0.10	3.38±0.12	2.87±0.22	2.76±0.17	3.36±0.16	3.06±0.34	2.78±0.13	3.56±0.34
2.43±0.61	3.80±1.05	5.5±0.64	4.63±0.87	3.73±1.04	4.70±0.97	4.55±0.85	3.90±1.00	4.67±0.95	4.71±0.82
1.72±0.49	2.63±0.76	3.86±0.68	3.25±0.70	2.58±0.75	3.35±0.86	3.19±0.68	2.69±0.73	3.32±0.85	3.31±0.67

Legend to Table 4:11

The cells isolated were incubated in MEM supplemented with 10%(v/v) dialysed, charcoal-treated FCS. Hormones were added at the start of the incubation period. Final concentrations were: 10^{-8}M glucagon; 10^{-5}M hydrocortisone; 10^{-5}M triamcinolone; 10^{-7}M dexamethasone.

Units of enzyme activity are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. A, B and C are the values from individual cell preparations, which are expressed as the means (3 incubations) $\pm$ S.D. The specific activities and relative specific activities, denoted *, are the mean value of the three cell batches $\pm$ S.E.M. Values in the columns headed $(\ )_{CALC}$ are the specific activities for treatment with glucagon and glucocorticoid combined calculated from the specific activities obtained after treatment with glucagon or glucocorticoid alone.

Incubation time (h)	ATP content nmoles.(mg dry wt cells)$^{-1}$	LDH activity μmoles.min^{-1}.(g wet wt cells)$^{-1}$
0	-	304$\pm$50
3	14.0$\pm$0.5	-
6	9.4$\pm$0.3	237$\pm$36

Values are the means $\pm$ S.E.M. (3 cell preparations) from unsupplemented incubations with the exception of the 6h LDH activity: this is the mean value of the LDH activity in the control and hormone-supplemented incubations $\pm$ S.E.M.

(Legend to Table 4:11 / Continued)

Statistical analysis

The significance of the treatments was tested using a 2-way analysis of variance as described in Chapter 2; the analysis was performed on the normalised (relative specific activities) data from the individual animals. A probability (p) of <0.05 was considered significant; N.S. = no significant difference.

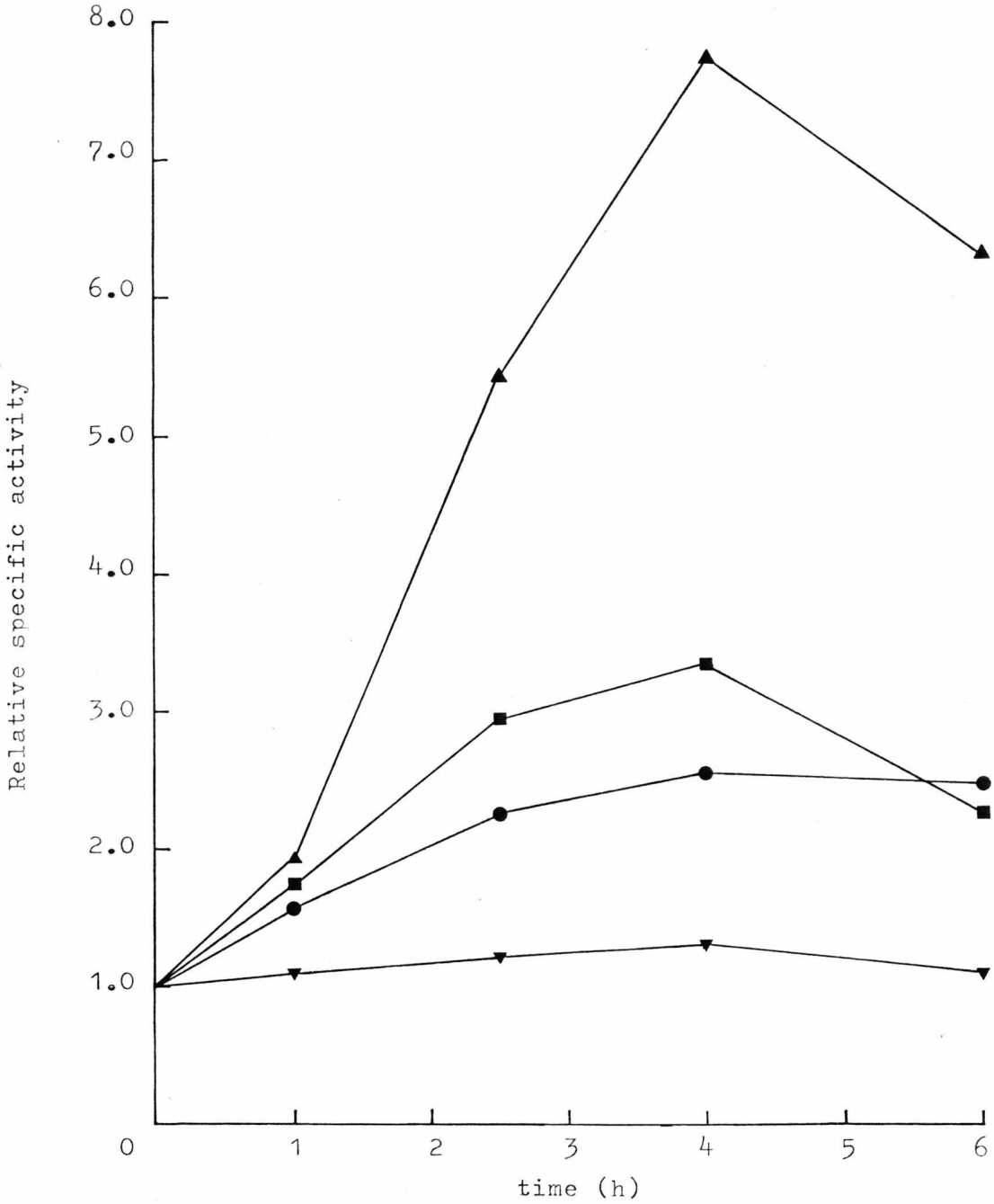
	Variance ratio	p
Between treatments	$F_{(7,14)} = 6.47$	<0.01
Between animals	$F_{(7,14)} = 25.86$	<0.001

From LSD :

Treatment	p (compared with no additions (6h))
Glucagon	N.S.
Hydrocortisone	<0.01
Hydrocortisone + Glucagon	<0.001
Triamcinolone	<0.02
Triamcinolone + Glucagon	<0.001
Dexamethasone	<0.01
Dexamethasone + Glucagon	<0.001

Treatment	p (compared with (glucocorticoid + glucagon (6h) calculated)
Hydrocortisone + Glucagon	N.S.
Triamcinolone + Glucagon	N.S.
Dexamethasone + Glucagon	N.S.

Fig 4: 8 The effect of hydrocortisone and 8x physiological amino acid mixture on TAT activity in hepatocytes isolated from a fed, adrenalectomized rat



The cells isolated were divided into two portions during the washing procedure (See Chapter 2 for details); one portion was resuspended in MEM supplemented with 10%(v/v) charcoal-treated, dialysed FCS and the second portion resuspended in the same medium supplemented additionally with 8x physiological

(Legend to Fig 4: 8/ Continued)

amino acid mixture. The cell suspensions were subsequently incubated in similarly supplemented media. Hydrocortisone (10^{-5}M) was added to the appropriate incubation vials at the start of the incubation period (Oh). All incubations were performed in triplicate; values presented are the means. Standard deviations were <5%. The specific activity of TAT at Oh was 0.47μmoles.min^{-1}.(g wet wt cells)$^{-1}$.

Incubation time (h)	Treatment	ATP content ($\pm$S.D.) nmoles.min^{-1}.(mg dry wt cells)$^{-1}$
0	None	8.5 ± 0.2
6	None	7.4 ± 0.7
6	Hydrocortisone	6.5 ± 0.1
6	Amino acids	8.1 ± 0.5
6	Hydrocortisone + amino acids	7.7 ± 0.2

▼ : No treatment

■ : Hydrocortisone

● : Amino acids

▲ : Hydrocortisone + amino acids

from exogenous protein and release into the plasma of endogenously-derived amino acids (Elwyn, 1970). The availability of amino acids to the liver has profound effects on hepatic protein metabolism. Livers perfused with medium devoid of amino acids exhibit increased rates of proteolysis (Mortimore and Mondon, 1970), disaggregation of polyribosomes and decreased protein synthesis (McGown et al., 1973).

The perfusion medium used routinely for the preparation of isolated liver cells is a simple, unsupplemented salts medium. Inclusion of an amino acid mixture in the perfusion medium does not significantly alter the initial level of TAT or the change in basal activity over a 4h incubation period (Table 4:12). After preparation, isolated liver cells are suspended and incubated in a complex nutrient medium which may rapidly reverse any changes in protein metabolism resulting from perfusion with a medium deficient in amino acids. This is supported by a number of observations. In perfused liver preparations, reaggregation of polysomes (McGown et al., 1973) and decreased proteolysis (Neely et al., 1977) occurred rapidly after supplementation of the perfusion medium with amino acids. Hepatocytes exhibit higher rates of protein turnover when isolated than in intact liver (Seglen, 1977a; Gan and Jeffay, 1971). Incubation of isolated hepatocytes in a complex nutrient medium decreases protein turnover and ammonia has been identified as a possible regulatory metabolite (Seglen, 1977b).

Elevated amino acid concentrations in the incubation medium induce a 2 - 3 fold increase in TAT activity in 4h which decreases slightly by 6h (Fig 4:8). In this laboratory, cysteine, glutamine, methionine, tryptophan and tyrosine individually, at the concentration present in this inducing amino acid mixture,

Table 4:12 Supplementation of the perfusion medium with amino acids: effect on cellular ATP levels, TAT and LDH activities

Incubation time (h)	ATP		TAT		LDH	
	-	+	-	+	-	+
0	6.6	7.5	1.20	0.91	191	215
1	10.2	10.9	1.15	1.08	198	151
2	10.6	10.9	1.60	1.13	179	182
3	10.1	10.4	1.82	1.29	197	172
4	9.5	10.9	1.33	1.34	212	151

Cells were isolated from fed, normal rats ; one liver was perfused with Krebs-Henseleit bicarbonate buffer and the other perfused with the same buffer supplemented with 6x a physiological amino acid mixture (East et al., 1973). All other procedures used in the isolation of the hepatocytes were as described in Chapter 2. The cells isolated were incubated in MEM supplemented with 10% (v/v) charcoal-treated, dialysed FCS. Values are the means of 3 incubations; standard deviations were $<5\%$.

have no effect on TAT activity (A.J. Dickson, unpublished observations). In vivo, the force feeding of adrenalectomized rats with amino acid mixtures induced hepatic TAT activity (Labrie and Korner, 1968a; Rosen and Milholland, 1963; Cihak et al., 1973a). Using cultured hepatoma cells, Lee and Kenney (1971) identified leucine as the amino acid, albeit at 5mM, responsible for inducing TAT.

The effect of the amino acid mixture on TAT activity is not representative of a general effect on protein synthesis, as LDH activity is unchanged (Table 4:13). Hydrocortisone in combination with the amino acid mixture induces an increase in TAT which is either additive or greater than additive (Fig 4:8; Table 4:13). Using a non-metabolised amino acid, δ-amino isobutyric acid, it has been demonstrated that steroid hormones stimulate amino acid uptake by isolated hepatocytes (Le Cam and Freychet, 1977). From this, it could be suggested that hydrocortisone and amino acids induce TAT by different mechanisms and that hydrocortisone enhances the amino acid effect by stimulating uptake. However, the regulation of transport differs between amino acids (Mallette et al., 1969) and increased uptake of a non-metabolisable amino acid does not always reflect an increase in amino acid transport (Manchester, 1960).

4) The effect of tryptophan on hepatic TAT activity.

In the inducing mixture of amino acids used, tryptophan is not responsible for the increase in TAT activity observed. However, intraperitoneal administration of tryptophan to intact rats has been shown to induce hepatic TAT activity (Lin et al., 1958a; Civen and Knox, 1959). Of a range of concentrations tested,

Table 4:13 The effect of amino acids and hydrocortisone on TAT activity in isolated liver cells

Additions (h)	TAT		LDH	
	Specific Activity	R.S.A.	Specific Activity	R.S.A.
None (0h)	0.88	1.00	152	1.0
None (6h)	1.13	1.26	140	0.92
10^{-5}M hydrocortisone (6h)	1.96	2.16	117	0.77
8x plasma amino acids (6h)	1.40	1.54	123	0.81
10^{-5}M hydrocortisone + 8x plasma amino acids (6h)	2.84	3.17	138	0.91

Cells isolated from fed, adrenalectomized rats were incubated in MEM supplemented with 10% (v/v) FCS. Hydrocortisone was added at the beginning of the incubation period. Values are the means from two separate cell batches.

Cellular ATP content: 3h = 8.5 ± 0.2 nmoles.(mg dry wt cells)$^{-1}$

6h = 7.4 ± 0.6 nmoles.(mg dry wt cells)$^{-1}$

only 2.5mM tryptophan induced a small, but significant, increase in TAT activity in cells isolated from a fed rat (Table 4:14). In vivo, a dose of 750 mg tryptophan/kg body weight elevated plasma tryptophan to between 2.5mM and 3.0mM in 1h; after a further 5h, hepatic TAT activity had increased 7-fold. The results presented here indicate that the effect of tryptophan on liver TAT in vivo is principally an indirect one (Smith et al.,1979).

Administration of tryptophan to adrenalectomized rats elicited an increase in TAT activity comparable with that obtained in livers from intact rats (Kenney and Flora, 1961; Cihak et al., 1973b); this indicated that the effect of tryptophan was not due to the stress-mediated release of adrenal cortical hormones. A similar level of induction is obtained in cells isolated from fed, normal and fed, adrenalectomized rats (Tables 4:14 and 4:15). In combination with glucagon, tryptophan induces a greater than additive increase in TAT activity (Table 4:15).

5) The effect of Cycloheximide and Cordycepin on TAT activity.

In isolated liver cells, cycloheximide prevents the induction of TAT by glucagon, glucocorticoids or tryptophan (Tables 4:16 and 4:17). In addition the basal activity of TAT decreases 60 - 70% in 6h. This is not the result of leakage from the cell as the activity of LDH is not significantly changed in the presence of cycloheximide. The normal half-life for TAT is 2h (Kenney, 1962b; Levitan and Webb, 1970); in the presence of cycloheximide this is apparently increased to 3 - 4h. The concentration of cycloheximide used will totally inhibit protein synthesis (Woodside, 1976) and must therefore also cause a slight inhibition of the degradation of TAT. This has previously been observed with perfused liver (Seglen, 1971) while in vivo and

Table 4:14 The effect of L-tryptophan on TAT activity in isolated hepatocytes: concentration dependence

L-tryptophan (mM)	Incubation time (h)	TAT			LDH		
		Specific activity	Relative specific activity	*p	Specific activity	Relative specific activity	*p
0	0	0.63±0.14	1.00	-	198±29	1.00	-
0	6	0.47±0.15	0.77±0.12	-	186±27	0.94±0.04	-
0.05	6	0.52±0.15	0.79±0.13	N.S.	194±25	0.99±0.11	N.S.
0.20	6	0.60±0.19	0.91±0.11	N.S.	176±25	0.89±0.01	N.S.
1.25	6	0.74±0.26	1.11±0.15	N.S.	169±28	0.85±0.05	N.S.
2.50	6	0.79±0.24	1.30±0.13	<0.05	173±25	0.88±0.03	N.S.

Cells were isolated from fed, normal rats and incubated in MEM (minus L-tryptophan) supplemented with 10%(v/v) charcoal-treated, dialysed FCS and tryptophan as indicated above. The values presented are means (3 separate cell preparations) ± S.E.M. Units of enzyme activity are μmoles.min^{-1}.(g wet wt. cells)$^{-1}$. *p: probability that the mean relative specific activities of the control and tryptophan-treated incubations (at 6h) differ significantly - calculated using Student's t test. Cellular ATP content at 6h: 9.8±2.0 (± L-tryptophan).

Table 4:15 The effect of tryptophan and glucagon on TAT activity in isolated liver cells

Additions	TAT activity	
	Specific Activity	Relative Specific Activity
None (0h)	0.61±0.14	1.0
None (6h)	0.46±0.16	0.72±0.15
2.5mM tryptophan (6h)	0.75±0.32	*1.09±0.15
10^{-8}M glucagon (6h)	0.66±0.19	*0.98±0.02
2.5mM tryptophan + 10^{-8}M glucagon (6h)	1.27±0.39	**2.03±0.16

Cells were isolated from fed, adrenalectomized rats. The values presented are the means from 4 separate cell preparations ± S.E.M. The significance of the increases in TAT activity induced was tested using a paired t test:

	probability (p)
*	<0.05
**	<0.002

Units of specific activity = μmoles.min^{-1}.(g wet wt cells)$^{-1}$

Incubation time (h)	ATP content (nmoles.(mg dry wt cells)$^{-1}$	LDH activity
0	-	308±70
3	9.0±0.94	-
6	7.4±0.52	224±53

in vitro other workers have obtained complete inhibition of degradation of TAT by cycloheximide (Kenney, 1967; Levitan and Webb, 1970; Barker *et al.*, 1971).

In the liver, cordycepin (3' deoxyadenosine) is phosphorylated forming 3' deoxy-ATP which is incorporated into the 3' end of an RNA molecule by RNA polymerase, terminating chain elongation (Truman and Klenow, 1968; Suhadolnik, 1970). By this mechanism, cordycepin inhibits the processing of ribosomal RNA (Siev *et al.*, 1969) and in particular, the synthesis of polyadenylic acid (poly(A)) which is involved in the processing and transport of messenger RNA from the nucleus to the cytoplasm (Lee *et al.*, 1971; Philipson *et al.*, 1971).

Induction of TAT activity by glucagon, glucocorticoids and tryptophan was prevented by cordycepin and basal TAT activity decreased by 65 - 75% (Tables 4:16 and 4:17). This was accompanied by a similar decrease in LDH activity. The normal half-life of LDH is 3 - 4 days (Don and Masters, 1976) and it seems unlikely that the rate of degradation would be increased by such a magnitude; a more likely explanation is that cordycepin affects the integrity of the plasma membranes. However this is not consistent with the fact that after 24h incubation with cordycepin (20 $\mu g.ml^{-1}$), electron micrographs reveal no damage to the plasma membranes of isolated hepatocytes (Puvion *et al.*, 1976). Cordycepin may specifically inactivate LDH, or may have a general toxic effect on the whole cell. Induction of TAT by steroids is prevented by cordycepin in cultured hepatoma cells (Butcher *et al.*, 1972; Dethlefsen, 1975). In both studies, the basal activity of TAT decreased by a similar magnitude to that seen in the experiments presented here, but no comment on this was made by the authors.

Table 4:16 The effect of cycloheximide and cordycepin on glucagon- and glucocorticoid-mediated induction of TAT activity in isolated liver cells

	TAT activity			LDH activity		
Additions	No inhibitor	Cycloheximide	Cordycepin	No inhibitor	Cycloheximide	Cordycepin
None (0h)	0.34±0.07	-	-	118±12	-	-
None (6h)	0.26±0.02	0.10±0.01	0.07±0.004	108±6	117±7	47±8
Glucagon (6)	0.62±0.05	0.095-0.001	0.072±0.012	112±3	101±6	40±14
Hydrocortisone (6)	0.61±0.05	0.092-0.008	0.113±0.009	97±3	109±2	66±2
Hydrocortisone + Glucagon (6)	0.74±0.09	0.090-0.03	0.090±0.01	93±6	112±10	50±4
Triamcinolone (6)	0.34±0.03	0.094±0.009	0.094±0.016	89±8	109±4	55±4
Triamcinolone + Glucagon (6)	0.58±0.07	0.083±0.013	0.083±0.006	102±6	105±0	54±2
Dexamethasone (6)	0.37±0.04	0.102±0.006	0.089±0.014	102±3	113±6	57±3
Dexamethasone + Glucagon (6)	0.52±0.04	0.074±0.05	0.078±0.006	105±2	106±5	46±0

Legend to Table 4:16

Cells were isolated from a fed, adrenalectomized rat. Inhibitors were added at the start of the incubation period, together with hormones where present. The final concentration of cycloheximide and cordycepin was 50$\mu g.ml^{-1}$. Values are the means of 3 determinations $\pm$ S.D. from a single cell batch. Units of enzyme activity are $\mu moles.min^{-1}$.(g wet wt cells)$^{-1}$.

Cellular ATP content = 3h: 8.5 ± 0.5 (nmoles.(mg dry wt cells)$^{-1}$)

6h: 5.1 ± 0.1 (nmoles.(mg dry wt cells)$^{-1}$)

Table 4:17 The effect of cycloheximide and cordycepin on glucagon- and tryptophan-mediated induction of TAT activity in isolated liver cells

	TAT activity			LDH activity		
Additions	No inhibitor	Cycloheximide	Cordycepin	No inhibitor	Cycloheximide	Cordycepin
None (0h)	0.41±0.02	-	-	267±3	-	-
None (6h)	0.33±0.06	-	-	210±2	-	-
Tryptophan (6)	0.43±0.01	0.16±0.03	0.18±0.004	210±2	156±19	146±5
Glucagon (6)	0.61±0.04	0.10±0.001	0.08±0.01	162±20	147±9	40±9
Tryptophan + Glucagon (6)	0.90±0.05	0.14±0.01	0.13±0.007	163±3	170±11	132±14

Cells were isolated from a fed adrenalectomized rat. Where present, inhibitors were added to the incubation medium before the addition of cells. The final concentrations of cycloheximide and cordycepin was 50$\mu g.ml^{-1}$. Values are the means ± S.D. from a single cell preparation. Units of enzyme activity are $\mu moles.min^{-1}.g$ wet $cells^{-1}$. Cellular ATP content: 3h = 11.6±0.9 $nmoles.(mg\ dry\ wt\ cells)^{-1}$

6h = 8.7±1.3 $nmoles.(mg\ dry\ wt\ cells)^{-1}$

GENERAL DISCUSSION

Inclusion of serum in incubation media may invite criticism because a number of undefined components are introduced. Evidence is presented in this chapter and by others (Crane and Miller, 1974; Jeejeebhoy et al., 1975) that one or more serum factors are essential to prolong the viability of hepatocytes in suspension. Charcoal treatment and dialysis render serum less undefined, while retaining the capacity to sustain viability. By removing any peptide or steroid hormone present, charcoal treatment enables accurate manipulation of exogenous hormone. Dialysis of FCS is of particular importance since FCS contains concentrations of fructose and lactate which are unphysiological for rat hepatocytes (Krebs, 1976); high concentrations of fructose have been shown to inhibit hepatic protein synthesis (Mäenpää et al., 1968), deplete cellular ATP levels, disaggregate polyribosomes (Seglen, 1974) and depress TAT activity (Ohisalo, 1977). Charcoal-treated, dialysed, FCS was therefore used routinely. The most acceptable alternative would involve the identification of the essential factor(s) in serum, and thus permit the use of a completely defined incubation medium.

The results presented show that there is a marked variation of both basal and induced TAT activity in hepatocytes prepared from rats in different hormonal and nutritional conditions, an observation which has been briefly reported previously (Crane and Miller, 1977). This is not parallelled by variation in LDH activity. Some inferences can be drawn from these results. An important overall observation is that the hormonal response of cells isolated from fed, adrenalectomized rats is reproducible

between cell batches while that of cells from fed, normal or fasted rats varies considerably between cell batches. This suggests that the hormones of the adrenal cortex are directly or indirectly responsible for the variability. It is likely that these hormones are secreted in response to stress during the preparative procedures.

The initial activity of TAT is 3 - 4 fold higher in hepatocytes from fasted rats than in those from fed rats. It is unlikely that such a large increase in activity occurred during the isolation procedure and must be primarily a result of altered hormone levels due to fasting. Since tyrosine is a glucogenic and ketogenic amino an increase in TAT activity during fasting is not unexpected. In 48h fasted rats, the concentration of plasma glucagon increases and that of insulin decreases (Van Lan *et al.*, 1974); exogenous administration of glucagon is known to increase hepatic TAT activity (Holten and Kenney, 1967; Hager and Kenney, 1968). Van Lan and co-workers also found the same pattern of changes in plasma glucagon and insulin 7 days after adrenalectomy; however the magnitude of the changes after fasting were 2-fold greater than after adrenalectomy. These results indicate that adrenal cortical hormones have a role in controlling plasma glucagon and insulin levels.

Basal TAT activity in cells from adrenalectomized rats decreased after 6h but was increased or maintained in cells from intact fed or fasted rats respectively. One possible explanation is that adrenal cortical hormones, produced in response to stress, interact with the liver cells *in situ* during the isolation procedure; as a result the activity of TAT is subsequently increased. It is also possible that adrenal cortical hormones exert their effect indirectly via other hormones or

metabolites.

A comparison of the normalised data shows that the magnitude of the increase in TAT activity elicited by glucagon is similar in adrenalectomized, intact, fed and intact, fasted rats. This is unexpected as a greater number of glucagon receptors are found on hepatocytes isolated from fed rats than on those isolated from fasted rats; in addition after the interaction of glucagon, a correspondingly greater increase in cyclic AMP is obtained (Fouchereau-Peron *et al.*, 1976). This inverse relationship between the plasma concentration of a hormone and the number of the receptors on liver cells has also been demonstrated for insulin (Soll *et al.*, 1975; Blackard *et al.*, 1978). The effect of exogenously-added glucagon may be limited if plasma glucagon has interacted with the liver cells before isolation. *In vivo*, administration of a second dose of glucagon to a pre-induced animal did not stimulate a further increase in hepatic TAT activity (Holten and Kenney, 1967).

The lack of response of hepatocytes from intact, fed rats to glucocorticoids is difficult to interpret. *In vivo* and *in vitro* the increase in TAT activity was shown to be directly proportional to the amount of steroid added; that is, unlike glucagon, further addition of steroid caused a further increase in TAT activity (Grossman and Mavrides, 1967). This lack of response in isolated cells has been noted previously (Crane and Miller, 1977; Ernest *et al.*, 1977). As mentioned earlier, Ernest and co-workers obtained a significant increase in TAT activity only when glucagon and glucocorticoids were present together; it was suggested that glucocorticoids have a "permissive" role in allowing glucagon to induce TAT activity. This phenomenon has not been observed *in vivo*; it has previously been observed in cultures of

hepatoma-derived cells (Wicks et al., 1974).

Unless the regulation of TAT activity in cells from intact, fasted and adrenalectomized rats is inherently different from that in cells from intact, fed rats, the results observed are the consequence of a more complex interaction of metabolites or hormones than the "permissive" effect proposed.

In the studies presented in this chapter, homogeneous suspensions of parenchymal cells were used, while in in vivo and in perfused liver, total hepatic TAT activity is measured. However, parenchymal cells contain 93% of total hepatic TAT activity while Kupffer cells contain only 6%; in addition TAT activity in Kupffer cells is not induced by either glucagon or hydrocortisone (Civen and Brown, 1973). It is difficult to compare the magnitude of the induction of TAT in this study with results obtained in vivo as doses administered vary and the resultant plasma concentrations of hormones were not measured. In the perfused liver, a comparable level of induction was obtained with glucagon (Hager and Kenney, 1968). The level of induction in the experiments presented here for glucagon and glucocorticoids compares well with previous studies using suspensions of isolated hepatocytes (Crane and Miller, 1977; Ernest et al., 1977) and also with results obtained using enzymically isolated parenchymal cells cultured on collagen discs (Michalopoulos et al., 1978). Michalopoulos and co-workers have also demonstrated catabolite or glucose repression of the induction of TAT in vitro. This phenomenon was first demonstrated in micro-organisms (Neidhart and Magasanik, 1956) and more recently has been demonstrated for rat liver TAT (Yuwiller et al., 1970; Sudilovsky et al., 1972). Glucose ($1 g.litre^{-1}$) is present in

MEM, which was used routinely, and at this concentration glucocorticoid or glucagon mediated induction of TAT is repressed by 30% in primary cultures of hepatocytes.

The half-lives of Serine Dehydratase and Phosphoenolpyruvate Carboxykinase, 4h and 5h respectively (Goldberg and St. John, 1976), are longer than that of TAT and this may account for the fact that no increase in their activity was obtained after 5h incubation with hydrocortisone. Tryptophan Aminotransferase activity was also unchanged by hydrocortisone. The level of induction of this enzyme *in vivo* is low (16%) compared with the increase in TAT (62%) (Civen and Knox, 1959). In addition, it has been noted that the levels of Tryptophan Aminotransferase and TAT respond differently to pyridoxal phosphate-deficiency (Lin *et al.*, 1958a). From these observations, it has been suggested that transamination of tryptophan and tyrosine are catalysed by two separate enzyme proteins. However, pure TAT was found to transaminate tryptophan to a limited extent (Jacoby and La Du, 1963); the use of a specific antibody directed against TAT would provide definitive evidence about the identity of the enzymes catalysing these reactions.

From the results presented in this chapter, the mechanisms by which glucagon (cyclic AMP) and glucocorticoids increase TAT activity appear to be independent. Immunological studies have shown that both hydrocortisone and glucagon stimulate an increase in the rate of synthesis of TAT; hydrocortisone additionally inhibits the rate of degradation (Levitan and Webb, 1970; Holten and Kenney, 1967). It was found that Actinomycin D inhibited the induction of TAT by hydrocortisone but not by cyclic AMP (Wicks *et al.*, 1972) and that pre-treatment

with glucocorticoid enhanced subsequent induction by cyclic AMP (Stellwagen et al., 1977; Leichtling et al., 1978). It was therefore suggested that hydrocortisone stimulates an increase in the level of TAT-mRNA, while cyclic AMP facilitates translation of TAT-mRNA. The transit time for translation of TAT-mRNA is decreased by cyclic AMP (Roper and Wicks, 1978), which supports this hypothesis. However, using cell-free protein synthesising systems the level of TAT-mRNA was shown to be similarly increased after induction by cyclic AMP or glucocorticoid (Ernest and Feigelson, 1978; Noguchi et al., 1978).

The effect of nutritional imbalance on TAT activity

In a number of experiments, the activity of basal and induced TAT in isolated hepatocytes decreased after 4 - 6h incubation; it was suggested earlier that this could be a rate-limiting effect of essential nutrients. TAT requires pyridoxal phosphate for activity, and an inadequate cofactor supply may result in a loss of activity. It has been proposed that enzyme-ligand interactions determine the rate of protein degradation in vivo (Dubnoff and Dimick, 1959); in addition proteases specific for the apoenzymes of pyridoxal-dependent enzymes have been identified (Kominami and Katanuma, 1976). However, under conditions of Vitamin B_6-deficiency, hepatic TAT activity decreases, but the apoenzyme concentration actually increases (Lin et al., 1958a; Tryfiates and Saus, 1975); in addition the TAT-pyridoxal interaction is not a major determinant of its rate of degradation (Lee et al., 1977).

While the physiological significance of using amino acids at concentrations above normal plasma levels is questionable, some indication of amino acids which have a regulatory role in protein turnover may be obtained. There are a number of mechanisms by which amino acids may exert a regulatory effect on protein turnover. In perfused liver, tryptophan has been found to be essential for the reaggregation of monomers to form polyribosomes (Sidransky et al., 1968) and recent evidence suggests that the mechanism involves increased translocation of messenger RNA from the nucleus to the cytoplasm (Murty et al., 1977).

In the absence of amino acids in the perfusate, the lysosomes and autophagic vacuoles in liver increase in size (Neely et al., 1977). Since lysosomes contain proteolytic enzymes it has been assumed that they have an important role in intracellular protein breakdown (Gordon, 1973); there is some direct evidence for this involvement (Dean, 1975), but the mechanism for controlling specificity is unknown.

A role for aminoacyl-tRNA in the regulation of protein turnover has been proposed (Lee and Kenney, 1971). The rate-limiting step in protein degradation may be the first peptide cleavage (Schimke, 1970) and unacylated tRNA may be required to accept the product of this cleavage. In this way, the concentration of an amino acid could regulate the rate of degradation of specific proteins. At present there is no experimental evidence to support this proposal.

The effect of tryptophan on hepatic TAT activity

Immunological studies have provided evidence that in vivo administration of tryptophan increases TAT activity by

inhibiting the rate of its degradation (Cihak *et al.*, 1973b). Neither tryptophan nor tryptophanyl-tRNA are limiting for protein synthesis (Ballard and Hopgood, 1973; Allen *et al.*, 1969). The studies in this chapter show that tryptophan elicits only a small increase in TAT activity in isolated hepatocytes; the effect of tryptophan *in vivo* must therefore be primarily dependent on extra-hepatic factors.

It has been suggested that metabolites of tryptophan, rather than tryptophan itself, are responsible for the increase in TAT activity (Deguchi and Barchas, 1971). While both tryptamine and 5-hydroxytryptamine increase TAT activity *in vivo*, their effects are distinct from that of tryptophan (Smith *et al.*, 1979).

CHAPTER 5

THE MULTIPLE FORMS OF TYROSINE AMINOTRANSFERASE

INTRODUCTION

For many years, the terms 'multiple forms' and 'isoenzymes' have been used synonymously to describe enzymes which catalyse the same reaction within an organism, but differ in certain physical or chemical properties. However the forms may arise either as a result of differences in the primary structure of the enzymes or as a result of modifications subsequent to translation. It has therefore been recommended (IUPAC - IUB Commission on Biochemical Nomenclature, 1971) that:

1) 'Multiple forms ..... should be a broad term covering all proteins possessing the same activity and occurring naturally in a single species.'

2) 'Isoenzyme or isozyme should apply only to those multiple forms of enzymes arising from genetically determined differences in primary structure and not to those derived by modification of the same primary sequence.'

Using ion-exchange or electrophoretic procedures, an extensive number of enzymes, in prokaryotic and eukaryotic organisms, have been shown to exist in more than one form. A comprehensive survey of their occurrence is beyond the scope of this chapter, but several reviews are available (Wieland and Pfleiderer, 1963; Markert and Whitt, 1968; Shaw, 1969; Scandalios, 1974; Ureta, 1978).

The heterogeneity of hepatic TAT was first noted by Kenney (1962b) during procedures for the purification of the enzyme. TAT in extracts from normal rat liver can be separated into three forms by ion-exchange chromatography (Johnson et al., 1973; Rodriguez and Pitot, 1975). Using

Sepharose or Sephadex chromatography, TAT activity elutes as a single peak (Iwasaki et al., 1973; Johnson et al., 1973); this suggests that the forms differ in net charge, but not molecular weight. Using polyacrylamide gel electrophoresis, two or three forms of TAT were observed in extracts from foetal or adult rat liver and hepatoma-derived cells (Holt and Oliver, 1969; Spencer and Gelehrter, 1974). The identity of the forms separated by these two chromatographic procedures has not been established.

Conditions have been defined which result in the interconversion of the multiple forms of TAT, without alteration in the total activity of the enzyme (Rodriguez and Pitot, 1975, 1976; Smith et al., 1975, 1976; Aviram and Hershko, 1977). A factor, associated with, or located in, a subcellular organelle, is involved in this process. The forms of TAT, therefore, arise from modifications of the same enzyme molecule and cannot be described as isoenzymes.

As discussed in Chapter 4, investigations in vivo and in vitro have shown that hepatic TAT activity is altered by a variety of hormonal and nutritional agents; many of these increase TAT activity by independent mechanisms. With the discovery of multiple forms of TAT, it was suggested that the activity of each form could be regulated by a distinct inducing mechanism; thus a role for the multiple forms of TAT in facilitating the complex regulation of its activity was proposed. In some investigations, administration of either glucagon, glucocorticoids, insulin, dibutyryl-cyclic AMP or a tryptophan-free amino acid mixture induced a distinct form of TAT (Holt and Oliver, 1969; Iwasaki and Pitot, 1971; Bourdel

et al., 1975); in contrast, Johnson and co-workers found that each hormone induced the same form of TAT. In addition, glucocorticoids induced the three forms of TAT equally in perfused liver (Iwasaki et al., 1973).

An alternative hypothesis is that multiple forms are intermediates in the degradation of TAT (Johnson et al., 1973). Form I, separated on carboxymethyl-Sephadex, was shown to be the immediate product of translation. Although conversion to forms II and III occurs with no loss of total activity, form III is inactivated more readily in vitro than form I (Beneking et al., 1977).

Since the forms of TAT are not separate gene products, it is possible that they are artificially produced during the extraction procedure. In liver cell extracts prepared by crushing liver slices between glass slides, only form III was found after chromatography on carboxymethyl-Sephadex (Johnson and Grossman, 1974). However, it is likely that this method of extraction releases only a proportion of the cytoplasmic contents of the liver cells. The relative proportions of the forms varied with the pH of the extraction buffer used (Rodriguez and Pitot, 1975); but this was consistent with the activity of the interconverting factor in crude homogenates at each pH (Smith et al., 1976).

In this chapter, the experiments presented compare the distribution and interconversion of the multiple forms of TAT in extracts from whole liver and isolated parenchymal cells. The use of ion-exchange chromatography and polyacrylamide gel electrophoresis in conjunction with the isolated parenchymal cell system is critically discussed. Glucagon and glucocorti-

coids have been shown to increase TAT activity in isolated parenchymal cells (Chapter 4); the relative proportions of the multiple forms of TAT, after induction, were measured. From these results, and from previous observations, the possible significance of multiple forms of TAT *in vivo* is considered.

MATERIALS AND METHODS

1) Animals and dosing procedures

Unless otherwise stated, animals were allowed access to food and water ad libitum. Adrenalectomy was performed as described in Chapter 2, and animals were used on the 6th day following adrenalectomy.

In some experiments, hepatic TAT activity was increased by glucocorticoid administration. Hydrocortisone (5mg.(kg body weight)$^{-1}$) was injected i.p. 3h before the preparation of crude liver extracts.

2) Preparation of liver extracts for chromatography

The composition of the extraction buffers used was as follows:

Carboxymethyl (CM)-Sephadex chromatography:

50mM phosphate (K^+), pH 6.5
2.5mM 2-oxoglutarate

Hydroxylapatite chromatography:

0.1M phosphate (K^+), pH 6.9
0.2mM pyridoxal phosphate
1.0mM dithiothreitol
1.0mg.ml^{-1} phenylmethylsulphonyl-fluoride (PMSF)

or

0.025M Tris-HCl, pH 8.7
0.2mM pyridoxal phosphate
1.0mM dithiothreitol
1.0mg.ml^{-1} PMSF

Polyacrylamide gel electrophoresis:

50mM phosphate (K^+), pH 6.5
0.2mM pyridoxal phosphate
1.0mM 2-oxoglutarate
1.0mM EDTA
1mg.ml^{-1} PMSF

A) Whole liver extracts

Rats were decapitated and bled. Livers were removed, rinsed in ice-cold 0.9%(w/v) NaCl, blotted and weighed. Using scissors, the livers were minced finely, then dispersed in 4 volumes (w/v) of ice-cold extraction buffer using a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 105,000g for 30 min at 4°C (MSE Prepspin 50) and the supernatant retained for chromatographic analysis.

B) Isolated parenchymal cell extracts

Immediately after isolation, or after incubation, cells were pelleted by centrifugation at 50g for 2 min and the supernatant removed by aspiration. On resuspension in the appropriate ice-cold extraction buffer, extracts were prepared by one of the following procedures:

i) Potter-Elvehjem homogenization - a concentrated cell suspension (approximately 50mg dry weight.ml^{-1}) was dispersed in a 5ml homogenizer.

ii) Freezing and thawing - as described in Chapter 3.

iii) Sonication - as described in Chapter 3.

iv) Extraction buffers contained 0.2%(w/v) digitonin. Cells were suspended in the appropriate buffer and retained on ice for 4 min, then centrifuged at 3,500g for 1 min. The supernatant was retained.

Extracts prepared by method i), ii), and iii) were centrifuged at 12,000g for 10 min at 4°C.

When pH 6.5 or 6.9 extraction buffers were used, crude supernatants were applied directly to columns or gels. The pH of crude supernatants at pH 8.7 was adjusted to 6.5 by the addition of 0.1M phosphate (K^+), pH 6.3, before chromatographic analysis.

3) Preparation of crude particulate extract from liver

This method was a modification of that of Rodriguez and Pitot, 1976 (using 0.25M sucrose instead of 0.44M sucrose). When used, this extract was freshly prepared. Fed rats were bled after decapitation, livers removed, rinsed in ice-cold 0.25M sucrose, blotted and weighed. The finely minced liver was dispersed in 2 volumes (w/v) ice-cold 0.25M sucrose. All subsequent procedures were performed at 4°C. The homogenate was centrifuged at 600g for 10 min and the supernatant transferred and centrifuged at 3,000g for 10 min. This supernatant was centrifuged at 12,500g for 20 min; the pellet was resuspended in 0.25M sucrose and centrifuged for a further 20 min at 12,500g. The pellet from this spin was resuspended in 7ml 0.25M sucrose, sonicated for 3 x 10 seconds at 4°C (MSE sonicator, 150W) and centrifuged at 105,000g for 30 min. The supernatant contained the interconverting factor and was described as the crude particulate extract or fraction.

Conditions for the interconversion of TAT

The crude particulate extract was incubated with the crude supernatant from a whole liver or isolated cells extract at 25°C for 60 min. Whole liver extract and particulate extract were mixed 1:1 (v/v); isolated cell extract and particulate extract were mixed 9:1 (v/v).

4) Chromatographic procedures

A) CM-Sephadex (Johnson et al., 1973)

CM-Sephadex (C-50) swollen in 50mM-phosphate (K^+), pH 6.5, was used to prepare a 2cm x 20cm column; this was equilibrated with 50mM-phosphate (K^+), pH 6.5, supplemented

with 2.5mM-2-oxoglutarate, 1mM-EDTA and 1mM-2-mercaptoethanol (buffer A) at 4°C for 2 days before use. All subsequent chromatographic procedures were performed at 4°C. Crude extract (containing approximately 250mg soluble protein) was applied to the column and washed through with 50ml buffer A. TAT was eluted from the column with a linear KCl gradient made from 200ml buffer A and 200ml buffer A containing 0.4M KCl. A flow rate of 25ml.h^{-1} was maintained using an LKB 12000 Varioperpex pump and 5ml fractions were collected (LKB Ultrorac 7000 fraction collector).

TAT was assayed spectrophotometrically (Lin et al., 1958b) and protein was assayed by the method of Lowry et al., (1951).

B) Hydroxylapatite (Smith et al., 1976)

Hydroxylapatite (Biogel HT) was obtained as a suspension in 0.001M phosphate (Na^+) pH 6.8. Resuspension of settled crystals was achieved by gentle swirling; a magnetic flea should not be used. The suspension was allowed to settle under gravity, the fines decanted and the hydroxylapatite resuspended in 0.1M phosphate (K^+)m pH 6.9. The de-fining procedure was repeated once, then columns were prepared in plastic syringes; glass fibre discs were used to support, and to protect the top of, the columns. Columns were equilibrated overnight at 4°C with 0.1M phosphate, pH 6.9, containing 0.2mM pyridoxal phosphate, 1mM dithiothreitol. All further chromatographic procedures were performed at 4°C. Crude extract was applied to the columns; the capacity of hydroxylapatite for protein is 1 - 5 mg.(ml bed volume)$^{-1}$. The columns were washed with 0.1M phosphate (K^+) and TAT eluted with successive batches of 0.17M phosphate (K^+); 0.23M phosphate (K^+), and 0.32M phosphate (K^+). All phosphate buffers were pH 6.9 and contained 0.2mM-

pyridoxal phosphate, 1mM-dithiothreitol. Equal volumes of the four buffers were used, determined by the size of the column used:

Column volume	Volume of each phosphate buffer used
6ml	21ml
3ml	11ml
1ml	5ml

Columns were allowed to flow under gravity; the flow rate varied considerably between columns. Unless otherwise stated, each of the four phosphate buffers was collected as a batch on elution from the column. TAT activity was assayed spectrophotometrically (Lin et al., 1958b) or radioactively (as described in Chapter 3). Protein was assayed by the method of Lowry et al., (1951).

C) Polyacrylamide gel electrophoresis (Gelehrter et al., 1972)

i) Buffers

Stacking gel buffer — 30mM Tris-HCl, 16mM phosphate, pH 6.9

Running gel and electrode buffer — 5mM Tris-HCl, pH 8.4, 38mM glycine

Sample buffer - the extraction buffer described earlier was supplemented with 10%(w/v) sucrose and 0.001%(w/v) bromophenol blue.

ii) Polyacrylamide Gel

22.2g acrylamide and 0.6g bis-acrylamide (37:1 w/w) were dissolved together in 100ml water. The concentration of the acrylamide gels used routinely was:

Stacking gel: 4% (w/v) acrylamide

Running gel: 7.5% (w/v) acrylamide

The stock acrylamide solution was diluted with the appropriate buffer and de-gassed under vacuum. Polymerisation was initiated by the addition of TEMED(N,N,N',N'' tetramethyl-ethylenediamine) and catalysed by ammonium persulphate (2% w/v).

Glass gel tubes of 0.5cm internal diameter were treated with 'Repelcote' before use. A running gel 6 cm in length and a stacking gel 1cm in length were prepared. Crude extract containing approximately 5mU TAT was applied to each gel. Electrophoresis (from cathode to anode) was carried out at 3mA per gel at 4°C until the bromophenol blue band reached the anode end of the gel ($1\frac{1}{2}$ - 2h). Gels were removed from the tubes by 'rimming'.

Histochemical stain for TAT (Schepartz, 1969; Thompson and Tomkins, 1971)

After electrophoresis, gels were rinsed in water and soaked in 3mM potassium ferricyanide for 5 min at room temperature. This procedure reduces non-specific background staining (Higgins and Barrnett, 1970). Gels were rinsed in water and placed in the staining mixture which contained:

16mM 3-monoiodotyrosine
20mM 2-oxoglutarate
40μM pyridoxal phosphate
5μg.ml^{-1} phenazine methosulphate (PMS)
0.2mg.ml^{-1} [3-(4,3-Dimethyl Thiazolyl-2)-2,5-diphenyl Tetrazolium Bromide] (MTT)

in 0.1M phsophate (K^+) pH 7.6. Identical results are obtained using tyrosine or 3-monoiodotyrosine, but the latter is more soluble (Thompson and Tomkins, 1971). Staining was carried out for 30min at 60°C. Gels were rinsed in water and fixed in 7.5% (w/v) acetic acid. The stained gels were scanned at

575nm in a Gilford 250 spectrophotometer equipped with a model 2520 gel scanner. The peak areas were integrated on a Kontron W + W 1100 recorder.

Elution of TAT from polyacrylamide gels

After electrophoresis, gels were rinsed in water, sliced with a scalpel and each slice placed in a tube containing 0.1ml 0.1M-phosphate (K^+), pH 7.6 supplemented with 0.2mM-pyridoxal phosphate and 0.5mM-2-oxoglutarate. Each gel slice was homogenised using a tight-fitting glass rod. Tubes were covered and left overnight at 4^oC for the enzyme to elute. Before assay, a further 0.1ml buffer was added to each tube. TAT was assayed radioactively using L-[side chain 2,3-^3H] tyrosine.

5) Cell incubation conditions

In a number of experiments, isolated parenchymal cells were incubated with hormones to increase TAT activity. Approximately 10mg dry weight cells were suspended in 20ml MEM supplemented with 10%(v/v) charcoal-treated, dialysed FCS in 100ml Ehrlenmeyer flasks. After gassing with O_2:CO_2 (95%:5%), flasks were incubated in an orbital shaking water bath at 37^oC at 130 rev min^{-1}. Hormones were added to the flasks in the water bath with micro-syringes.

At the appropriate time-points, cell suspensions were centrifuged at 50g for 2 min at room temperature. The cell pellets were washed once in extraction buffer, then resuspended to a known volume in the same buffer. Extracts were prepared as described earlier.

RESULTS AND DISCUSSION

1) Characterisation of the multiple forms of TAT

Throughout the literature, a variety of systems have been used to number the multiple forms of TAT. For clarity, the conventions outlined in Table 5:1 have been used throughout this chapter, regardless of the conventions used by the authors cited.

Multiple forms of TAT have not previously been separated in extracts from isolated liver cells. Therefore, using a variety of chromatographic techniques, profiles obtained from whole liver and isolated cell extracts were compared.

A) Ion-exchange chromatography

Crude liver extracts contain considerable amounts of protein; this restricts the amount of extract which can be adsorbed on to the ion-exchange column. TAT represents only a small percentage of the total protein in a crude extract and is greatly diluted after elution. Heat-treatment or ammonium sulphate precipitation could be used to increase the relative percentage of TAT in the extract; however, these procedures could alter the distribution of the multiple forms.

i) Chromatography on carboxymethyl-Sephadex (CM-Sephadex)

Isolated cells were incubated with hydrocortisone to increase TAT activity and facilitate its measurement in the column eluate. Hydrocortisone was also administered to the intact animal to enable a comparison of the multiple form profiles in whole liver and isolated parenchymal cell extracts.

Table 5:1 Conventions used for numbering the multiple forms of TAT

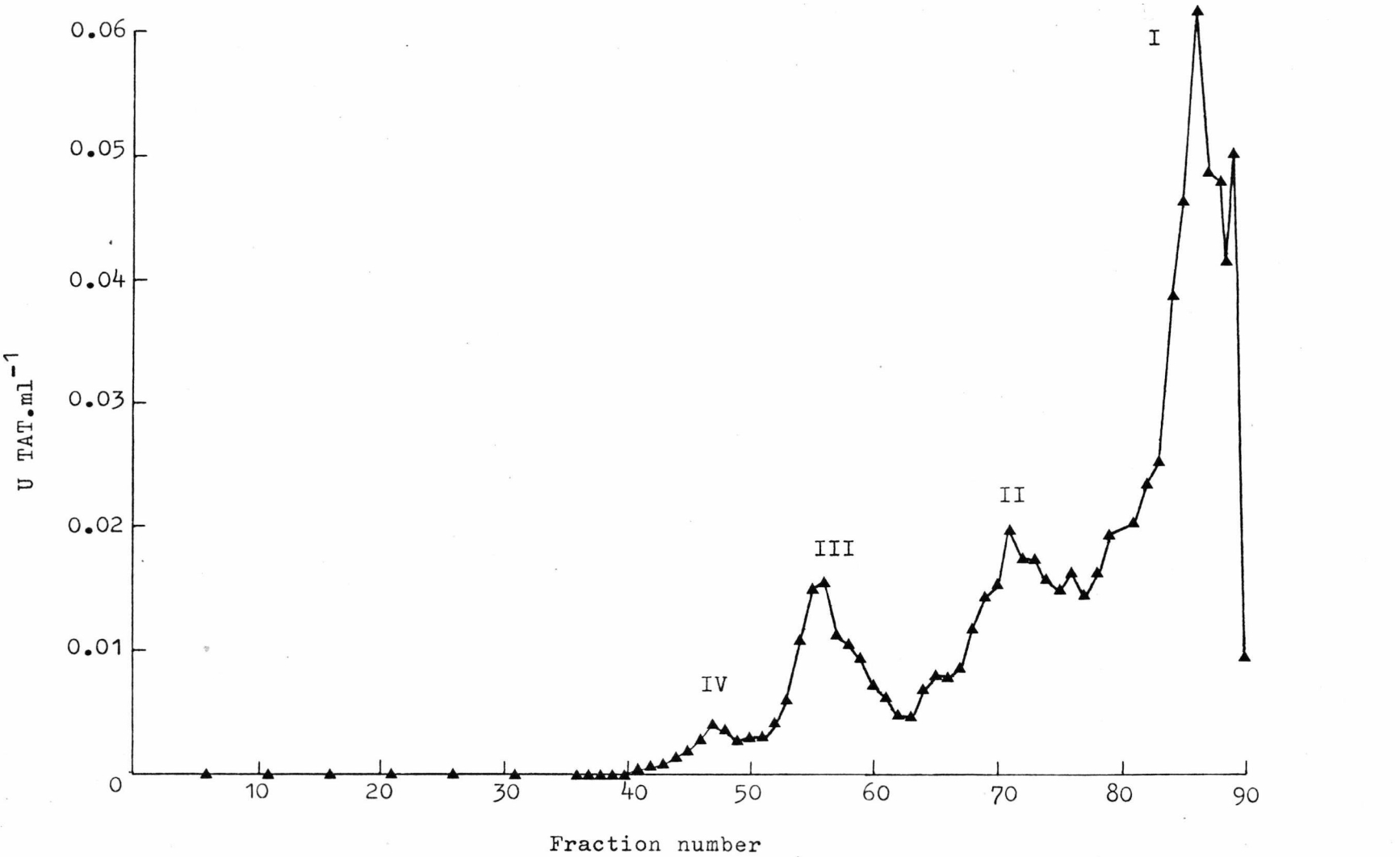
Chromatographic Procedure	Order of elution from column	
CM-Sephadex	III ; II ; I	(Johnson et al., 1973)
Hydroxylapatite	1 ; 2 ; 3	(Smith et al., 1975)
	Anode ← cathode	
Polyacrylamide gel electrophoresis	P1 ; P2 ; P3	

TAT in these extracts separates into three major forms (III, II and I) after chromatography or CM-Sephadex (Figs 5:1 and 5:2). A fourth form (IV) was also observed in extracts from whole liver (see Fig 5:5 also). In whole liver extracts, TAT appears to be predominantly in form I; this profile is comparable with that obtained by Johnson et al., (1973). In extracts from isolated liver cells, TAT is more equally distributed between the three forms.

It has been suggested that forms II and III of TAT are more susceptible to degradation than form I (Johnson et al., 1973). The protein catabolic state of parenchymal cells is increased after enzymic isolation (Seglen, 1975); the rate of protein turnover is 3% - 5%.h^{-1} compared to 1%.h^{-1} in vivo (Gan and Jeffay, 1971). The differences in the relative proportions of the forms in whole liver and isolated cell extracts are consistent with their proposed role in the degradation of TAT (Johnson et al., 1973).

A fourth form of TAT has not been separated on CM-Sephadex previously, but has been separated on hydroxylapatite (Iwasaki and Pitot, 1971; Iwasaki et al., 1973). From studies on the interconversion of the multiple forms of TAT it appears that forms III, II and I correspond to forms 1, 2 and 3 respectively. The fourth form of TAT eluted from hydroxylapatite before the three major forms as did form IV in the current studies. The physical and kinetic properties of this form differ markedly from those of the three other forms (Iwasaki et al., 1973; Table 5:2). Glutamate-oxaloacetate transaminase (GOT) elutes from hydroxylapatite just before form 1 of TAT (Smith et al., 1976); transamination of tyrosine by

Fig 5:1 Separation of the Multiple Forms of TAT in a crude liver extract on CM-sephadex



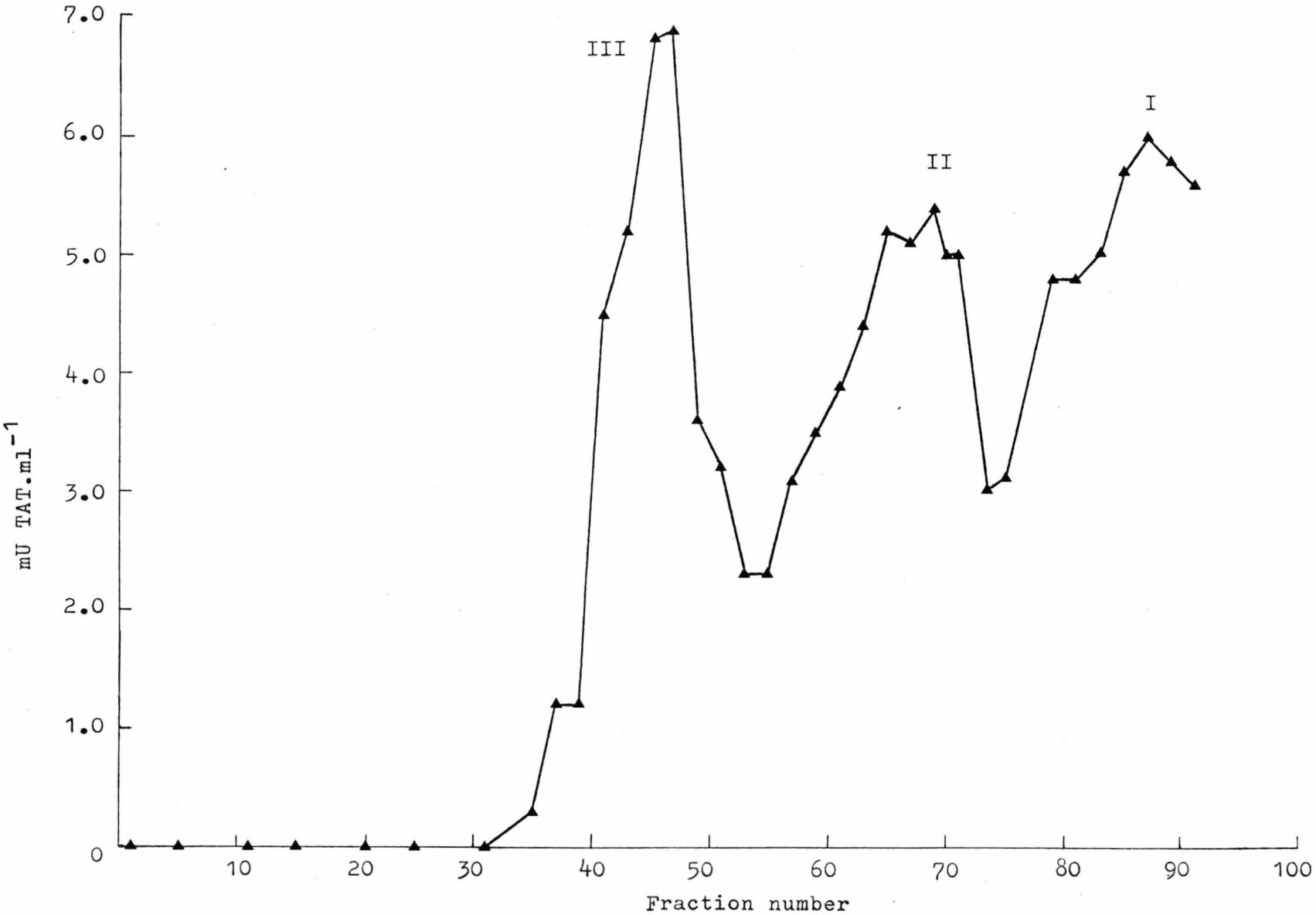
Legend to Fig 5:1

Rats were adrenalectomized, 6 days before use. The liver extract was prepared, as described in Materials and Methods, 3h after the i.p. administration of hydrocortisone (5mg.(kg body weight)$^{-1}$). 6ml of the 105,000g supernatant was applied to the column, and the enzyme eluted with a linear KCl gradient (0 - 0.4M). A flow rate of 25ml.h^{-1} was used and fractions of 5ml collected.

TAT activity in 105,000g supernatant = 670 mU.ml^{-1}.

93% TAT activity recovered from the column.

Fig 5:2 Separation of the multiple forms of TAT in an isolated cell extract on CM-sephadex



Legend to Fig 5:2

Cells were isolated from an adrenalectomized rat and incubated with hydrocortisone for 3h as described in Materials and Methods. 14ml cell extract was applied to the column and eluted with a linear KCl gradient (0 - 0.4M). A flow rate of 25ml.h^{-1} was used and fractions of 5ml collected.
TAT activity in original cell extract = 43.5 mU.ml^{-1}.
98.5% TAT activity recovered from the column.

Table 5:2 Properties of the multiple forms of TAT separated by hydroxylapatite chromatography

FORM	HEAT STABILITY	Km (mM)			MOLECULAR WEIGHT	OPTIMUM pH
		TYROSINE	2-OXOGLUTARATE	PYRIDOXAL PHOSPHATE		
1	60°C	1.4	1.1	1.3	123.10^3	7.6
2	60°C	1.5	1.0	1.6	120.10^3	7.6
3	60°C	1.5	1.2	1.1	126.10^3	7.6

Data from Iwasaki *et al.*, 1973

this enzyme could be responsible for the fourth form of activity observed.

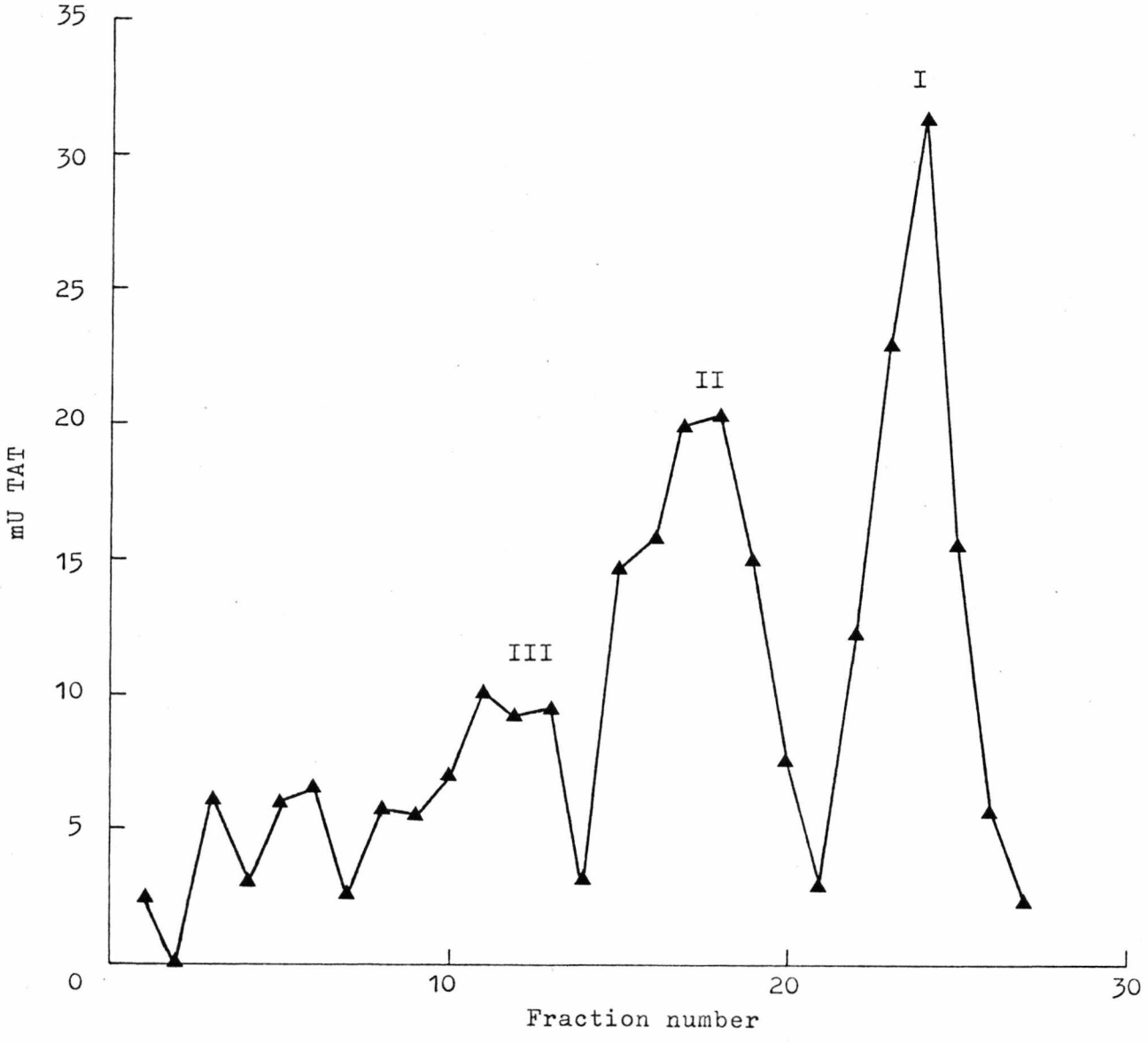
ii) Chromatography on hydroxylapatite

Hydroxylapatite is a form of $CaHPO_4$; proteins are adsorbed as a result of ionic interactions between the Ca^{2+} ions and negatively charged groups in the surface of the protein molecules (Bernardi, 1973). Adsorbed proteins can be eluted by phosphate ions which 'compete' for the Ca^{2+} binding sites; this is therefore a form of ion-exchange chromatography.

TAT in crude liver extracts separated into three forms after chromatography on hydroxylapatite (Rodriguez and Pitot, 1975; Smith et al., 1975). The fourth form of TAT reported earlier by Pitot's group was not observed in more recent reports (Rodriguez and Pitot; 1975, 1976). The three forms can be eluted using, in turn, 0.17M, 0.23M and 0.32M phosphate buffers (Smith et al., 1976); the proportions of the multiple forms are similar to those obtained after gradient elution of the same liver extract.

Using this batch-elution technique, the multiple forms of TAT in extracts from whole liver and isolated cell preparations were separated (Fig 5:3; Table 5:4). When 25mg protein was applied to a 6ml column, only 80% of the TAT was adsorbed (Fig 5:3); in subsequent experiment, by applying less than 3mg protein.(ml bed volume)$^{-1}$, 95% - 100% of the TAT was adsorbed. In all experiments, greater than 80% of the protein applied eluted from the columns. An unexpected finding was that the amount of TAT eluted from the column was consistently 2-fold - 3-fold greater than the calculated amount applied to the column. The same assay was used to measure TAT activity in crude extracts and in column eluates. The hydroxylapatite

Fig 5:3 Separation of the multiple forms of TAT in a crude liver extract on hydroxylapatite



Legend to Fig 5:3

A liver extract was prepared in 0.1M phosphate (K^+), pH 6.9 containing 0.2mM pyridoxal phosphate and 1.0mM dithiothreitol as described in Materials and Methods. 0.25ml of the 105,000g supernatant was applied to a 6ml hydroxylapatite column. TAT was eluted under gravity; 3ml fractions were collected. TAT was assayed spectrophotometrically (Lin et al., 1958).

Recovery from the column: protein : 80%

TAT : 211%

Table 5:3 A comparison of the recoveries and multiple form profiles of TAT after chromatography of different amounts of crude liver extract on hydroxylapatite.

mU TAT		% recovery of	% total TAT eluted		
applied to column	eluted from column	TAT from columns	Peak 1	Peak 2	Peak 3
3.9	9.1	233	63	4	33
11.7	24.0	205	58	0	42

A crude liver extract was prepared as described in Materials and Methods. 100µl and 300µl aliquots were applied to separate 6ml hydroxylapatite columns and TAT eluted with 11mls portions of the appropriate phosphate buffers. The values presented are means from duplicate columns.

Table 5:4 The effect of protease inhibitors on the multiple forms of TAT separated by hydroxylapatite chromatography

Method of extraction	Protease inhibitors	TAT activity in supernatant ($\mu moles.min^{-1}.ml^{-1}$)	% total TAT eluted		
			Peak 1	Peak 2	Peak 3
Freezing and thawing	-	0.09	20	38	42
	+	0.08	28	39	34
Digitonin	-	0.07	20	39	42
	+	0.06	29	37	34
Potter-Elvehjem	-	0.05	47	21	32
	+	0.05	55	24	21

Cells were isolated from a fed rat. Extracts were prepared, as described in Materials and Methods, in 0.1M phosphate (K^+), pH 6.9 containing 0.2mM-pyridoxal phosphate, 0.1mM-dithiothreitol.

Protease inhibitors: - = no additions

+ = extraction buffer contained pepstatin ($20\mu g.ml^{-1}$), leupeptin ($20\mu g.ml^{-1}$) and PMSF ($1mg.ml^{-1}$)

had not been used previously and was therefore not contaminated. However, that this anomaly is a property of the crude extract and not the hydroxylapatite is indicated by the results shown in Table 5:3; when different amounts of crude extract were applied to separate columns, similar percentage recoveries and multiple form profiles of TAT activity were obtained. This increased activity of TAT could be a result of the removal of an inhibitor, present in crude extracts, during the chromatographic procedure. The recovery of TAT from hydroxylapatite has previously been reported as greater than 85% (Iwasaki et al., 1973; Smith et al., 1976); but there has been no indication of recoveries exceeding 100%.

The protease inhibitor PMSF inhibits trypsin and chymotrypsin, in particular, by reacting with a serine residue necessary for the activity of these proteases (Fahrney and Gold, 1963; Gold, 1967). The effect of including PMSF in the extraction buffer, together with leupeptin, a serine/thiol protease inhibitor, and pepstatin, an inhibitor of acid proteases (Umezawa, 1977) was investigated. The multiple form profile of TAT was unchanged by the inclusion of this combination of protease inhibitors (Table 5:4). However, PMSF has been found to prevent interconversion of the multiple forms (Aviram and Hershko, 1977) and was subsequently included in all extraction buffers.

The relative proportions of the multiple forms of TAT in crude extracts, immediately after preparation, varies. In general, TAT in extracts from whole liver and isolated parenchymal cells exist predominantly in form 1 (Tables 5:4; 5:7 and 5:9). The variability may reflect the extent to

which interconversion occurs during the extraction procedure or may result from the differences in the animals themselves.

B) Polyacrylamide gel electrophoresis

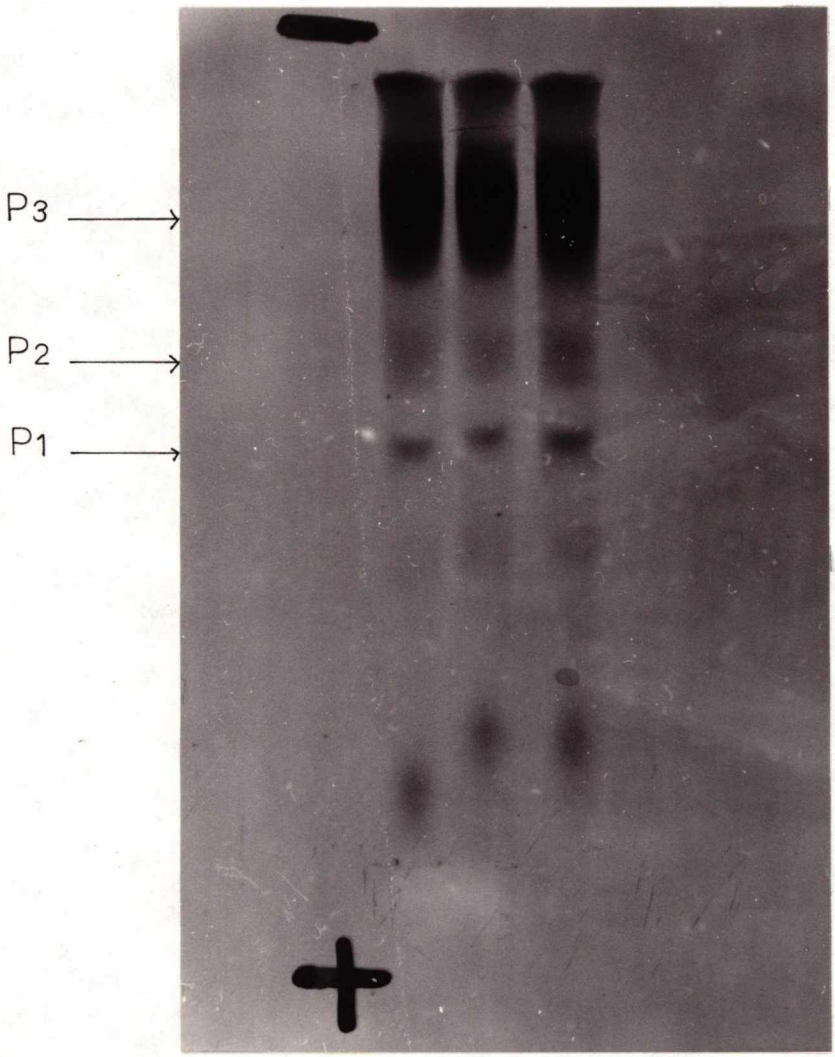
Methods for detecting TAT activity in polyacrylamide gels

After electrophoretic separation of proteins in a crude liver extract in polyacrylamide gels an enzyme may be assayed *in situ* using a specific histochemical stain; alternatively, gels may be sectioned, proteins eluted and the enzyme assayed spectrophotometrically or radioactively. The basis of the histochemical staining reaction for TAT is the transfer of electrons from *p*-HPP to phenazine methosulphate (PMS); subsequently the PMS reduces the tetrazolium dye, MTT to form a blue, formazan compound (Schepartz, 1969; Thompson and Tomkins, 1971). The mechanism of the electron transfer in this reaction is not known.

When crude liver extracts are subjected to electrophoresis, then stained for TAT activity, three bands are visualized (Plate 5:1); these are numbered P1, P2 and P3. The bands are characterised by the distance moved relative to the bromophenol blue marker. In the absence of substrate, no bands are obtained. A similar pattern of bands was obtained with either 6.5%, 7.0%, 7.5% or 8.0% acrylamide (ratio of acrylamide:methylene-bis-acrylamide = 37.5:1). 7.5% acrylamide gels were used throughout. A 4% stacking gel was also used in order to obtain sharp, defined bands. Inclusion of PMSF in extraction buffers had no effect on the number of bands obtained; buffers were, however, routinely supplemented with this protease inhibitor.

PLATE 5:1

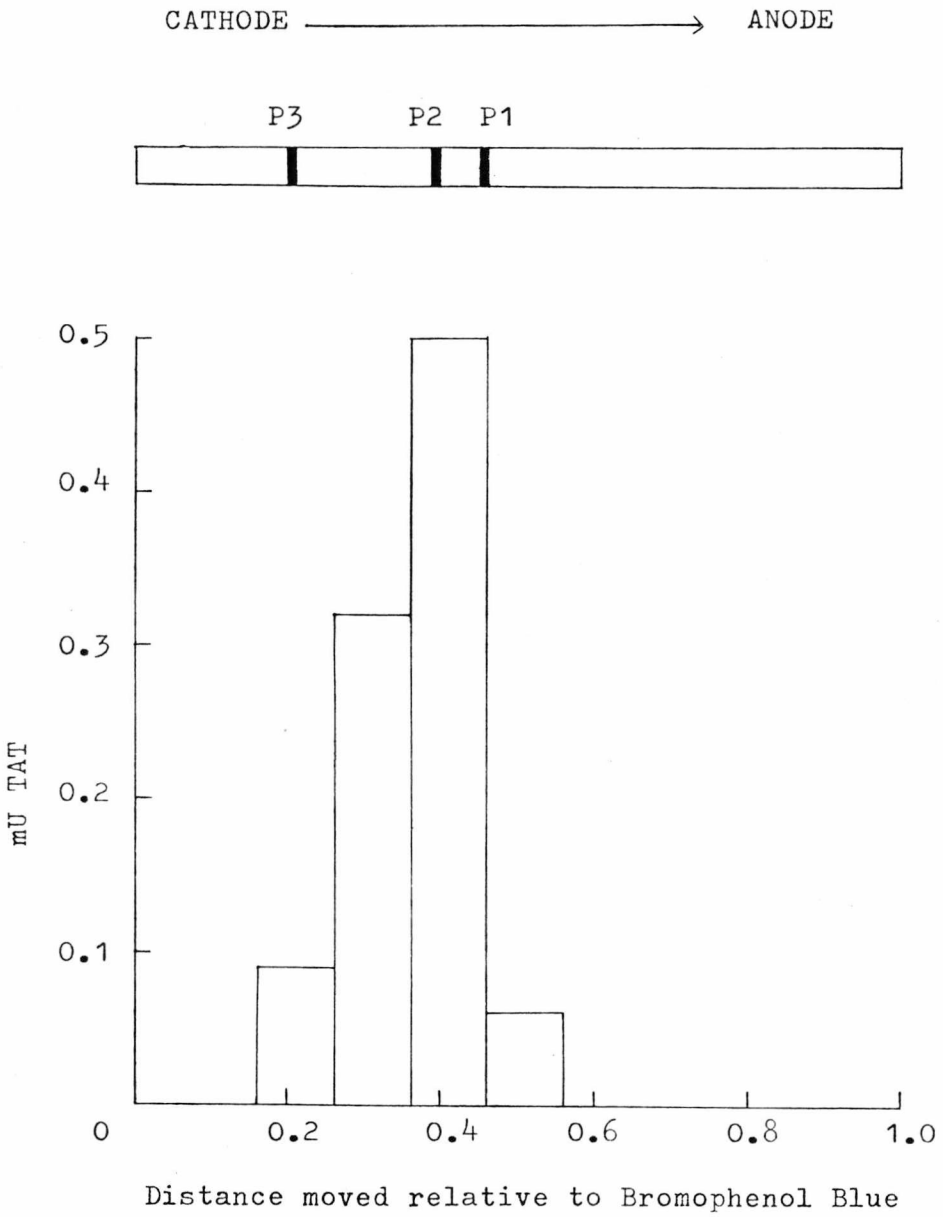
Multiple forms of TAT separated using polyacrylamide gel electrophoresis



After elution of the proteins from gel slices, overnight, at $4^{o}C$, TAT was assayed radioactively using L-[side chain 2,3-^{3}H] tyrosine. It appears that TAT elutes only in the region of the stained bands (Fig 5:4). A more precise correlation of the eluted and stained activity could be obtained by preparing smaller gel slices with a mechanical slicer. Only 30% of the total TAT activity applied to the gel was recovered by this procedure. A better recovery could be achieved with a longer period of elution, but during this time, the TAT may be inactivated. By mounting each slice on a support gel, proteins can be eluted by electrophoresis (Braatz and M^{c}Intire, 1977). Although total recovery of the protein is possible, this procedure is cumbersome to employ when several gels are to be analysed.

N,N'-diallyl-tartardiamide can replace methylene-bis-acrylamide to cross-link gels, and this compound is broken down by 2%(w/v) periodic acid (Anker, 1970). On gels prepared with this cross-linking reagent, a profile of bands comparable with that shown in plate 5:1 was obtained after staining; however, TAT was inactivated by periodic acid. Solubilizable gels were also prepared using a disulphide-containing analogue of bis-acrylamide, bis-acrylylcystamine (Hansen, 1976); these gels can be dissolved by reduction of the disulphide bonds. Again, a standard pattern of bands was obtained with these gels, after staining for TAT activity; but gel slices would not dissolve completely in 2-mercaptoethanol. This procedure has previously only been used with 2.5% gels; it is possible that alterations in the concentrations of ammonium persulphate and TEMED used will facilitate the solubilization of 7.5% gels (Hansen, personal communication).

Fig 5:4 Correlation of TAT activity eluted from polyacrylamide gels and assayed histochemically in situ



P3 : 0.21 ± 0.03

P2 : 0.40 ± 0.04

P1 : 0.46 ± 0.04

Cells were isolated from a fed rat and a crude extract prepared, as described in Materials and Methods. 3 mU TAT applied to each gel; recovery on elution: 32%.

2) Interconversion of the multiple forms of TAT

A particulate factor from rat liver promotes the interconversion of form 1 to forms 2 and 3 in crude extracts from whole liver and from isolated cells (Table 5:5). The supernatant obtained after the centrifugation of crude extracts at 12,000g, was incubated with the particulate preparation; no interconversion occurred after incubation with sucrose alone. The total activity of TAT was unaltered after this incubation, therefore the alteration in the multiple form profile is not a result of inactivation of the enzyme. These results are in agreement with previous observations made using whole liver extracts (Rodriguez and Pitot, 1976; Smith et al., 1976).

The factor(s) responsible for interconversion is non-dialysable, heat-labile, and the optimum pH for its activity is 7.0. By the preparation of pure sub-fractions, the factor(s) was shown to be associated with the lysosomes (Rodriguez and Pitot, 1976; Smith et al., 1976). Sonication is required to solubilise the factor(s); it is not known whether it is located within the lysosomes or associated with the lysosomal membrane.

Aviram and Hershko (1977) have suggested that a more accurate indication of the distribution of the multiple forms of TAT in vivo may be obtained by preparing liver extracts under conditions which prevent interconversion in vitro. The distribution of the multiple forms of TAT in isolated parenchymal cell extracts prepared at pH 6.9 and pH 8.7 were compared (Table 5:6). At both pH 6.9, and 8.7, three forms of TAT are present in the extracts. Interconversion is inhibited at pH 8.7; in extracts prepared at this pH, a greater proportion of form 1 was present than in extracts prepared at pH 6.9.

Table 5:5 Interconversion of multiple forms of TAT by a particulate extract - separation by hydroxylapatite chromatography

WHOLE LIVER EXTRACT

	TAT activity (μmoles.min^{-1}.ml^{-1})	% total TAT activity eluted		
		Peak 1	Peak 2	Peak 3
Initial profile	0.27	90	10	-
60 min + sucrose buffer	0.13	80	9	11
60 min + particulate extract	0.14	17	32	51

ISOLATED PARENCHYMAL CELL EXTRACT

	TAT activity (μmoles.min^{-1}.ml^{-1})	% total TAT activity eluted		
		Peak 1	Peak 2	Peak 3
Initial profile	0.041	50	36	14
60 min + sucrose buffer	0.042	64	20	16
60 min + particulate extract	0.041	32	25	43

Isolated parenchymal cells were prepared from fed rats. Crude extracts from whole liver and isolated cells were prepared, as described in Materials and Methods, in 0.1M phosphate (K^+), pH 6.9, containing 0.2mM-pyridoxal phosphate, 1.0mM-dithiothreitol and PMSF (1mg.ml^{-1}). Values are means from two independent experiments. Duplicate multiple form profiles differed by 10%.

TAT activity in particulate extracts was 0.028 ± 0.006 U/ml (mean from 4 preparations $\pm$ S.E.M.)

Recovery from columns: Protein: 80 - 85%

TAT : 173 - 273%

Table 5:6 Effect of the pH of extraction on the multiple forms of TAT - separation by hydroxylapatite chromatography

pH of extraction	TAT activity (μmoles.min.ml^{-1})	% total TAT activity eluted		
		Peak 1	Peak 2	Peak 3
pH 6.9	0.019	26	35	39
pH 8.7	0.010	49	22	29

Cells were isolated from a fed rat; extracts were prepared as described in Materials and Methods in buffers containing 0.2%(w/v) digitonin. The pH of the extract prepared at pH 8.7 was adjusted to pH 6.9 by the addition of an equal volume of 0.1M phosphate (K^+), pH 6.3.

Recovery from the columns: protein: 80 - 82%

TAT : 185 - 193%

In extracts from hepatoma-derived cells, prepared at pH 8.7, only form III of TAT was found after chromatography on CM-Sephadex (Aviram and Hershko, 1977).

3) The distribution of the multiple forms of TAT after induction by glucagon and glucocorticoids

Extracts, in this investigation, were prepared in pH 8.7 buffers, supplemented with PMSF. Glucagon, hydrocortisone and dexamethasone each increased TAT activity during a 6h incubation period. After chromatography on hydroxylapatite, the relative percentages of the forms were unchanged in the glucagon and glucocorticoid treated cells (Table 5:7).

4) Characterisation of the bands obtained on polyacrylamide gels after staining histochemically for TAT activity

The relative proportions of each stained band was found to vary with the method used for preparing liver extracts (Table 5:8). Most of the TAT is in form P3 in extracts prepared by sonication, or freezing and thawing, while there is a preponderance of form P1 in extracts prepared using a Potter-Elvehjem homogeniser. As the concentration of TAT extracted is similar with each method used, this difference may be the result of interconversion during the extraction procedure.

It has previously been shown that form 1 (hydroxylapatite) of TAT is converted to forms 2 and 3 in a crude liver extract; interconversion was prevented by 10mM KCNO (Smith et al., 1975; Rodriguez and Pitot, 1976). A crude liver extract was incubated under conditions known to promote interconversion, and the multiple forms of TAT were then separated, simultaneously by polyacrylamide gel electrophoresis and hydroxylapatite

Table 5:7 Induction of TAT activity by glucagon and glucocorticoids - separation of multiple forms by hydroxylapatite chromatography

Additions	Incubation time (h)	TAT activity ($nmoles.min^{-1}.ml^{-1}$)	% total TAT activity eluted		
			Peak 1	Peak 2	Peak 3
None	0	4.7 ± 0.40	54	13	33
None	6	2.5 ± 0.15	51	18	31
10^{-8}M glucagon	6	3.3 ± 0.07	53	14	33
10^{-5}M hydrocortisone	6	5.6 ± 0.32	52	13	37
10^{-7}M dexamethasone	6	5.0 ± 0.16	57	19	34

Cells were isolated from an adrenalectomised rat and incubated as described in Materials and Methods. Extracts were prepared in a pH 8.7 buffer containing 0.2% digitonin; the pH of the extracts was adjusted to 6.9 before separation of the multiple forms. TAT activity is expressed as the mean $\pm$ S.D. (3 incubations). The values of the multiple forms are the means from triplicate columns, which varied by 5 - 10%.

Recovery from columns: 240%

Table 5:8 The effect of extraction procedure on the multiple forms of TAT separated on polyacrylamide gels

Method of extraction	TAT activity (μmoles.min^{-1}.ml^{-1})	% total TAT activity stained		
		Form P1	Form P2	Form P3
Potter-Elvehjem	0.050	54	4	41
Freezing and thawing	0.030	14	7	79
Sonication	0.043	19	3	79

Cells were isolated from a fed rat and extracts prepared as described in Materials and Methods. 0.1ml crude supernatant was applied to each gel. All gels were run in triplicate and relative percentages varied by 5%.

chromatography (Table 5:9). Interconversion of the forms is clearly demonstrated after hydroxylapatite chromatography. The bands separated by electrophoresis were altered; after incubation with the particulate extract the percentage of P1 decreased, while that of P3 increased relative to the incubated control. In a separate experiment, the effect of including KCNO in the extraction buffer was investigated (Table 5:10). The initial profiles of TAT activity indicate that KCNO may be preventing the conversion of form P1 to form P3 during the extraction procedure. However, when the same homogenates were incubated for 60min at 25°C before electrophoresis, only form P1 was obtained, in the absence or presence of KCNO. These results do not consistently demonstrate that the bands of TAT separated on polyacrylamide gels correspond to the forms separated by ion-exchange chromatography.

To correlate the forms of TAT separated by electrophoresis and ion-exchange chromatography, TAT in a crude liver extract was separated on a CM-Sephadex column. Four peaks of activity were obtained (Fig 5:5); the fractions containing each form were pooled and concentrated as described (see Materials and Methods). A period of 10 - 12h was required to obtain solutions sufficiently concentrated to apply to polyacrylamide gels. It is assumed that no interconversion occurred during this concentration procedure. This is substantiated by the observation that the interconverting factor(s) in crude liver homogenates is associated predominantly with the particulate fraction after centrifugation (Smith et al., 1975); also after chromatography on CM-Sephadex, no interconverting factor elutes with the fractions containing TAT activity (Aviram and Hershko, 1977).

Table 5:9 Interconversion of the multiple forms of TAT: a comparison of chromatography on hydroxylapatite and polyacrylamide gels

HYDROXYLAPATITE

	TAT activity (μmoles.min^{-1}.ml^{-1})	% total TAT activity eluted		
		Peak 1	Peak 2	Peak 3
Initial profile	0.292	79	21	-
60 min + sucrose buffer	0.131	72	18	10
60 min + particulate extract	0.155	11	24	65

POLYACRYLAMIDE GEL ELECTROPHORESIS

	% total TAT activity stained		
	Form P1	Form P2	Form P3
Initial profile	67	14	19
60 min + sucrose buffer	81	11	8
60 min + particulate extract	60	11	30

Crude extract and particulate extract were prepared from the livers of fed rats as described in Materials and Methods. 0.25ml initial extract and 0.5ml incubated extracts were applied to 6ml hydroxylapatite columns. 20μl and 40μl of these extracts were applied to the polyacrylamide gels. TAT activity in particulate extract = 18mU.ml^{-1}; an equal volume of crude liver extract and particulate extract (or sucrose) were incubated together. Recovery from columns: Protein = 79%; TAT = 197%. Polyacrylamide gels were run in triplicate.

Table 5:10 The effect of KCNO on the profile and interconversion of the multiple forms of TAT separated by polyacrylamide gel electrophoresis

INITIAL PROFILE

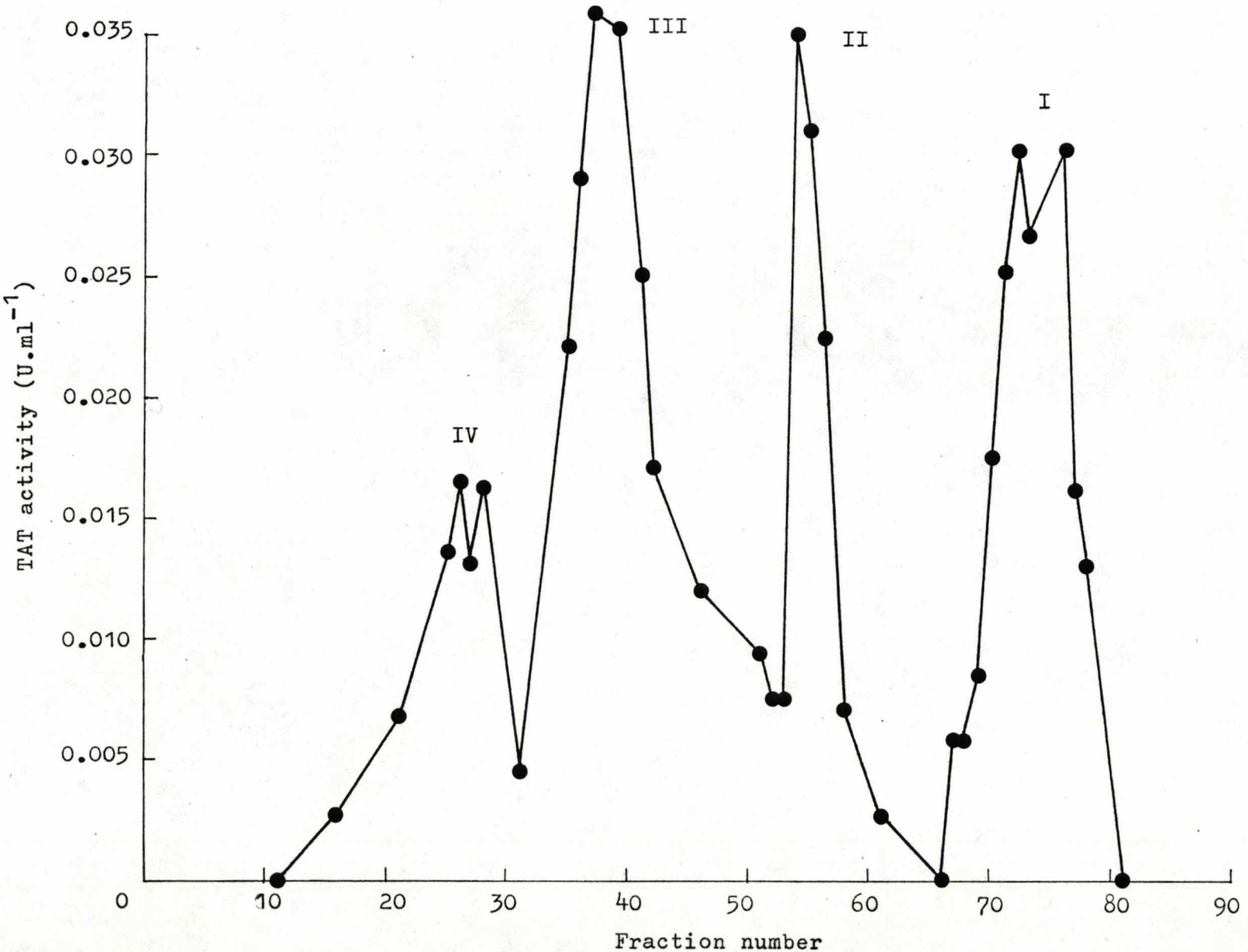
Method of extraction	KCNO	% total TAT activity stained		
		Form P1	Form P2	Form P3
Potter-Elvehjem	-	71	-	29
	+	100	-	-
Freezing and thawing	-	25	-	75
	+	63	-	37

AFTER INCUBATION OF CRUDE HOMOGENATE FOR 60 MIN AT 25°C

Potter-Elvehjem	-	100	-	-
	+	100	-	-
Freezing and thawing	-	100	-	-
	+	100	-	-

Cells were prepared from a fed rat; TAT activity in extracts was 0.053 Units.ml^{-1} and did not vary significantly after incubation for 60 min. 10µl extract was applied to each gel. Values are the means of duplicate gels, which varied by <5%. + = 10mM KCNO, - = no KCNO added.

Fig 5:5 Multiple forms of TAT in a crude liver extract separated by chromatography on CM-sephadex



Legend to Fig 5:5

A liver extract was prepared from a normal, fed rat and 12ml of the 105,000g supernatant applied to a CM-sephadex column. TAT was eluted with a linear KCl gradient (0 - 0.4M) at a flow rate of 25ml.h^{-1}. 5ml fractions were collected. Fractions 20 - 30, 34 - 46, 52 - 58 and 67 - 80 were pooled and assayed for TAT activity (Lin et al., 1958), and concentrated (Millipore immersible molecular separator) to a concentration of approximately 100mU.ml^{-1}.

After electrophoresis of forms III, II and I, one band of activity was observed; in each instance, however, this band corresponded to form P1 in a crude liver extract (Table 5:11). It appears therefore, that the forms of TAT separated by ion-exchange chromatography are not separated by polyacrylamide gel electrophoresis.

Electrophoresis separated form IV into three bands of activity, corresponding to P1, P2 and P3. It was suggested earlier that form IV may be GOT activity. Pure GOT can transaminate tyrosine in the histochemical staining medium; after electrophoresis, a single band is visualized, which corresponds to band P2 in a crude liver extract (Plate 5:2). Form IV and band P3 appear, at least in part, to be GOT activity.

The histochemical stain for TAT used in these experiments has previously been used to visualize the multiple forms of TAT in hepatoma cells on polyacrylamide gels (Gelehrter et al., 1972; Spencer and Gelehrter, 1974). Three bands of activity were detected, but, using specific antibody preparations, one was shown to be GOT. It was suggested that the two remaining bands were monomers and dimers of TAT. On the basis of its movement on polyacrylamide gel, form P3 may be an oligomer of TAT; however, the elution of form IV, from CM-Sephadex, before forms III, II and I is not consistent with this suggestion. Band P3 may be a form of TAT which separates on polyacrylamide gel but not on ion-exchange columns; this would be possible if the conformation of the protein was altered without loss of activity. It is also possible that band P3 results from the transamination of tyrosine by an enzyme other than TAT or GOT.

Table 5:11 Polyacrylamide gel electrophoresis of the multiple forms of TAT separated by chromatography on CM-Sephadex

Peaks separated on CM-Sephadex	Bands stained on polyacrylamide gels		
	Form P1	Form P2	Form P3
I	+	-	-
II	+	-	-
III	+	-	-
IV	+	+	+

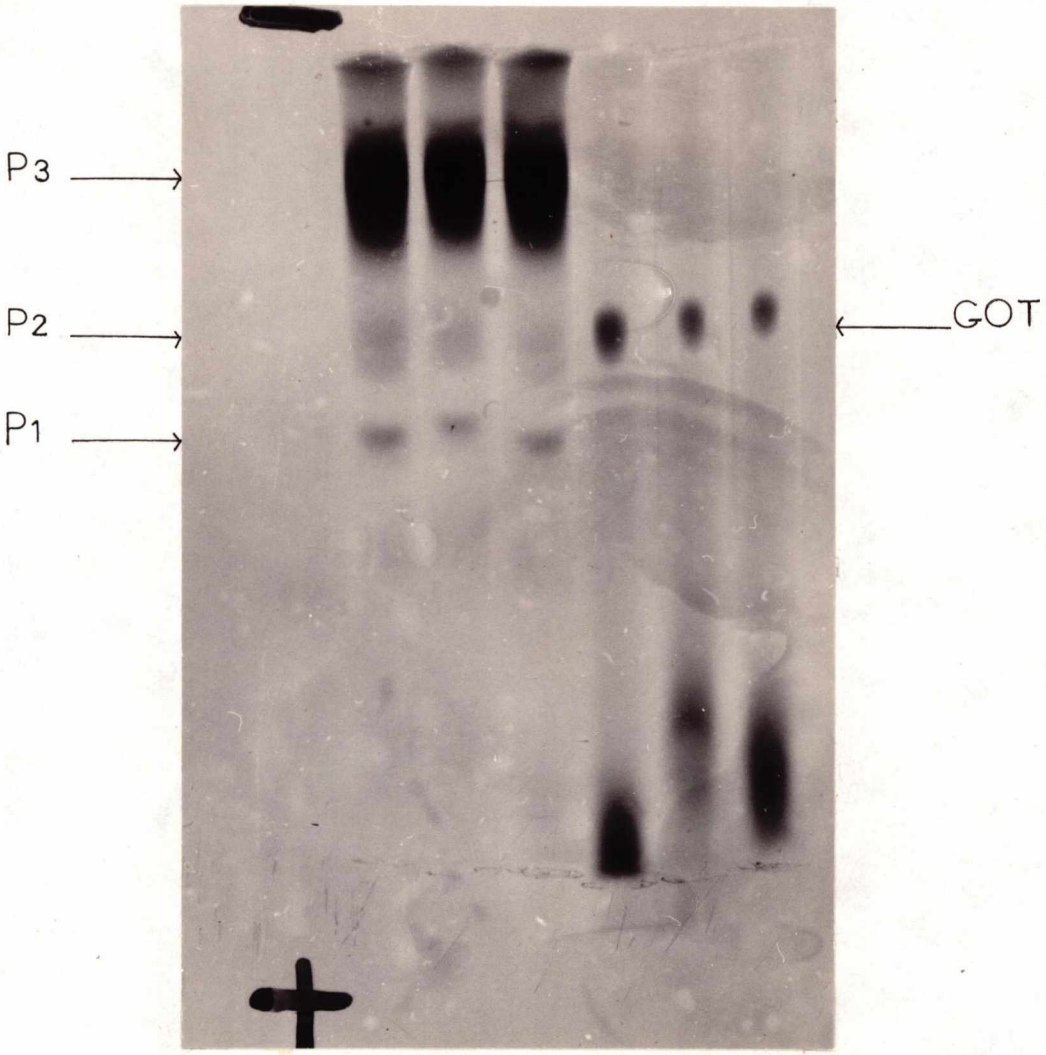
\+ indicates band obtained

\- indicates no band

0.1ml concentrated eluate from the CM-Sephadex column was applied to each gel and electrophoresis was carried out as described in Materials and Methods. The bands were identified from the position of bands obtained from the original crude extract.

PLATE 5:2

A comparison of the position of pure GOT and the multiple forms of TAT on polyacrylamide gels



GENERAL DISCUSSION

The separation of multiple forms of TAT in extracts from isolated liver cells by ion-exchange chromatography and polyacrylamide gel electrophoresis

One objective of these experiments was to find a chromatographic procedure suitable to use in conjunction with isolated liver cells; a system was required for the analysis of small amounts of crude extracts, which could be applied to several samples simultaneously. It was shown that ion-exchange chromatography can be used for the analysis of the multiple forms of TAT in extracts from isolated cells; activity profiles were obtained which compare well with those from whole liver extracts. Batch elution of TAT from the column is preferred to gradient elution; the latter process must be coupled with fraction collecting which severely restricts the number of samples which can be analysed simultaneously.

A disadvantage of ion-exchange chromatography is the dilution incurred on elution of the protein. This is particularly evident with the CM-Sephadex procedure used in this chapter. It was necessary to develop a more sensitive assay for TAT (Chapter 3) to enable accurate measurement of the activity eluted from the columns. To overcome this dilution problem, after the chromatographic analysis of hepatoma cell extracts, ammonium sulphate was added to the eluate, to concentrate the TAT (Aviram and Hershko, 1977). However, some enzyme may be inactivated by this process.

A refinement of the CM-Sephadex procedure for the separation of multiple forms of TAT has recently been published, which may be more suitable for the analysis of isolated parenchymal cell extracts (Boctor and Grossman, 1978). Crude extract

was added directly to tubes containing known amounts of CM-Sephadex; after adsorption, appropriate concentrations of KCl were added to different tubes to elute all the TAT or form III only. By subtraction, the relative amounts of form III and forms (II and I) were determined. This method could be further refined to enable the measurement of all three forms. Alternatively, small amounts of TAT in eluates from ion-exchange columns could be measured by pre-labelling proteins and using a specific antibody directed against TAT. The forms of TAT are antigenically identical (Johnson et al., 1973), therefore antibodies raised against total hepatic TAT could be used. However, for routine experimental work, large amounts of antibody may be required.

Of the chromatographic procedures used, polyacrylamide gel electrophoresis is most suitable for the analysis of crude extracts from isolated parenchymal cells. Small amounts of extract are used and several gels can be run simultaneously. However, the results obtained suggest that the multiple forms of TAT do not separate after polyacrylamide gel electrophoresis. It is possible that this is a result of the conditions used for electrophoresis. The pH of the buffer system used was 8.7 while ion-exchange was performed at pH 6.5 or 6.9.

The detection of TAT in polyacrylamide gels, after electrophoresis has proved to be difficult. The results emphasise the caution required in the use and interpretation of histochemical stains. A more reliable method is to assay the enzyme after elution from gel slices. Gels cross-linked with bisacrylylcystamine would be appropriate for this purpose; but detailed investigation is required to determine the conditions necessary for solubilization. As with ion-exchange chromato-

graphy, antibodies directed against TAT could also be used to assay TAT either *in situ* or after elution from the gels. The latter alternative would provide a more accurate estimate, as the antibody preparation is unlikely to diffuse efficiently into the gels.

Significance of the multiple forms of TAT.

The experiments in this chapter demonstrate that TAT in crude liver extracts can be separated into three or possibly four forms. A factor (or factors) associated with the particulate fraction of the cells can promote the interconversion of these forms. This factor is either membrane-bound or may be located within the lysosomes.

The forms of TAT are therefore not separate gene products, and it is possible they do not exist *in vivo*. In hepatoma-derived cells, TAT appears to exist only in one form; under the same conditions, three forms of TAT are still observed in extracts from isolated parenchymal cells. That the multiple forms of TAT are not artifacts of extraction is supported by the observation that only one form of TAT is present in extracts from heart and kidney; an identical extraction and chromatographic procedure demonstrated three separable forms of TAT in liver (Iwasaki *et al.*, 1973).

One hypothesis for a possible role of the multiple forms of TAT *in vivo* is that the activity of each form is controlled by different hormonal or nutritional inducers of TAT. The original objective of the studies presented here was to extend the observations reported in Chapter 4 to an analysis of the multiple forms of TAT. Only a limited investigation of this aspect has been possible as a result of the considerable technical

difficulties encountered in the analysis of multiple forms in cell extracts. The results obtained indicate that neither glucagon nor glucocorticoids specifically increase one form of TAT; the activities of the forms are equally increased, so that no change in their relative percentages are observed. The results are at variance with previous reports (Holt and Oliver, 1969; Iwasaki et al., 1973; Johnson et al., 1973); but these reports themselves are not in agreement with each other.

As the factor which promotes the interconversion of the multiple forms of TAT was found to be associated with or within the lysosomes (Smith et al., 1976), it was suggested that the forms may have a role in the turnover of TAT. However, there is little direct evidence for the involvement of the lysosomes in the intracellular degradation of proteins (Dean, 1975). Interconversion in the opposite direction to that promoted by the particulate factor(s) has also been implicated in the turnover of TAT (Johnson et al., 1973). There is support for this hypothesis, as form III is more readily inactivated in vitro than form I (Beneking et al., 1978). Also consistent with a multistep degradation mechanism is the observation that TAT can be reversibly inactivated in vivo (Grossman and Boctor, 1972). The irreversible degradation of ornithine aminotransferase appears to be preceded by limited proteolysis without loss of activity (Kominami et al., 1972; Kominami and Katanuma, 1976). However there is evidence to suggest that the factor responsible for in vitro inactivation of TAT is not a protease (Reynolds, 1978).

Rodriguez and Pitot (1976) have suggested that the multiple forms of TAT differ in the number of free sulphydryl groups; they have shown that forms 1, 2, and 3 contain 17, 15, and 14 free sulphydryl groups respectively. These figures do not provide very convincing evidence as it is difficult to measure such small differences in sulphydryl content accurately. However, assuming that there were significant differences in sulphydryl content, the authors suggested that glutathione-insulin transhydrogenase may be implicated in interconversion. This transhydrogenase is membrane-bound; recent evidence obtained by immunoferritin labelling of isolated liver cells suggests that the enzyme is located extensively on the smooth endoplasmic reticulum (Varandani et al., 1978).

As well as preventing interconversion by a particulate fraction, KCNO promoted the conversion of form I to form III (Johnson et al., 1973). Carbamylation may therefore be implicated in interconversion. An alternative suggestion is that phosphorylation may be involved in interconversion. The activation and inactivation of enzymes by phosphorylation is a widespread phenomenon (Rubin and Rosen, 1975). TAT can be phosphorylated in vivo and in vitro (Lee and Nickol, 1974; Hamm and Seubert, 1977); there is evidence to suggest that such phosphorylation is not related to the regulation of TAT by cyclic AMP (Wicks and Su, 1978). If this modification is implicated in interconversion the forms would separate on hydroxylapatite on the basis of their state of phosphorylation.

The existence of more than one form of an enzyme per se appears energetically wasteful. However theoretical consideration suggests that reversible interconversion could

be advantageous as a regulatory mechanism (Stadtman and Chock, 1978). In contrast to regulatory mechanisms which either totally activate or inactivate an enzyme, interconversion could allow a progressive, reversible change in activity. Such a mechanism could increase the number of steps which could be controlled; therefore an increased number of hormonal and nutritional agents could have independent regulatory roles. Overall, interconversion would allow a rapid and gradual variation in enzyme activity in response to changes in the environment. The evidence suggests that interconversion alters the susceptibility of TAT to degradation rather than its activity; by an analagous mechanism the multiple forms of TAT could facilitate the regulation of the turnover of this enzyme.

CHAPTER 6

METABOLISM OF TYROSINE BY ISOLATED PARENCHYMAL CELLS

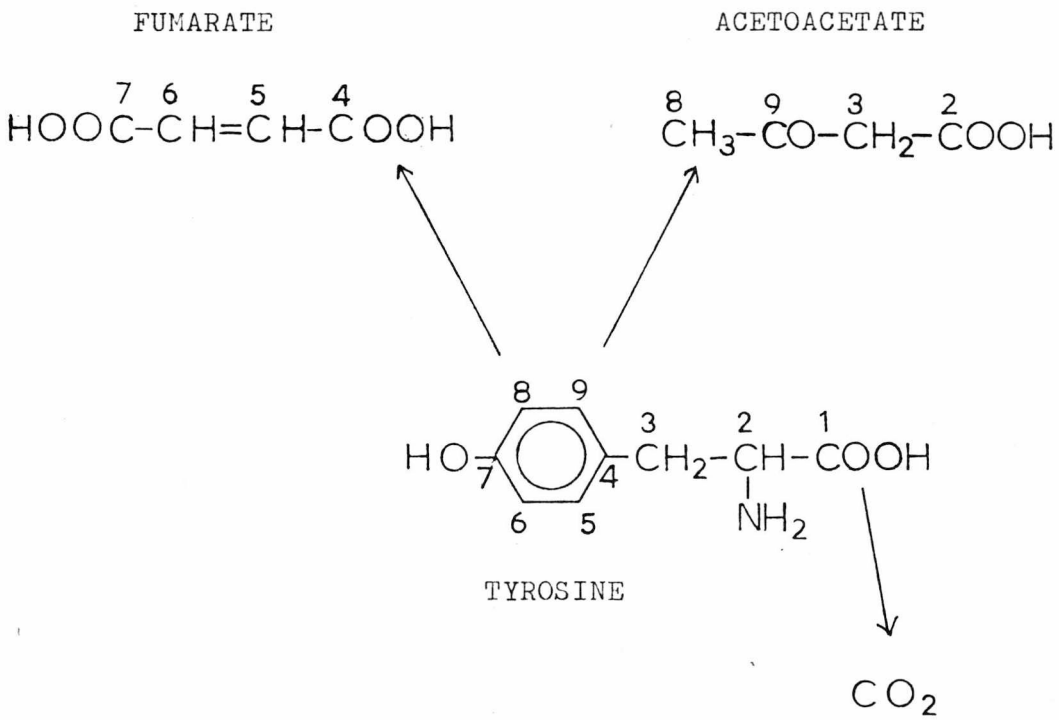
INTRODUCTION

Catabolism of tyrosine via transamination is the major route of degradation for this amino acid in mammals (Greenberg, 1961). The hypothesis that decarboxylation of tyrosine to tyramine, in the liver, is of quantitative significance (David et al., 1971a and b) has been conclusively refuted (Fellman et al., 1976). From these studies it was suggested that oxidative degradation is the only catabolic pathway for tyrosine in the liver.

The detailed metabolism of the carbon skeleton of tyrosine was elucidated using ^{14}C-labelled phenylalanine and tyrosine. When these compounds were administered in vivo, or included in liver slice incubations, the ultimate products isolated were ^{14}C-acetoacetate and ^{14}C-malate (Weinhouse and Millington, 1948; Lerner, 1949; Schepartz and Gurin, 1949). From the labelled intermediates isolated, the fate of each carbon atom in tyrosine was proposed, as illustrated in Fig 6:1. These studies also demonstrated that rearrangement of the amino acid side chain occurs during the formation of homogentisate, accompanied by the loss of the C-1 atom as CO_2 (Lerner, 1949; Schepartz and Gurin, 1949).

Since the flux through a metabolic pathway is determined by the rate of the slowest reaction, enzymes which catalyse such reactions have important regulatory roles. The evidence to suggest that transamination of tyrosine is the rate-limiting step of hepatic tyrosine metabolism is largely circumstantial. TAT catalyses the first step in the oxidative degradation of tyrosine (Knox and Le May-Knox, 1951; La Du and Greenberg, 1951) and transamination is irreversible (Bücher and Kirberger, 1952). Using pure TAT, the kinetic

Fig 6:1 The metabolic fate of the carbon atoms of tyrosine



properties of the enzyme were investigated. Simple Michaelis-Menten kinetics were observed (Rosenberg and Litwack, 1970), the relationship between the rate of reaction and tyrosine concentration being a rectangular hyperbola. The K_m for tyrosine is high, compared with intrahepatic tyrosine concentrations (Jacoby and La Du, 1963; Hayashi *et al.*, 1967), indicating that substrate availability could limit the rate of transamination *in vivo*.

It is perhaps the regulatory properties of TAT which provide most support for the hypothetical rate-limiting role. As described earlier, TAT activity is induced by a variety of hormones, as well as by tryptophan and amino acid mixtures (Chapter 4; Table 4:1). However, in all these investigations TAT activity was quantitated *in vitro* by measuring its catalytic activity, or by immunological quantitation of enzyme protein. The physiological significance of these measurements is questionable, particularly with reference to the role of TAT in regulating the rate of hepatic tyrosine catabolism. An increase in the potential maximum activity of TAT is not necessarily paralleled by an increase in enzyme activity *in vivo*.

The experiments presented in this chapter were performed to develop a system for measuring rates of tyrosine metabolism in cells. Isolated liver cells are physiologically comparable with liver cells *in situ* (Seglen, 1976b) and therefore constitute a model *in vivo* system. Thus enzymically isolated parenchymal cells were incubated in medium containing L-[*side chain* 2,3-^{3}H] tyrosine and TAT activity in cells measured from the rate of exchange of ^{3}H with the medium.

The results obtained indicate that there is some correlation between rates of transamination measured in cells and *in vitro*, under conditions which induce TAT activity. The use of this system for studies of tyrosine metabolism in cells is critically discussed.

MATERIALS AND METHODS

1) Animals

Animals were maintained under the conditions described in the General Materials and Methods section. Unless otherwise stated, all animals were allowed access to food and water *ad libitum*. Adrenalectomized animals were used on the 6th day following removal of the adrenal glands.

2) Solutions

In experiments where the concentration of individual amino acids, or the total amino acid mixture, was to be altered, a concentrated stock solution of MEM was prepared with the appropriate amino acid(s) omitted (Chapter 2). The media being compared in one experiment, were thus derived from the same original stock solution, by dilution with either water or amino acid(s) in solution. Where a variety of medium tyrosine concentrations were being compared, media were prepared by serial dilution of a stock solution, which contained 2.5mM tyrosine.

3) Cell incubation conditions

A) Incubations for the measurement of cellular tyrosine metabolism and *in vitro* enzyme activity

The standard procedure described in Chapter 2 (Section 4(C)) was used, with one exception: 0.2ml aliquots of cell suspension were added to 20ml glass vials containing 1.0ml incubation medium. A final cell concentration of <1mg dry wt.ml^{-1} was used in all experiments, and incubations were performed in triplicate.

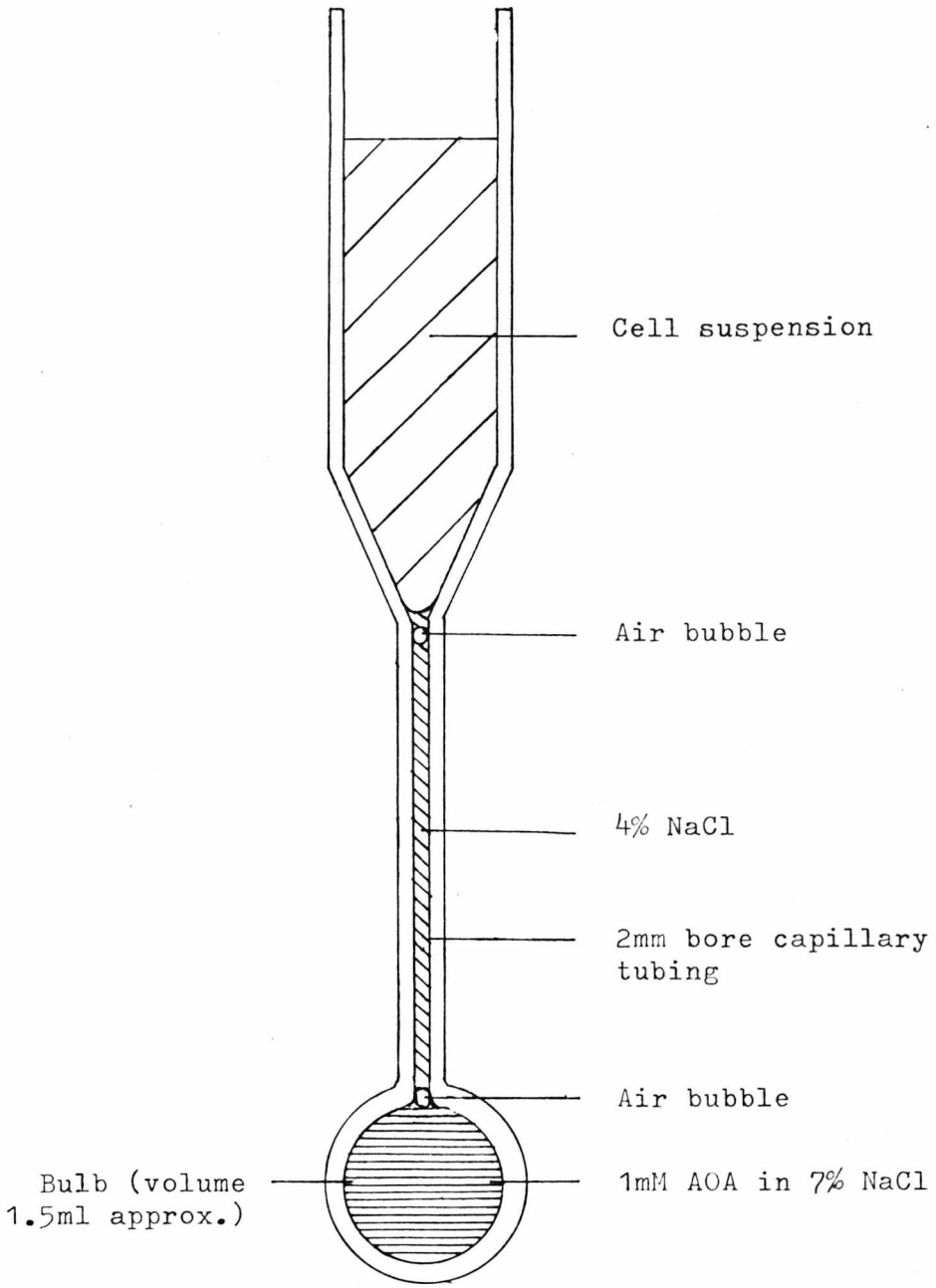
Suspensions in which rates of tyrosine metabolism were to be determined were pre-incubated for 30min in the presence of the appropriate level of cold tyrosine. L-[side chain 2,3-^{3}H] tyrosine was then added to each vial; this time-point is referred to as Oh in the text. Incubations were terminated by the addition of amino-oxyacetate (AOA; final concentration 1mM). Separate incubation vials were prepared for LDH and TAT measurements and the method used to terminate cell metabolism was as described in Chapter 4.

B) Incubations for the determination of extracellular and intracellular tyrosine concentrations

0.5ml aliquots of cell suspension were added to 25ml Erhlenmeyer flasks, each containing 2.5ml medium; thus the ratio of cells : medium was identical to that used in incubations for enzyme measurements. Cells were pre-incubated in the presence of cold tyrosine for 30min, as described above, at which point [^{3}H] tyrosine was added.

In order to measure the extracellular and intracellular levels of tyrosine, the cells must be separated from the suspending medium. A rapid and efficient separation can be achieved using the special centrifuge tubes designed by Hems et al., (1975). These tubes each consist of a bulb connected to an upper chamber by a capillary (Fig 6:2). The bulb contained 7%(w/v) NaCl supplemented with 1mM AOA and the capillary contained 4%(w/v) NaCl. Tubes were filled using Pasteur pipettes, and care was taken to avoid cross-contamination of the solutions. While filling the tubes, air bubbles were introduced at each end of the capillary, as shown (Fig 6:2); these prevent mixing and allow the cell

Fig 6:2 Tube for rapid separation of hepatocytes from incubation medium



suspension to be poured rapidly into the upper chamber. The tubes were centrifuged at 1,500g for 1 min at room temperature (M.S.E. Super Minor Centrifuge). Since the specific gravity of the solutions increases from the top to the bottom of the tubes the cells move rapidly through the capillary into the bulb, while the medium remains in the upper chamber. The AOA in the bulb immediately terminates transamination.

A sample of the suspension medium was taken, and the remaining medium in the upper chamber and capillary removed by aspiration. After drying the capillary with a pipe cleaner, 7%(w/v) NaCl supplemented with 1mM AOA was added to a pre-determined volume. The cell pellet was resuspended using a motor-driven stirring rod and the resultant suspension was transferred to an Eppendorf centrifuge tube.

Some suspending medium is inevitably carried down with the cells, therefore 0.5μCi [^{14}C] inulin (specific activity 0.05μCi.mg^{-1}) was added to the flasks prior to centrifugation. Since the medium and cell extract each contained ^{3}H and ^{14}C, samples were counted using windows pre-set for the measurement of ^{3}H in the presence of ^{14}C and vice versa. After counting the samples were each 'spiked' with 0.005μCi [^{14}C] Na acetate and recounted to allow for the overlap from the ^{14}C-channel into the ^{3}H-channel to be estimated. All samples were counted in suspension in PCS scintillation fluid.

4) Preparation of cell extracts

Cells in which enzyme activities and tyrosine were to be measured were precipitated and resuspended by the procedures described. In contrast, cell extracts for metabolism measurements were prepared from the incubated suspensions, with

no further manipulations except AOA addition. Since there is a vast excess of H_2O in the medium, ^3H_2O produced in the cell is likely to appear rapidly in the medium; therefore concentration of the cells is unnecessary.

Sequential freezing (-70°C) and thawing (30°C), three times, was used to lyse the cells. Cell debris was removed by centrifugation at 12,000g for 2.5min at room temperature.

A) Isolation of intracellular tyrosine using ion-exchange chromatography

Cell extracts were deproteinized by the addition of $HClO_4$ (final concentration 0.2M) and the precipitated protein removed by centrifugation. The supernatant was neutralized with 0.5M triethanolamine, pH 7.4, containing 2N-KOH and the $KClO_4$ allowed to precipitate at 4°C. An aliquot of this supernatant was applied to an Amberlite CG 120 (H^+) column, washed with water, and the tyrosine was eluted with 2M ammonium formate. Cellular tyrosine concentrations were determined from extracellular specific activities, and on the basis of a cell volume of 0.7ml.(g wet wt cells)$^{-1}$ (Seglen, 1976b).

B) Tyrosine metabolism: measurement of ^3H_2O formed in cells

50µl aliquots of cell extract were transferred to Eppendorf centrifuge tubes, 9mg charcoal (0.45ml of a 20mg. ml^{-1} suspension) was added to each tube, mixed and centrifuged for 2min at 12,000g. 0.25ml of the resultant supernatant was counted in 1ml PCS.

Cell extracts and samples of medium obtained from separation tubes were treated in a similar manner, but the

scintillation counting was performed as previously described. During the course of experiments it was found that charcoal-treatment completely removes inulin from the extracts.

5) Enzyme assays

TAT was assayed using L-[side chain 2,3-^{3}H] tyrosine and LDH was assayed spectrophotometrically (Stinson and Gutfreund, 1971). These procedures were described in Chapters 3 and 4 respectively. TAT assays were performed at 37°C, not 30°C, to allow a comparison between in vitro and cellular rates of activity.

6) Fluorimetric assay for tyrosine (Waalkes and Udenfriend, 1957)

Cell extracts and medium samples were acidified and deproteinized before being assayed, by the addition of trichloroacetic acid (TCA; final concentration 5%(w/v)). Precipitated protein was removed by centrifugation. The tyrosine standard was prepared as a solution in 5%(w/v) TCA.

The assay mixture is stable for only 30min and was therefore prepared immediately before use:

15mM HNO_3
0.044%(w/v) $NaNO_2$
0.025%(w/v) 1-nitroso-2-naphthol (in methanol A.R.)

An equal volume of tyrosine (standard or unknown) was added to initiate the reaction. Tubes were mixed, covered, and incubated at 55°C for 30min. After the tubes had cooled, 1.0ml dichloroethane was added to each tube and mixed thoroughly. Unreacted 1-nitroso-2-naphthol is extracted into the organic phase, while the nitroso-naphthol derivative of tyrosine remains in the aqueous phase. Tubes were centrifuged at 1,500g for 5min to

ensure a complete separation of the two phases. Blanks, in which $NaNO_2$ was omitted, were performed simultaneously. The nitroso-naphthol derivative of tyrosine was activated at 460nm, and fluorescence measured at 570nm (Perkin-Elmer 1000 Fluorescence Spectrometer).

RESULTS AND DISCUSSION

In all the experiments presented, AOA was used to terminate the exchange of [^{3}H] with the medium during transamination. This metabolite competitively inhibits transamination of amino acids by reacting with the aldehyde group of the cofactor pyridoxal phosphate (Braunstein, 1973). Transaminases have high affinities for AOA, because of its structural resemblance to amino acids (Oehme, 1968). At a concentration of 1mM, AOA completely inhibited release of ^{3}H from L-[side chain 2,3-^{3}H] tyrosine, whether added 30min before or, simultaneously with, the [^{3}H] tyrosine.
In addition to its specific effect on transaminases, AOA inhibits urea synthesis (Rognstad and Clark, 1974) and protein synthesis (Seglen and Solheim, 1978b) in isolated liver cells. The use of AOA is preferred to acidification, because non-specific exchange of ^{3}H from [side chain-^{3}H] tyrosine is stimulated at low pH (Chapter 3).

While developing the radioactive assay for TAT, using L-[side chain 2,3-^{3}H] tyrosine, crude liver extracts were found to promote exchange of the 3-C-^{3}H with the medium, subsequent to transamination. Therefore, the results in this chapter have been calculated on the basis of the exchange of both the 2-C-^{3}H and the 3-C-^{3}H with the medium.

1) Estimation of intracellular tyrosine levels in isolated parenchymal cells

To measure rates of tyrosine metabolism in isolated liver cells, the intracellular specific activity of tyrosine must be known. A measure of the intracellular tyrosine concentration ($[tyr]_{int}$) is also required to allow

Table 6:1 Extracellular and intracellular concentrations of tyrosine

$[tyr_{ext}]$	$[tyr_{int}]$
0.045±0.005	0.24±0.01
0.22±0.02	0.22±0.02
1.01±0.08	1.07±0.02
2.4±0.20	2.80±0.45

Cells were isolated from a fed, normal rat. $[tyr]_{ext}$ was assayed fluorimetrically, $[tyr]_{int}$ was measured as described in Materials and Methods. The unit of concentration is mM; values are means ± S.D. (3 determinations).

an estimation of the K_m for tyrosine, in cells. As described in Materials and Methods, cells isolated from a fed rat were incubated for 30min with various concentrations of tyrosine, then separated from the medium using Hems' tubes. The fluorimetric assay (Waalkes and Udenfriend, 1957) provided values for medium tyrosine concentration ($[tyr]_{ext}$) which were expected from the amount of tyrosine added (Table 6:1). But the estimates of $[tyr]_{int}$ greatly exceeded the corresponding medium concentrations. Further experiments demonstrated that this fluorescence was almost entirely due to contaminants in the cell extracts: omission of either $NaNO_2$ or HNO_3 from the assay mixture had no effect on the fluorescence readings obtained.

Recent reports have demonstrated that the extracellular and intracellular specific activities of [^{14}C] valine equilibrate within 5min, at 37°C, in isolated hepatocytes (Ricca et al., 1978; Seglen and Solheim, 1978a); extracellular valine concentrations of 0.4mM - 10mM were used. It is equally probable that extracellular and intracellular tyrosine specific activities equilibrate rapidly. On the basis of this assumption, $[tyr]_{int}$ were calculated from extracellular specific activities measured. Tyrosine, in liver cell extracts, was separated from its metabolites by ion-exchange chromatography, using Amberlite CG 120 (H^+); $[tyr]_{int}$ was estimated as described in Materials and Methods. When cells were incubated in medium containing either 0.2mM, 1.0mM or 2.5mM tyrosine, the $[tyr]_{int}$ approximated to the corresponding $[tyr]_{ext}$ (Table 6:1). But, at a $[tyr]_{ext}$ of 0.05mM, the tyr_{int} was estimated as 0.24mM, which suggests concentrative uptake of tyrosine by the cells. Using a

comparable method to measure valine distribution, Seglen and Solheim (1978a) found that intracellular valine levels did not exceed extracellular levels; the intracellular : extracellular ratio approached unity. In these studies, however, the minimum extracellular valine concentration used was 0.5mM.

2) Rates of tyrosine metabolism in isolated parenchymal cells

When TAT activity is assayed *in vitro*, using L-[*side chain* 2,3-^{3}H] tyrosine, a low rate of tritium exchange with the solvent occurs in the absence of cell extract (Chapter 3). Therefore, in preliminary experiments, incubations with cells omitted were included, but no loss of tritium occurred. In Chapter 3, it was suggested that spontaneous formation of a Schiff base aldimine complex, in the presence of excess pyridoxal phosphate, may be responsible for this *in vitro* blank rate. The observation that no blank rate occurs in the cell incubation medium, which contains physiological amounts of pyridoxal-HCl, supports this hypothesis.

A) Concentration dependence of the rates of tyrosine metabolism

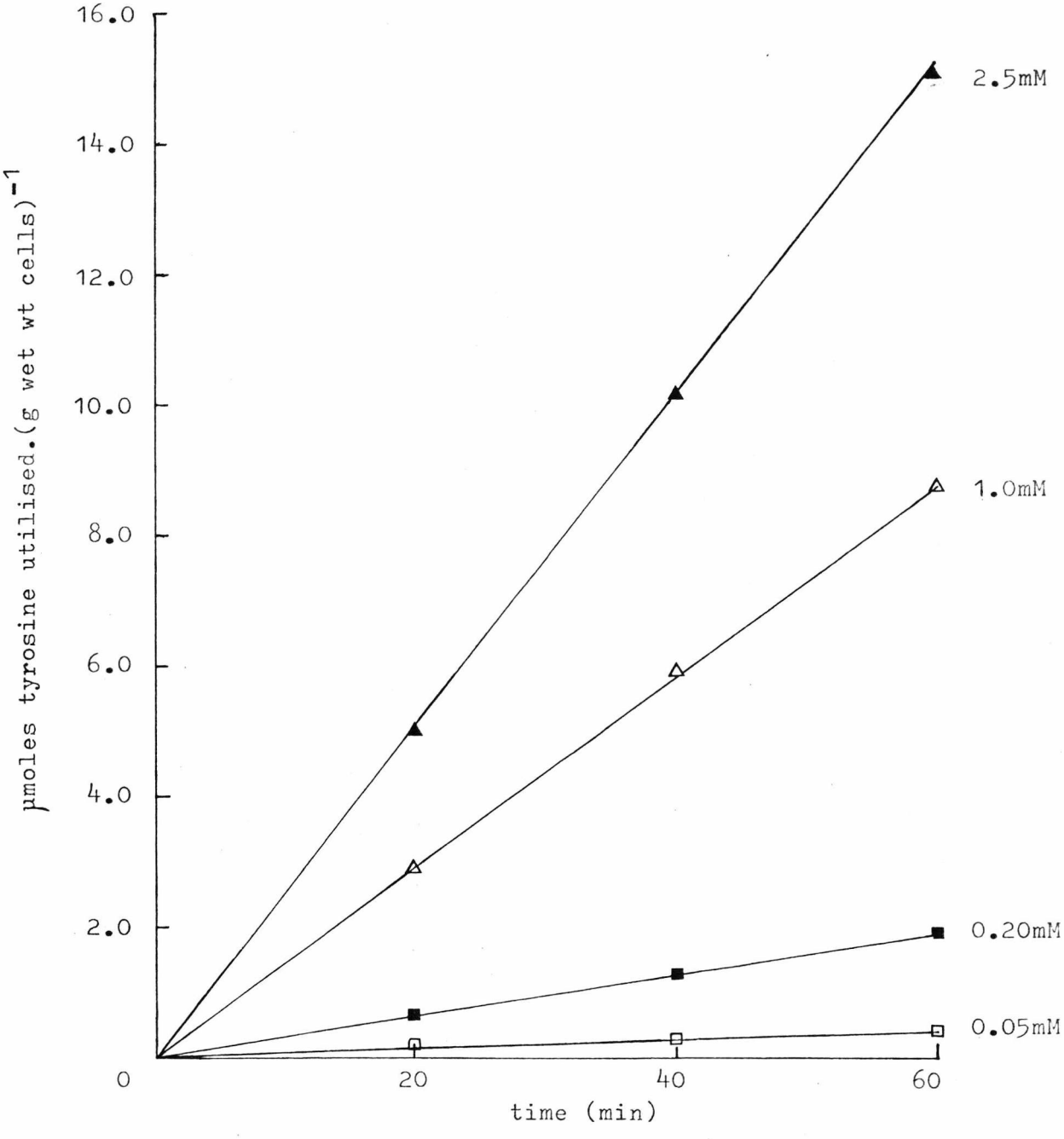
The reported values for hepatic tyrosine levels are between 0.05mM and 0.1mM (Herbert *et al.*, 1966), while plasma concentrations vary, diurnally, between 0.05mM and 0.1mM (Wurtman, 1970). Therefore rates of tyrosine metabolism were measured at extracellular concentrations of 0.05mM and 0.1mM; in addition, two unphysiological medium concentrations were used, 1.0mM and 2.5mM, to allow the K_m for tyrosine, in cells, to be measured. Using purified TAT, the K_m for tyrosine *in vitro* was found to be 1.7 - 2.2mM (Jacoby and La Du, 1963; Weinstein *et al.*, 1968; Hayashi *et al.*, 1967). A similar

value of 1.92mM was estimated,using crude extracts, in this thesis (Chapter 3). Because of the limited solubility of tyrosine at 37°C, a medium concentration much greater than 2.5mM could not be achieved.

In cells isolated from fed, normal rats, tyrosine is transaminated at a constant rate for 60min, throughout the concentration range (Fig 6:3). The relationship between the rates of metabolism measured and the estimated $[tyr]_{int}$ was examined (Figs 6:4 and 6:5). The reciprocal values of the rates and $[tyr]_{int}$ fit a straight line (Fig 6:5), with the exception of those obtained at the lowest $[tyr]_{ext}$ used. If, however, it is assumed that this $[tyr]_{int}$ approaches the $[tyr]_{ext}$, then the reciprocal of the rate measured falls on the straight line as shown. The double-reciprocal plot (Lineweaver and Burk, 1934) yields a K_m for tyrosine, in cells of 2.5mM, which is similar to the values estimated *in vitro*. A linear relationship between the rate of metabolism and tyrosine concentration would be expected at concentrations $\leq K_m$ for tyrosine. This was found to hold only when the lowest $[tyr]_{int}$ was assumed to be 0.05mM, rather than 0.24mM (Fig 6:4).

The rates of tyrosine metabolism observed are thus consistent with $[tyr]_{int}$ being the same as, or approaching, the corresponding $[tyr]_{ext}$ at all concentrations of tyrosine. The cell preparation used for $[tyr_{int}]$estimation was also used for tyrosine metabolism measurements. Rates of 0.007, 0.026, 0.115 and 0.232μmoles tyrosine utilised.min^{-1}.(g wet wt cells)$^{-1}$ were obtained at tyr_{ext} of 0.05mM, 0.2mM, 1.0mM and 2.5mM respectively. These results suggest that the value for $[tyr]_{int}$ estimated at a $[tyr]_{ext}$ of 0.05mM is an anomaly, rather than an error, of the method used; further results are calculated assuming a $[tyr]_{int}$ of 0.05mM.

Fig 6:3 Time course of tyrosine transamination in liver cells isolated from fed, normal rats



The values presented are the means from 3 separate cell preparations. L-[side chain 2,3-^{3}H] tyrosine was added after the 30min preincubation period and the specific activities used were:

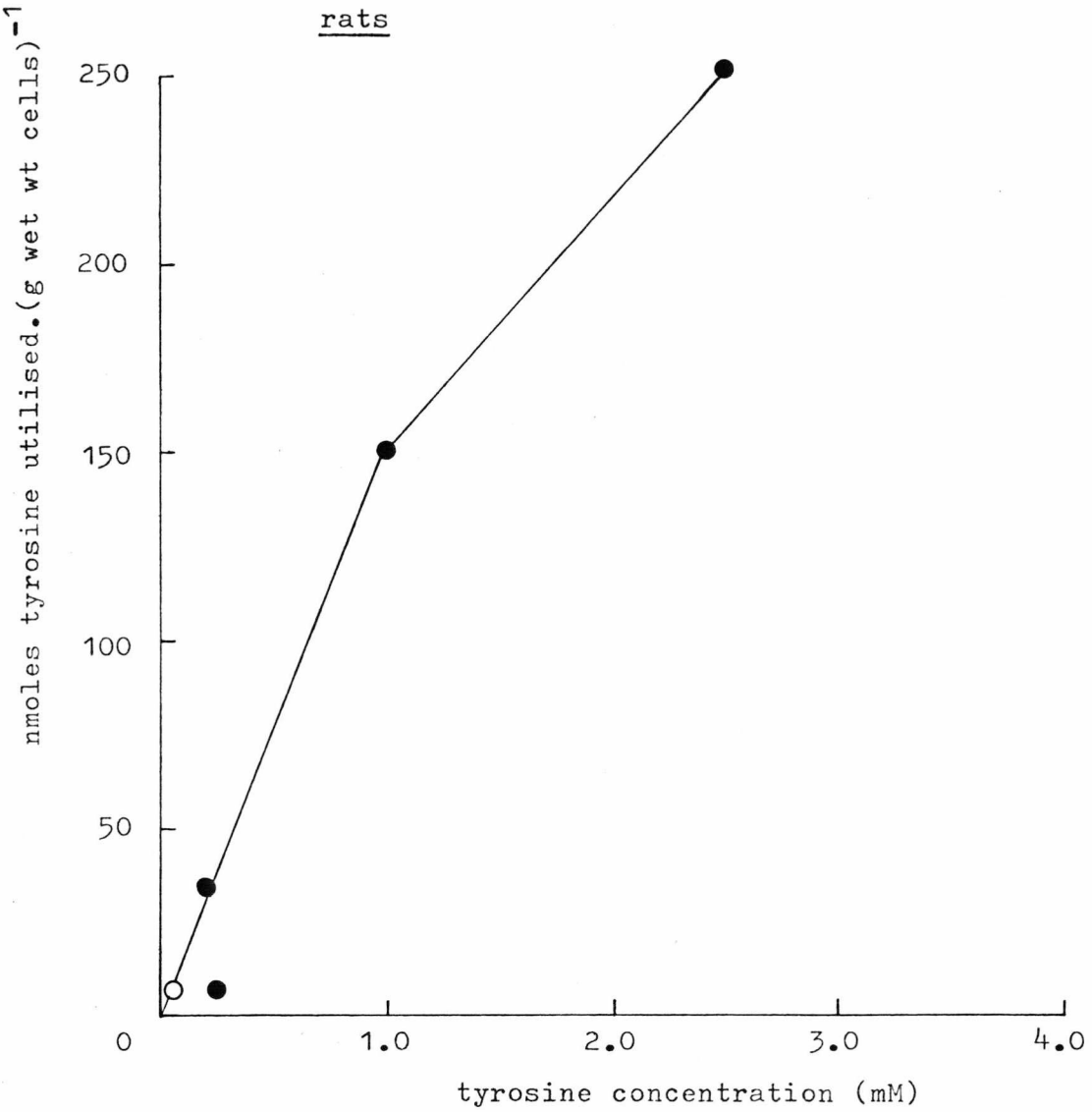
Legend to Fig 6:3 / Continued

medium 'tyrosine	$\mu Ci.(\mu mole\ tyrosine)^{-1}$
0.05mM	0.8
0.20mM	0.8
1.0mM	0.16
2.5mM	0.064

Cellular ATP content: 14.4 ± 0.43 nmoles.(mg dry wt cells)$^{-1}$

Tyrosine (mM)	Rate of tyrosine metabolism (nmoles tyrosine utilised. (g wet wt cells)$^{-1}$.min^{-1}) $\pm$S.E.M.
0.05	6.9 ± 0.7
0.20	33.0 ± 4.0
1.0	150.0 ± 31.0
2.5	251.0 ± 75.0

Fig 6:4 The relationship between tyrosine concentration and tyrosine metabolism in cells isolated from fed, normal rats



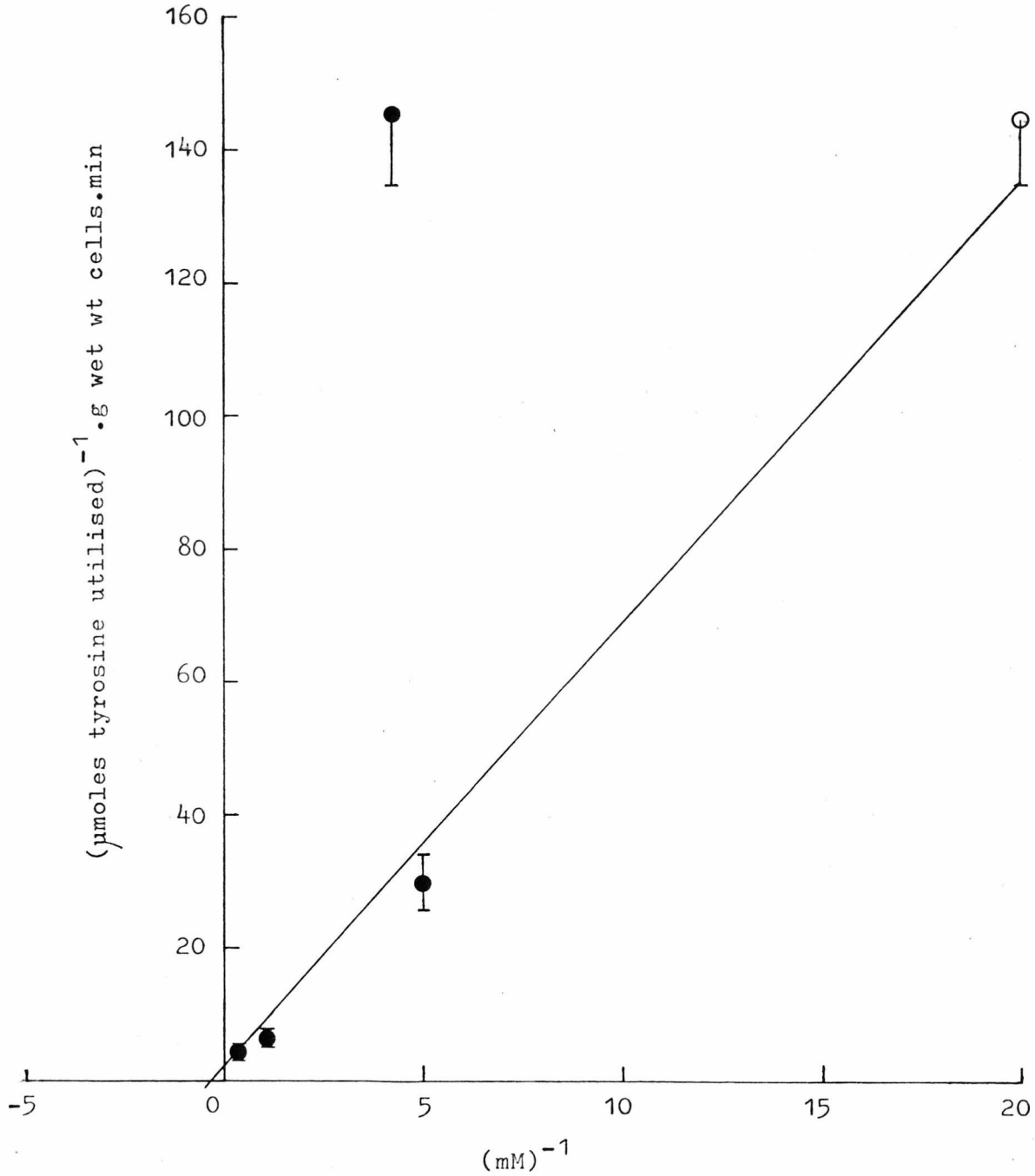
The values presented are taken from Fig 6:3.

—●—: rates plotted relative to $[tyr]_{int}$

—○—: rates plotted relative to $[tyr]_{ext}$

	V_{max} (μmoles.min^{-1}.(g wet wt.)$^{-1}$)
in vitro	0.82 ± 0.16
intact cells	0.38 ± 0.08

Fig 6:5 A Lineweaver-Burk plot of rates of tyrosine metabolism in cells isolated from fed, normal rats



The reciprocal values are taken from Fig 6:3.

-●-: reciprocal rates plotted relative to $1/[tyr]_{int}$

-O-: reciprocal rates plotted relative to $1/[tyr]_{ext}$

From the graph: K_m for tyrosine = 2.5mM.

The value of V_{max} calculated from the Lineweaver-Burk plot is <50% of V_{max} measured in cell-free extracts *in vitro* (Fig 6:4). A comparison of V_{max} measured spectrophotometrically and radioactively revealed an isotope effect of between 2 and 3 (Chapter 3). A correction for this is included in the calculation of V_{max} *in vitro*, but not in the calculation of V_{max} in cells. Thus, allowing for an isotope effect the values of V_{max} measured in cells and *in vitro* are similar.

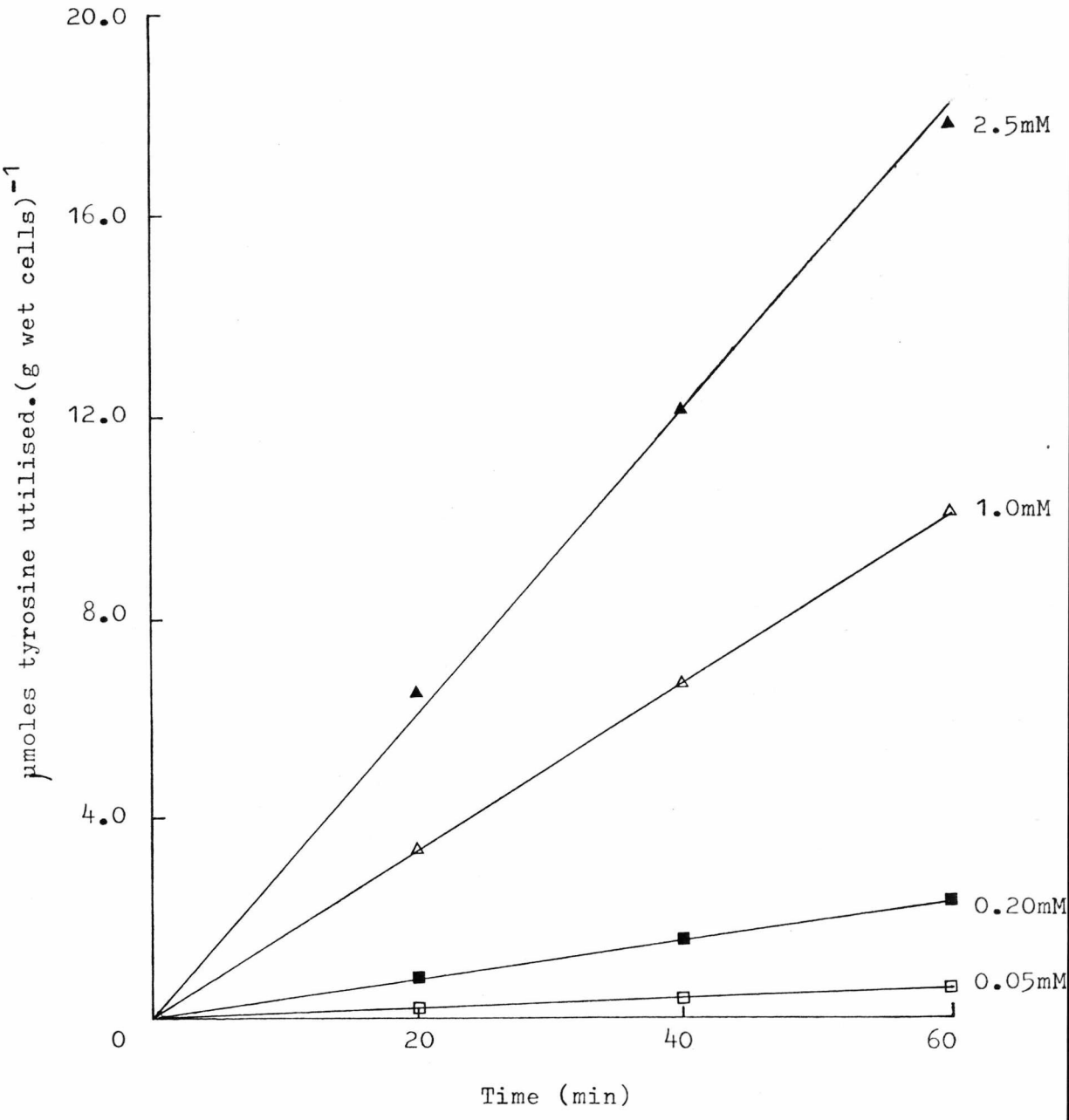
B) Tyrosine metabolism in parenchymal cells isolated from rats differing in hormonal and nutritional status

Results presented earlier in this thesis (Chapter 4) indicated that basal TAT activity and its susceptibility to induction varied in cells isolated from fed or fasted, normal rats and fed adrenalectomized rats. Therefore comparative studies of the rates of tyrosine metabolism were performed.

As with cells isolated from fed, normal rats, tyrosine is metabolised in a linear fashion, for the 60min incubation period, in cells isolated from fasted, normal and fed, adrenalectomized (adex) rats (Figs 6:6 and 6:7). The rates of tyrosine metabolism are distinct, at each tyrosine concentration used, but do not differ significantly between the different cell types (compare Figs 6:3; 6:6 and 6:7). In previous experiments (Chapter 4), *in vitro* TAT activity was greater in cells isolated from fasted rats than in those isolated from fed (normal or adrenalectomized) rats. However, in these experiments, *in vitro* TAT activity is comparable in the three cell types.

Lineweaver-Burk plots yield values of the K_m for tyrosine of 2.2mM and 2.4mM in cells isolated from fasted rats and fed, adex rats respectively (Figs 6:8 and 6:9).

Fig 6:6 Time course of tyrosine transamination in liver cells isolated from 48h fasted rats



The values presented are the means from 3 separate cell preparations. L-[side chain 2,3-^{3}H] tyrosine was added after the 30min preincubation period and the specific activities used were:

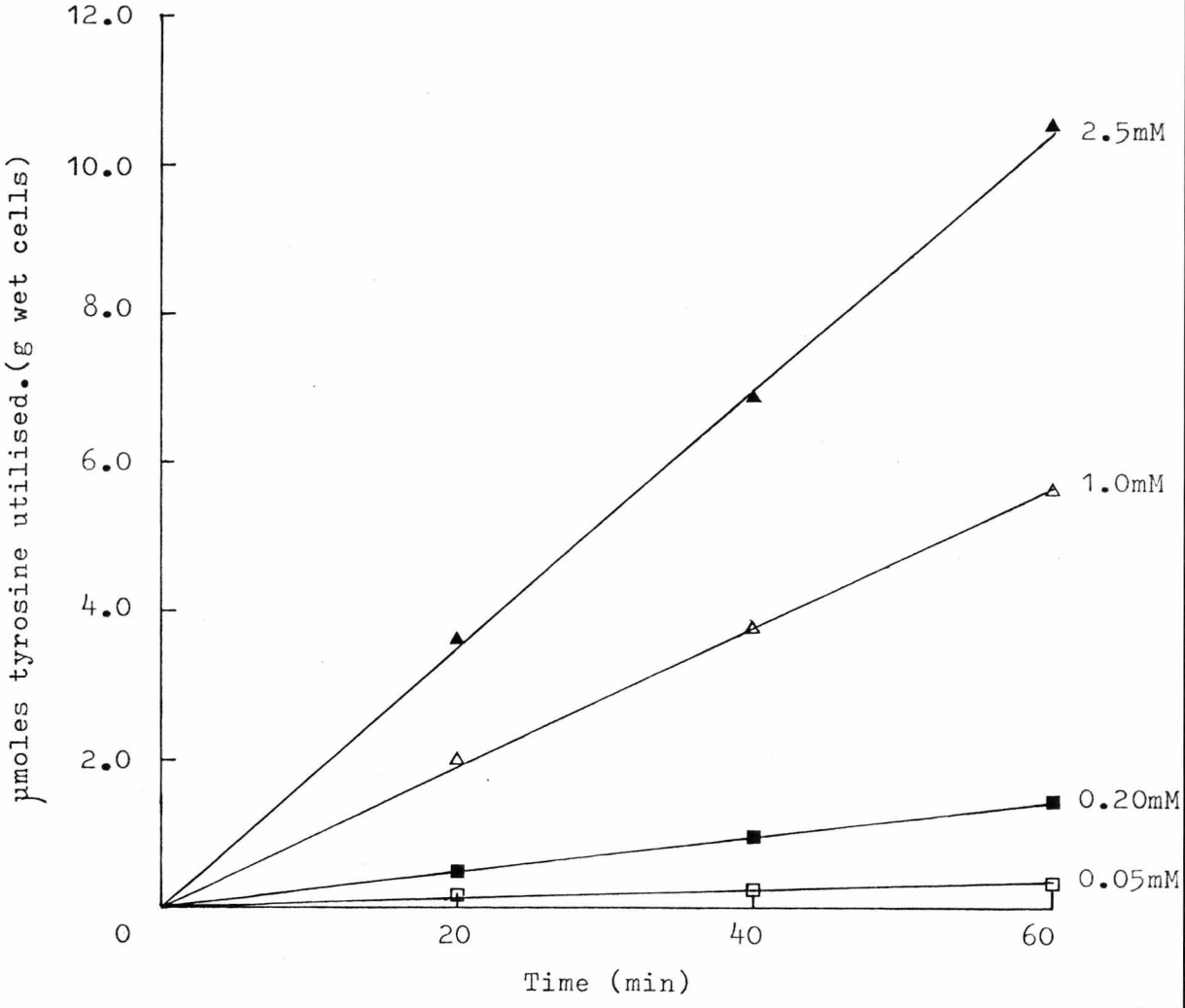
medium tyrosine	μCi.(μmole tyrosine)$^{-1}$
0.05mM	0.8
0.20mM	0.8
1.0mM	0.16
2.5mM	0.064

Legend to Fig 6:6 / Continued

Cellular ATP content: 9.0 ± 1.5 nmoles.(mg dry wt cells)$^{-1}$

Tyrosine (mM)	Rate of tyrosine metabolism (nmoles tyrosine utilised.(g wet wt cells)$^{-1}$.min^{-1}) $\pm$S.E.M.
0.05	10.5 ± 2.0
0.20	39.0 ± 7.0
1.0	156.0 ± 46.0
2.5	307.0 ± 60.0

Fig 6:7 Time course of tyrosine transamination in liver cells isolated from fed, adrenalectomized rats



The values presented are the means from 3 separate cell preparations. L-[side chain 2,3-^{3}H] tyrosine was added after the 30min preincubation period and the specific activities used were:

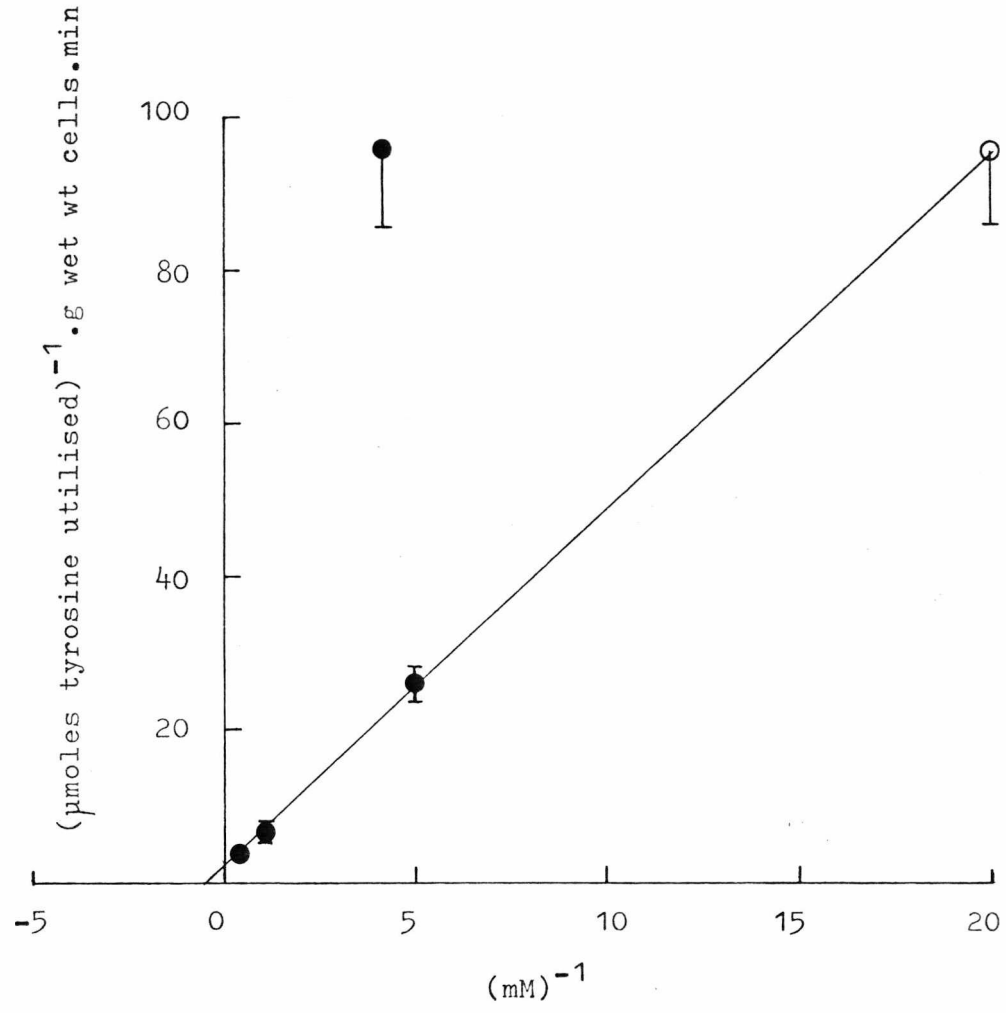
medium tyrosine	μCi.(μmole tyrosine)$^{-1}$
0.05mM	0.8
0.20mM	0.8
1.0mM	0.16
2.5mM	0.064

Legend to Fig 6:7 / Continued

Cellular ATP content: 10.8 ± 0.8 nmoles.(mg dry wt cells)$^{-1}$

Tyrosine (mM)	Rate of tyrosine metabolism (nmoles tyrosine utilised.(g wet wt cells)$^{-1}$.min^{-1}) $\pm$ S.E.M.
0.05	5.6 ± 0.7
0.20	24.0 ± 1.4
1.0	89.0 ± 9.0
2.5	172.0 ± 10.0

Fig 6:8 A Lineweaver-Burk plot of rates of tyrosine metabolism in cells isolated from 48h fasted rats



The reciprocal values are taken from Fig 6:6.

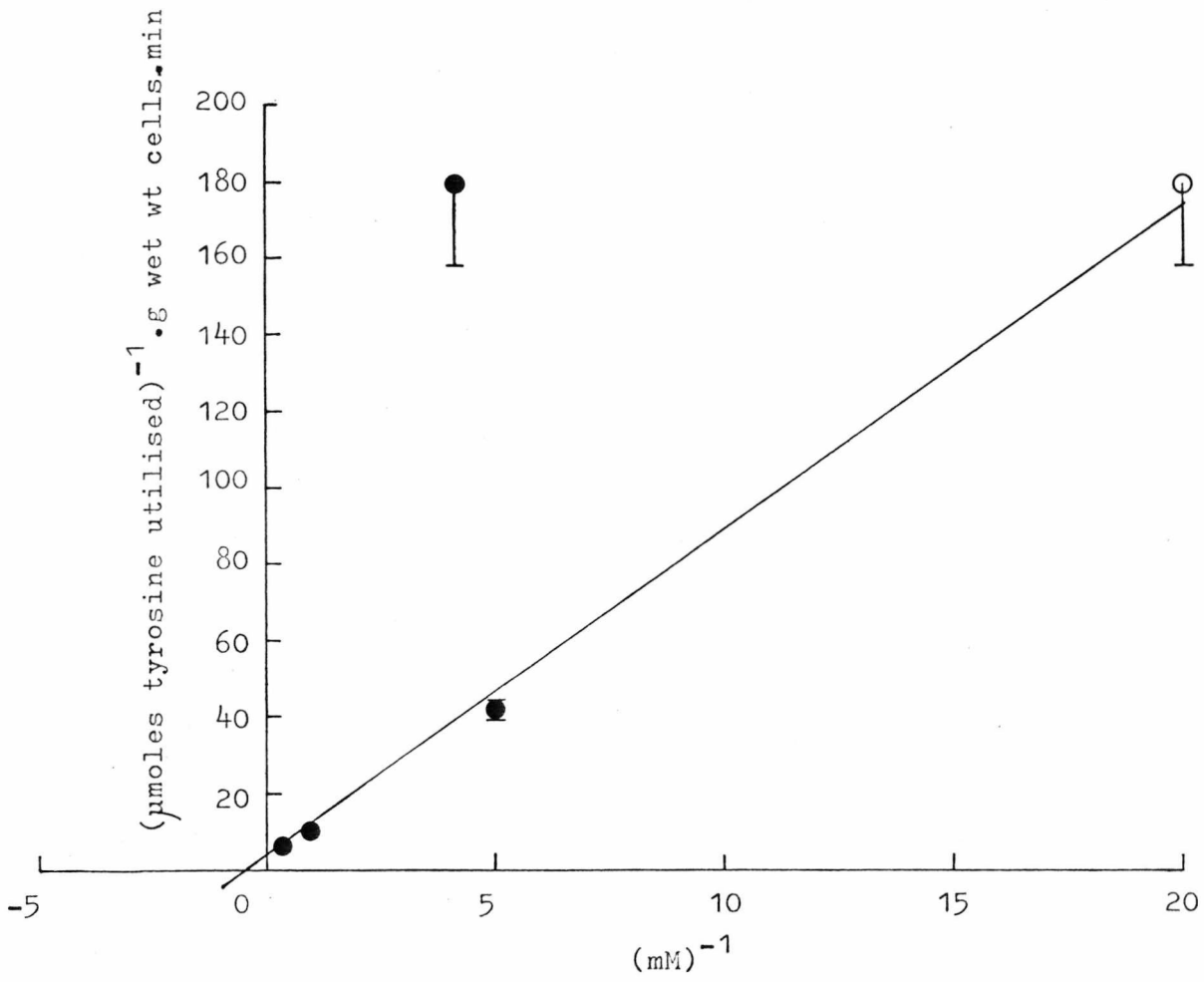
—●— : reciprocal rates plotted relative to $1/[tyr]_{int}$

—○— : reciprocal rates plotted relative to $1/[tyr]_{ext}$

From the graph: K_m for tyrosine = 2.2mM

	V_{max} (μmoles.min^{-1}.(g wet wt.)$^{-1}$)
in vitro	0.89±0.12
intact cells	0.46±0.06

Fig 6:9 A Lineweaver-Burk plot of rates of tyrosine metabolism in cells isolated from fed, adrenalectomized rats



The reciprocal values are taken from Fig 6:7.

—●—: reciprocal rates plotted relative to $1/[tyr]_{int}$

—○—: reciprocal rates plotted relative to $1/[tyr]_{ext}$

From the graph: K_m for tyrosine = 2.4mM.

$V_{max} = 0.26 \pm 0.03$umoles.min^{-1}.(g wet wt.)$^{-1}$

TAT activity in vitro = 1.03 ± 0.2umoles.min^{-1}.(g wet wt.)$^{-1}$

Again these values are similar to those estimated *in vitro*. As noted previously, cellular measurements provide a lower estimate of V_{max} than assays *in vitro*.

C) The effect of glucagon and hydrocortisone on rates of tyrosine metabolism

In Chapter 4, it was demonstrated that glucagon and hydrocortisone each induce TAT activity *in vitro*, in isolated liver cells. Significant increases in response to both of these hormones were obtained only in cells isolated from fed, adrenalectomized rats. In the current experiments, the procedure used in these earlier experiments was modified to allow tyrosine metabolism measurements from the time of hormone addition. The protocol used was:

i) isolated cells were incubated for 15min, at 37°C in a medium containing 0.2mM tyrosine

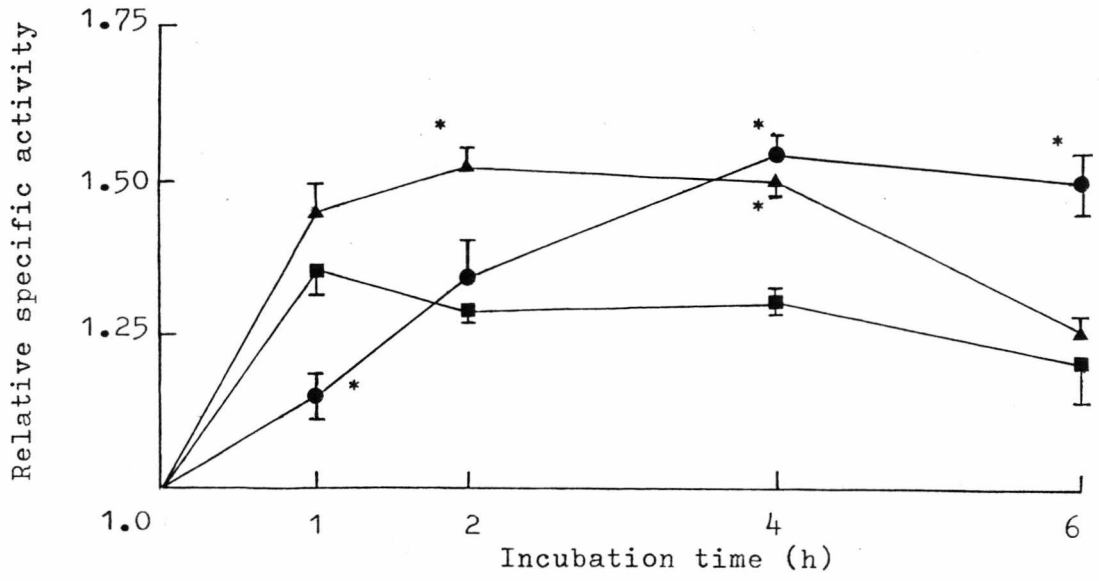
ii) [^{3}H] tyrosine was added to the vials

iii) after a further 15min at 37°C, hormones were added; this was the initial, 0h, time point.

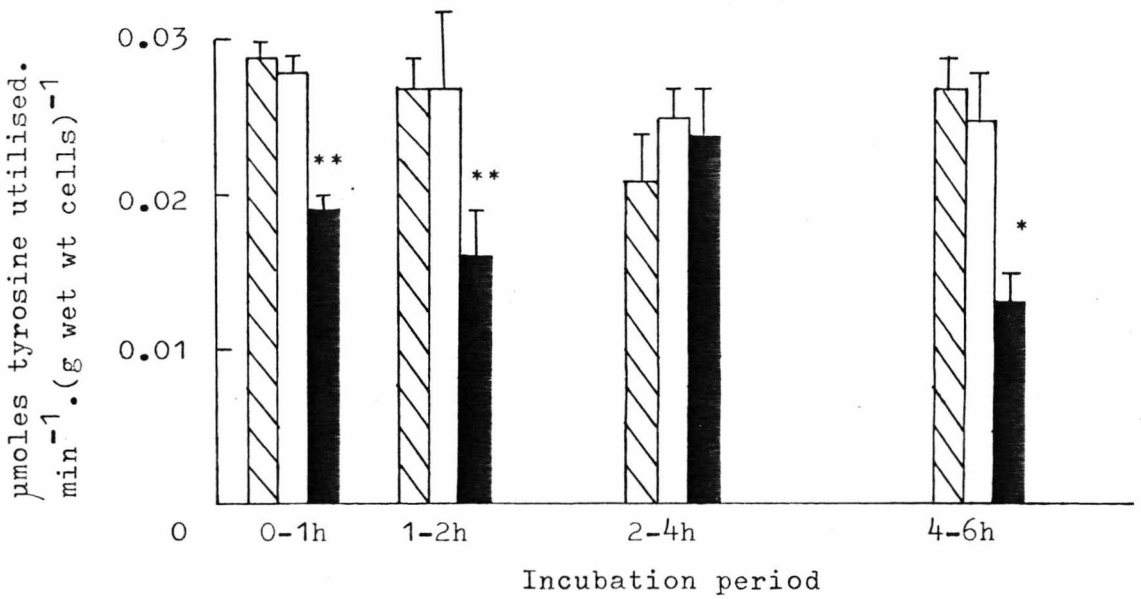
Fig 6:10 (a and b) shows the activity of TAT measured *in vitro* and the rates of tyrosine metabolism in cells. The rates of metabolism are presented in a histogram because the values are the mean rates over the time period indicated. TAT activity *in vitro* is significantly increased by both glucagon and hydrocortisone. The effect of glucagon is more immediate than that of the glucocorticoid; TAT activity is substantially increased within one hour. This is expected as cyclic AMP increases maximally within 3-6min in isolated liver cells (Pilkis *et al.*, 1975). The more

Fig 6:10 A comparison of tyrosine transamination in vitro and in cells during glucagon- and glucocorticoid mediated induction

a) in vitro TAT activity



b) cellular TAT activity



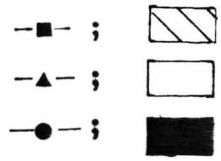
Values are means ± S.D. (3 determinations). Significance was tested using Student's t test.

	p
*	< 0.05
**	< 0.01

Values not marked with asterisks were not significantly different from control values.

Cellular ATP content: 3h = 7.3±0.1 nmoles.(mg dry wt cells)$^{-1}$
6h = 7.5±0.4 nmoles.(mg dry wt cells)$^{-1}$

Legend to Fig 6:10 contd.



Cells isolated from a fed, adrenalectomized rat were incubated in MEM, containing 0.2mM tyrosine, supplemented with 10% (v/v) charcoal-treated, dialysed FCS. The incubation conditions used for measurements of <u>in vitro</u> TAT activity were as described in Chapter 4 (Materials and Methods). Parallel incubations were performed to measure TAT activity in intact cells.

gradual increase apparent with hydrocortisone reflects the time required for hormone uptake, interaction with cytosolic receptors, transport into the nucleus and interaction with chromatin. Thus a statistically significant increase in TAT activity is induced within 2h by glucagon compared with a period of 2 - 4h in the presence of hydrocortisone. Glucocorticoid-mediated, but not glucagon-mediated induction is maintained at this level until 6h.

TAT activity increases rapidly in the first hour of incubation, both in the absence of hormones and in the presence of glucagon; however, this initial rise appears to be prevented by hydrocortisone (Fig 6:10). Atypically the increased level of activity in control incubations was sustained. In the original induction experiments, this initial increase was found to be transient (Chapter 4).

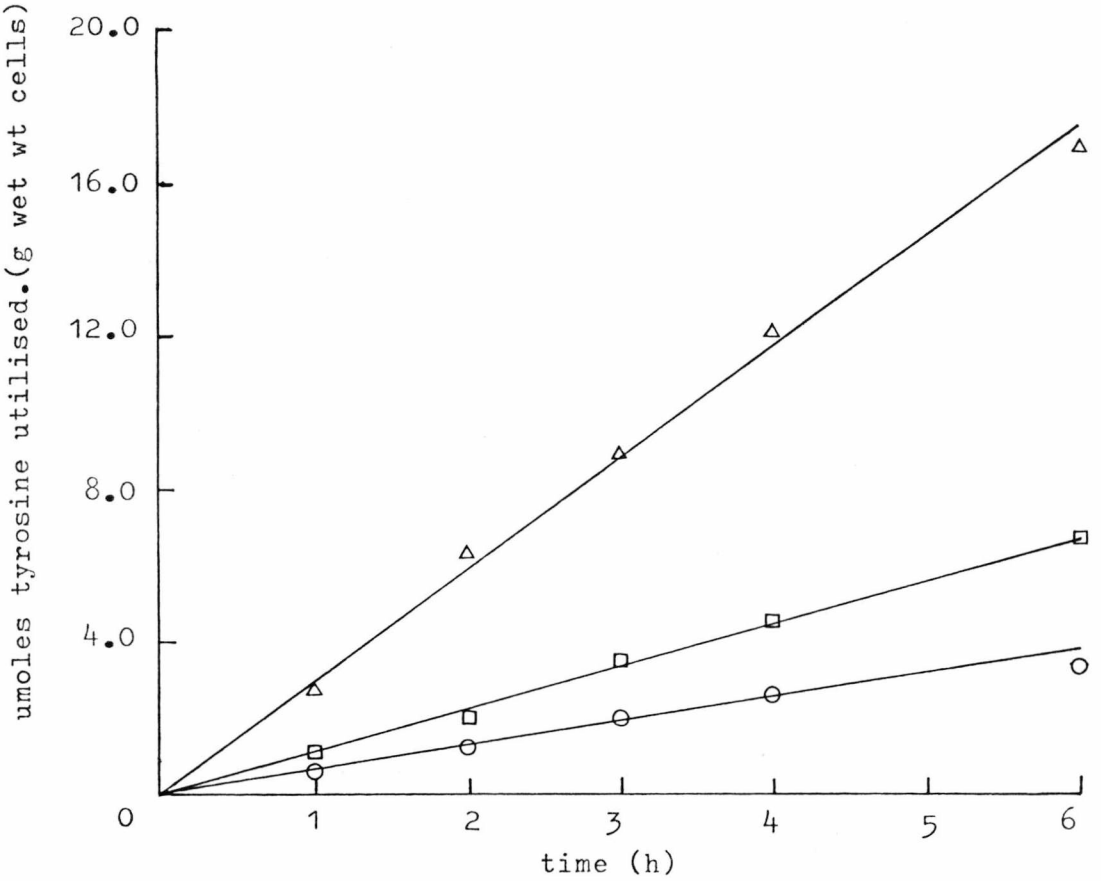
A comparison of Figs 6a and 6b shows that there is some direct correlation between changes in TAT activity *in vitro* and changes in the rate of tyrosine metabolism in cells. Thus in the first hour of incubation the cellular rates of transamination in untreated and glucagon-treated cells are similar and are each significantly greater than the rate observed in the presence of hydrocortisone. These rates of metabolism are unchanged between 1h and 2h, but at the end of this incubation period an increase in TAT activity *in vitro* was measured in cells isolated from both glucagon-treated and hydrocortisone-treated cells. In the next two hours, hydrocortisone stimulates a significant increase in TAT activity *in vitro*, which is paralleled by an increase in the rate of transamination in cells. As a result, the rates of metabolism in this time period are similar in control and hormone-treated cells. The level of TAT activity *in vitro* is maintained

between 4h and 6h in the presence of glucocorticoid hormone, but the corresponding rate of cellular tyrosine metabolism is decreased to a level significantly different from that in control and glucagon-treated incubations. In contrast to hydrocortisone-treated cells, there is no apparent correlation between TAT activity *in vitro* and rates of tyrosine transamination in cells, in either untreated or glucagon-treated cells. Glucagon had no apparent effect on the rates of metabolism in cells, which remained constant throughout the incubation period.

D) The effect of amino acids on rates of tyrosine metabolism in cells

Tyrosine is metabolised at a constant rate during the 6h incubation period in untreated, tryptophan-treated and amino acid-treated cells (Fig 6:11). *In vitro* TAT activity is significantly increased by 2.5mM tryptophan at 6h (Table 6:2). In contrast the rate of tyrosine transamination in cells is decreased by tryptophan. The decreased rate is apparent from the start of the incubation period indicating an immediate effect of tryptophan. It is possible that this decreased rate of metabolism in cells results because tryptophan competes with tyrosine for TAT. Jacoby and La Du (1963) found that pure TAT could also transaminate tryptophan. The K_m of pure TAT for tryptophan was reported as 30 - 50mM (Jacoby and La Du, 1963; Weinstein *et al.*, 1967). The K_m of TAT for tyrosine in these cells is 2.5mM and the concentration of tyrosine in the medium 0.2mM. From these figures, if tryptophan is competitively inhibiting transamination of TAT in cells, a rate 50 - 70% of the control rate would be expected in the presence of 2.5mM

Fig 6:11 The effect of amino acids on TAT activity in cells and in vitro



The values presented are taken from Fig 6:2.

-□-: control

-○-: 2.5mM tryptophan

-△-: 8x [plasma] amino acid mixture

Table 6:2 The effect of amino acids on TAT activity in cells and in vitro.

Incubation time (h)	μmoles tyrosine utilised.(g wet wt. cells)$^{-1}$		
	no additions	2.5mM tryptophan	8x amino acid mixture
1	1.10±0.03	0.66±0.05	2.72±0.20
2	1.98±0.17	1.36±0.04	6.23±0.19
3	3.42±0.08	2.06±0.10	8.91±0.66
4	4.57±0.25	2.68±0.08	12.10±0.47
6	5.74±0.08	3.39±0.01	16.90±0.78
† Rate of transamination 'in vivo'	0.018 ± 0.001	0.011 ± 0.001	0.049 ± 0.003
Rate of transamination in vitro	0.46±0.0.	*0.50±0.01	**0.78±0.05

Cells were isolated from a fed, normal rat and incubated in MEM supplemented with 10% charcoal-treated, dialysed FCS, containing 0.2mM tyrosine. Values are the means ± S.D. from a single cell preparation. †: values are the mean rates over the 6h incubation period and are μmoles.min^{-1}.(g wet wt cells)$^{-1}$. TAT activity in vitro was measured initially and at 6h; the initial specific activity was 0.56±0.03 μmoles. min^{-1}.(g wet wt cells)$^{-1}$.

Cellular ATP content: 13.2±0.3 nmoles.(mg dry wt cells)$^{-1}$.

Significance was tested using Student's t test:

	probability
*	<0.05
**	<0.01

tryptophan. The observed rate is 61% of the rate of tyrosine metabolism in the presence of physiological concentrations of tryptophan. An alternative explanation is that tryptophan competes with tyrosine for uptake into the cells, since both amino acids are transported by the L system.

In the presence of 8x plasma concentrations of amino acids, TAT activity *in vitro* is 60% greater than the corresponding control value, but the rate of metabolism in cells is increased 3-fold. The results presented in Chapter 4 showed that the increase in TAT activity *in vitro* occurred after a lag of 1 - 2h. However the increased rate of tyrosine metabolism in cells is evident from the beginning of the incubation period. The concentration of tyrosine in the medium containing 8x plasma concentration of amino acids is 0.8mM, and this probably accounts for the increased rate of metabolism observed.

GENERAL DISCUSSION

The inconsistencies observed in the relationship between the $[tyr]_{int}$ estimated and cellular rates of tyrosine metabolism evidently result from the method used to estimate $[tyr]_{int}$. If, as was shown for valine (Seglen and Solheim, 1978a), extracellular and intracellular specific activities of amino acids equilibrate rapidly in suspensions of isolated liver cells, a knowledge of the $[tyr]_{int}$ is not essential for estimating cellular rates of tyrosine metabolism. However this information is required for investigations of the mechanisms by which rates of metabolism are altered; such changes, in response to hormonal and nutritional stimuli, could simply result from alterations in the $[tyr]_{int}$. Tyrosine levels in isolated cells have previously been measured using the fluorimetric assay of Waalkes and Udenfriend (1957) (Jones and Mason, 1978). At a $[tyr]_{ext}$ of 0.22mM, $[tyr]_{int}$ was 0.4mM. However, the method used to separate the cells and medium was straightforward centrifugation. No mention was made of the method used to correct for contaminating medium or of the level of the cell blank. It is possible that the concentrative uptake reported is apparent rather than real.

The results in this chapter clearly demonstrate that when hydrocortisone induces TAT activity *in vitro* there is a corresponding increase in the rate of transamination in cells (Fig 6:10a and b). However, an increase in TAT activity between 1 and 2h is not accompanied by a change in the rate of cellular metabolism. Also the rate of transamination in cells decreases after 4 - 6h, while TAT activity *in vitro* remains at a constant level. The former, but not the latter, observation could be explained if the change in TAT

activity in cells occurred rapidly compared with the period of time over which tyrosine metabolism is measured, and towards the end of this period.

The high rate of cellular metabolism evident in untreated and glucagon-treated cells is consistent with the rapid increase in TAT activity *in vitro* in the initial hour of incubation (Fig 6:10a and b). However, the subsequent increase in TAT activity *in vitro* induced by glucagon, and the return of this activity towards basal levels is not reflected in the rates of transamination measured in cells. Thus the results obtained with the glucocorticoid and glucagon suggest that an increase in the activity (or amount) of an enzyme measured *in vitro* is not necessarily paralleled in cells; intracellular factors appear to regulate the catalytic activity of the enzyme available.

The rates of tyrosine metabolism in cells in the presence of high concentrations of tryptophan or total amino acids were explained on the basis of the altered amino acid levels themselves. Nonetheless, both tryptophan and the amino acid mixture induced small but significant increases in TAT activity *in vitro* and corresponding effects on the cellular rates of metabolism could have been masked.

One other possibility to consider is that tyrosine transamination may not be the rate-limiting step in this pathway under all physiological conditions. An analogous method to the one in this chapter was used by Kaufman and co-workers to measure phenylalanine hydroxylase activity *in vivo*: [ring-^{2}H] phenylalanine was administered *in vivo* or incubated with liver slices and the rate of ^{2}H release determined (Milstein and

Kaufman, 1975a and b). When [ring-^{2}H] tyrosine was administered *in vivo*, the rate of ^{2}H release was greater than that observed using [^{2}H] phenylalanine (Milstein and Kaufman, 1975b); this was proposed as evidence that phenylalanine hydroxylase and not TAT was the rate-limiting enzyme in this metabolic pathway. However hepatic phenylalanine hydroxylation is the only reaction which releases the [ring-^{2}H] from this amino acid. In contrast ^{2}H release from the aromatic ring of tyrosine would occur not only in reactions subsequent to transamination but also in the synthesis of thyroid hormones, melanin and the catecholamines; this accounts for the increased rate of ^{2}H release observed.

There is consequently scope for further studies of the relative roles of phenylalanine hydroxylase and TAT in this catabolic pathway. The results in this chapter show that isolated liver cells provide an excellent system for such investigations of the mechanism regulating the catalytic activity of enzymes in an intact *in vitro* system.

CHAPTER 7

CONCLUDING REMARKS

The isolated liver cell system has proved to be suitable for studies of the regulation of TAT activity. Cells must be incubated in a complex, partly undefined, medium to maintain cell viability for the periods of time necessary to observe increases in enzyme activity. This may be regarded as a retrograde step, since it introduces unknown factors which complicate the interpretation of data. A perfect illustration of this is the transient and sustained increases in TAT activity observed in control cell incubations. Isolated cells in monolayer culture on collagen membranes can be maintained in serum-free medium (Oliver et al., 1978), and may therefore provide a more suitable system in vitro. However, considerable cell losses are incurred in establishing cultures, and with medium changes. The cells remaining are unlikely to be a representative sample of the original liver cell population.

In agreement with the results of previous investigations, a variety of hormonal and nutritional agents were found to induce hepatic TAT activity. Glucagon and glucocorticoid hormones stimulate significant increases in TAT activity and appear to act by independent mechanism. The response obtained, however, is influenced by the hormonal and nutritional state of the donor animal. Adrenalectomy reduces the variability of the responses, implying that adrenocorticotrophic hormones are directly or indirectly involved. There is some evidence in the literature to support this observation (Crane and Miller, 1977). In recent studies with isolated hepatocytes maintained in monolayer culture, it was found necessary to maintain the cells for 24 - 48h before hormone addition to obtain an optimum level of induction (Oliver et al., 1978; Savage and Bonney, 1978). This may represent the time required for processes initiated prior to, or during, isolation to cease.

Tryptophan is known to have diverse effects on a humber of metabolic processes, including carbohydrate, lipid and protein metabolism (Smith, 1977). However the physiological significance of these observations is questionable since high concentrations of tryptophan are required to obtain an effect. The studies in this thesis show that tryptophan directly stimulates a small, but significant increase in TAT activity. Again, this is unlikely to represent a role for tryptophan *in vivo*, since a concentration of 2.5mM was required to elicit a response.

The multiple forms of TAT in extracts from isolated cells can be separated by ion-exchange chromatography, but not by polyacrylamide gel electrophoresis. No evidence was found for specific hormonal induction of these forms, rather, each increased to a similar extent. The physiological role for the multiple forms remains to be elucidated.

Exchange of ^{3}H from L- *side chain* 2,3-^{3}H tyrosine during transamination allows the measurement of TAT activity *in vitro* and in cells. Isolated liver cells provide a simple physiological system for these measurements. The changes in the rate of transamination in cells, stimulated by hydrocortisone, appear to correlate with changes in TAT activity *in vitro*. However, further information is required to determine whether transamination is the rate-limiting step in this pathway. An estimate of the overall flux could be obtained by measuring the rate of formation of ^{14}C-malate and ^{14}C-acetoacetate from ^{14}C-tyrosine. If the rate of transamination *in vivo* is greater than the overall flux, then a reaction subsequent to transamination is rate-limiting. Simultaneous measurement of the rates of hydroxylation of phenylalanine and transamination of tyrosine would determine which, if any, of these reactions is rate-limiting.

REFERENCES

Abita J-P., Milstien S., Chang N. and Kaufman S. (1976) J. Biol. Chem. 251 5310-5314.

Allen R.E., Raines P.L. and Regen D.M. (1969) Biochim. Biophys. Acta 190 323-330.

Alwen J. and Lawn A.M. (1974) Exp. Cell Res. 89 197-205.

Amsterdam A. and Jamieson J.D. (1974) J. Cell Biol. 63 1037-1056

Anderson N.G. (1953) Science 117 627-628.

Anker H.S. (1970) Febs Lett. 7 293.

Ap Gwynn I., Jones B., Jones B.M. and Kemp R. (1970) Cytobios 2 181-185.

Ash R., Elliott K.R.F. and Pogson C.I. (1976) Proc. Nutr. Soc. 35 A29.

Ash R. and Pogson C.I. (1977) Biochim. Biophys. Acta 496 475-583.

Aubin P.M.G. and Bücher N.L.R.(1952) Anatom. Record 112 797-809.

Auld D.S. and Bruice T.C. (1967) J. Amer. Chem. Soc. 89 2098-2106.

Auricchio F., Valeriote F., Tomkins G.M. and Riley D.J. (1970) Biochim. Biophys. Acta 221 307-313.

Aviram M. and Hershko A. (1977) Biochim. Biophys. Acta 498 83-90.

Axelrod J. (1962) J. Biol. Chem. 237 1657-1660.

Axelrod J. (1966) Pharmacol. Rev. 18 95-113.

Axelrod J. and Lerner A.B. (1963) Biochim. Biophys. Acta 71 650-655

Ayuso-Parilla M.S., Martin-Requero A., Perez-Diaz J. and Parilla R. (1976) J. Biol. Chem. 251 7785-7790.

Babu U.M. and Johnston R.B. (1974) Biochem. Biophys. Res. Commun. 58 460-466.

Bach S.J. and Holmes E.G. (1937) Biochem. J. 31 89-95.

Ballard F.J. (1977) Essays in Biochem. 13 1-38.

Ballard F.J. and Hopgood M.F. (1973) Biochem. J. 136 259-264.

Barker K.L., Lee K-L. and Kenney F.T. (1971) Biochem. Biophys. Res. Commun. 43 1132-1138.

Barnabei O., Leghissa G. and Tomasi V. (1974) Biochim. Biophys. Acta 362 316-325.

Barranger J.A., Geiger P.J., Huzino A. and Bessman S.P. (1972) Science 175 903-905.

Baur H., Kasperek S. and Pfaft E. (1975) Hoppe-Seylers Z. Physiol. Chem. 356 827-838.

Beato M., Schmid W., Braendle W. and Sekeris C.E. (1970) Steroids 16 207-216.

Belarbi A., Bollack C., Befort N., Beck J.P. and Beck G. (1977) FEBS Lett. 75 221-225.

Bender D.A. (1975) Amino Acid Metabolism, Wiley, London, U.K.

Beneking M., Schmidt H. and Weiss G. (1978) Eur. J. Biochem. 82 235-243.

Benkert O. and Matussek N. (1970) Nature 228 73-75.

Berg T., Bowman D. and Seglen P.O. (1972) Exp. Cell Res. 72 571-573.

Berlin C.M. and Schimke R.T. (1965) Mol. Pharmacol. 1 149-156.

Bernardi G. (1971) Methods Enzymol. XXII 325-335.

Berry M.N. (1974) Methods Enzymol. 32 625-632.

Berry M.N. and Friend D.S. (1969) J. Cell Biol. 43 506-520.

Besmer P. and Anigon D. (1968) Chimia 22 494-501.

Bissell D.M., Hammaker L.E. and Meyer U.A.(1973) J. Cell Biol. 59 722-734.

Blackard W.G., Guzelian P.S. and Small M.E. (1978) Endocrinology 103 548-533.

Bloom S. (1973) Postgrad. Med. J. 49 607-611.

Bloxham D.P. and Akhtar M. (1972) Int. J. Biochem. 3 294-307.

Bloxham D.P. and Klaipongpan A. (1979) Int. J. Biochem. 10 1-5.

Boctor A. and Grossman A. (1973) Biochem. Pharmacol. 22 17-28.

Boctor A. and Grossman A. (1978) Biochim. Biophys. Acta 543 137-148.

Bonney R.J. (1974) In Vitro 10 130-142.

Bonney R.J., Becker J.E., Walker P.R. and Potter V.R. (1974) In Vitro 9 399-413.

Bourdel G., Girard-Globa A., Forestier M.and Gouchot-Nubel B. (1975) Biochim. Biophys. Acta 399 339-344.

Braatz J.A. and McIntire K.R. (1977) Prep. Biochem. 7 495-509.

Branster M.V. and Morton R.K. (1957) Nature 180 1283-1284.

Braunstein A.E. and Kritzman M.G. (1937) Enzymologia 2 129-134.

Braunstein A.E. (1947) Adv. in Protein Chem. 3 1-52.

Braunstein A.E. (1973) The Enzymes 9B 379-481.

Braverman L.E., Ingbar S.H. and Stirling K. (1970) J. Clin. Invest. 49 855-864.

Briggs S. and Freedland R.A. (1976) Biochem. J. 160 205-209.

Bruice T.C. and Topping R.M. (1963) J. Amer. Chem. Soc. 1480-1487.

Buchanan D.L. (1961) Arch. Biochem. Biophys. 94 500-506.

Buchanan J.M., Hastings A.B. and Nesbett F.B. (1942) J. Biol. Chem. 145 715-718.

Bücher T. and Kirberger E. (1952) Biochim. Biophys. Acta 8 401-406.

Bu' Lock J.D. and Harley-Mason J. (1951) J. Chem. Soc. 43 703-712.

Butcher F.R., Becker J.E. and Potter V.R. (1971) Exp. Cell Res. 66 321-328.

Butcher F.R., Bushnell D.E., Becker J.E. and Potter V.R. (1972) Exp. Cell Res. 74 115-123.

Cammarata P.S. and Cohen P.P. (1950) J. Biol. Chem. 187 439-452.

Canellakis Z.N. and Cohen P.P. (1956) J. Biol. Chem. 222 53-62.

Capuzzi D.M., Rothman V. and Margolis S. (1974) J. Biol. Chem. 249 1286-1294.

Carlson C.A. and Kim K. (1974) Arch. Biochem. Biophys. 164 478-489.

Chambers J.W., Georg R.H. and Bass A.D. (1965) Mol. Pharmacol. 1 66-76.

Chambers J.W., Georg R.H. and Bass A.D. (1968) Endocrinol. 83 1185-1192.

Chapman G.S., Jones A.L., Meyer U.A. and Bissell D.M. (1973) J. Cell Biol. 59 735-747.

Chee P.Y. and Swick R.W. (1976) J. Biol. Chem. 251 1029-1034.

Chen C-P. and Lee R.H. (1977) Life Sci. 21 577-584.

Chen R.F. (1967) J. Biol. Chem. 242 173-181.

Chessebeuf M., Olson A., Bournot P., Desgres J., Guiget M., Maume B.F., Perissel G. and Padieu P. (1974) In Gene. Expression Carcinog. Cult. Liver, (Proc. Int. Symposium) 94-118 (Eds. Gerschenson L.E. and Thompson E.B.), Academic Press New York.

Cheung C., Cotropia J.P. and Sallach H.J. (1969) Arch. Biochem. Biophys. 129 672-682.

Christensen H.N. (1975) In Current Topics In Membranes And Transport 6 227-258 (Eds. Kleinzeller A. and Bronner F.)

Christensen H.N.(1977) Nutrition Rev. 35 129-133.

Christensen H.N. and Handlogten M.E. (1968) J. Biol. Chem. 243 5428-5438.

Church R.B. and McCarthy B.J. (1967) J. Mol. Biol. 23 477-481.

Ciarenello R.D., Barchas R.E., Byers G.S., Stemmle D.W. and Barchas J.D. (1969) Nature 221 368-369.

Cihak A., Inoue H. and Pitot H.C. (1975) Arch. Biochem. Biophys. 166 237-244.

Cihak A., Lamar C. and Pitot H.C. (1973a) Arch. Biochem. Biophys. 156 176-187.

Cihak A., Lamar C. and Pitot H.C. (1973b) Arch. Biochem. Biophys. 156 188-194.

Cihak A., Vesely J., Inoue H. and Pitot H.C. (1972) Biochem. Pharmacol. 21 2545-2553.

Cihak A., Wilkinson D. and Pitot H.C. (1971) Adv. Enz. Regul. 9 267-289.

Civen M. and Brown C.B. (1973) Reticuloendothelial Soc. 14 522-529.

Civen M. and Knox W.E. (1959) Science 129 1672-1673.

Clark D.G., Rognstad R. and Katz J. (1974) J. Biol. Chem. 249 2028-2036.

Clarke G.M. (1969) In Statistics and Experimental Design (Eds. Barrington E.J.W. and Willis A.J.) pp 149-154, Edward Arnold Ltd., London.

Cohen P. (1973) Eur. J. Biochem. 34 1-14.

Cohen P. (1976) In Control of Enzyme Activity, Chapman and Hall.

Coman D.R. (1954) Cancer Res. 14 519-521.

Constantsas N.S. and Knox W.E. (1966) Arch. Biochem. Biophys. 117 59-64.

Cori C.F. and Shine W.M. (1936) J. Biol. Chem. 114 xxi-xxv.

Cornell N.W., Lund P., Hems R. and Krebs H.A. (1973) Biochem J. 134 671-672.

Corrin B. and Aterman K. (1968) Amer. J. Anatomy 122 57-72.

Costa E. and Meek J.L. (1974) Annu. Rev. Pharmacol. 14 491-511.

Crane L.J. and Miller D.L. (1974) Biochem. Biophys. Res. Commun. 60 1269-1277.

Crane L.J. and Miller D.L. (1977) J. Cell Biol. 72 11-25.

Cunningham B.A. and Kirkwood S. (1961) J. Biol. Chem. 236 485-489.

Daoust R. (1958) in 'Liver Function', Ed. R.W. Brauer, pp 3-10, Amer. Inst. Biol. Sci., Washington D.C.

David J.C., Dairman W. and Udenfriend S. (1974a) Arch. Biochem. Biophys. 160 561-568.

David J.C., Dairman W. and Udenfriend S. (1974b) Proc. Natl. Acad. Sci. U.S.A. 71 1771-1775.

Davidson E.H. (1964) Adv. Genetics 12 143-281.

Dean R.T. (1975) Nature 257 414-416.

De Groot L.J. (1965) New Engl. J. Med. 272 243-260.

Deguchi T. and Barchas J. (1971) J. Biol. Chem. 246 7217-7222.

Dethlefsen L. (1975) J. Cell Physiol. 86 155-165.

Diamondstone T.I. (1966) Analyt. Biochem. 16 395-401.

Dickson A.J. and Pogson C.I. (1977) FEBS. Lett. 83 27-32.

Dickson J.A. (1970) Exp. Cell Res. 61 235-239.

Don M. and Masters C.J. (1976) Int. J. Biochem. 7 215-220.

Donlon J. and Kaufman S. (1977) Biochem. Biophys. Res. Commun. 78 1011-1017.

Donlon J. and Kaufman S. (1978) J. Biol. Chem. 253 6657-6660.

Donner D.B.,Nakayama K., Lutz U. and Sonnenberg M. (1978) Biochim. Biophys. Acta 507 322-326.

Dubnoff J.W. and Dimick M. (1959) Biochim. Biophys. Acta 31 541-545.

East A.G., Louis L.N. and Hoffenberg R. (1973) Exp. Cell Res. 76 41-46.

Edwards A.M. and Elliott W.H. (1974) J. Biol. Chem. 249 851-855.

Edwards A.N., Michalopoulos G., Sattler C.A. and Pitot H.C. (1977) Proc. Aust. Biochem. Soc. 10 32.

Eisenstein A.B. and Strack I. (1968) Enocrinol. 83 1337-1347.

Ekren T., Jervill K.F. and Seglen P.O. (1971) Nature New Biol. 229 244-245.

Elliott K.R.F., Ash R., Crisp D.M., Pogson C.I. and Smith S.A. (1976) in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Eds. Tager J.M., Soling, H-D. and Williamson J.R.) pp. 139-143, North-Holland, Amsterdam.

Elwyn D.H. (1966) Fed. Proc. 25 854-861.

Elwyn D.H. (1970) in: Mammalian Protein Metabolism IV pp 523-558. Academic Press, New York.

Elwyn D.H., Parikh H.C. and Shoemaker W.C. (1968) Amer. J. Physiol. 215 1260.

Ernest M.J., Chen C-L. and Feigelson P. (1977) J. Biol. Chem. 252 6783-6791.

Ernest M.J. and Feigelson P. (1978) J. Biol. Chem. 253 319-322.

Exton J.H. (1975) Methods Enzymol. XXXIX 25-36.

Exton J.H. and Park C.R. (1967) J. Biol. Chem. 242 2622-2636.

Exton J.H. and Park C.R. (1968) J. Biol. Chem. 243 4189-4196.

Exton J.H. and Park C.R. (1969) J. Biol. Chem. 244 1424-1433.

Exton J.H., Robinson G.A., Sutherland E.W. and Park C.R. (1971) J. Biol. Chem. 246 6166-6177.

Exton J.H., Corbin J.G. and Harper S.C. (1972) J. Biol. Chem. 247 4996-5003.

Fahrney D.E. and Gold A.M. (1963) J. Amer. Chem. Soc. 85 997-1000.

Fasella P.M. (1967) Annu. Rev. Biochem. 36 185-210.

Fawcett J.K. and Scott J.E. (1960) J. Clin. Pathol. 13 156-159.

Feigelson M., Gross P.R. and Feigelson P. (1962) Biochim. Biophys. Acta 55 495-502.

Feigelson P. and Greengard O. (1962) J. Biol. Chem. 237 3714-3717.

Felig P., Owen O.E., Wahren J. and Cahill G.F. Jnr. (1969) J. Clin. Invest. 48 584-593.

Fellman J.H., Roth E.S. and Fujita T.S. (1976) Arch. Biochem. Biophys. 171 562-567.

Fitzpatrick T. (1960) in The Metabolic Basis of Inherited Disease p 428, McGraw-Hill, New York.

Florini J.R., Peets E.A. and Buyske D.A. (1961) J. Pharmacol. Exp. Ther. 131 287-293.

Foster D.O., Ray P.D. and Lardy H.A. (1966) Biochemistry 5 555-562.

Fouchereau-Peron M., Rancon F., Freychet P. and Rosselin G. (1976) Endocrinol. 98 755-760.

Frank H., Hardeland R. and Frank A. (1976) Arch. Int. Physiol. Biochem. 84 471-477.

Freedland R.A. (1963) Biochim. Biophys. Acta 73 71-76.

Freedland R.A., Wadzinski I.M. and Waisman H.A. (1961) Biochem. Biophys. Res. Commun. 5 94-102.

Fry J., Jones C.A., Wiebkin P., Bellemann P. and Bridges J.W. (1976) Anal. Biochem. 71 341-351.

Fuller R.W. and Hunt J.M. (1967) Life Sci. 6 1107-1112.

Gabay S. and George H. (1967) Anal Biochem. 21 111-118.

Gallop P.M., Seifter S. and Meilman E. (1957) J. Biol. Chem. 227 891-906.

Gan J.C. and Jeffay H. (1971) Biochim. Biophys. Acta 252 125-135.

Garcia-Ruiz J.P., Ingram R. and Hanson R.W. (1978) Proc. Natl. Acad. Sci. U.S.A. 75 4189-4193.

Garcia-Sancho J., Sanchez A. Handlogten M.E. and Christensen H.N. (1977) Proc. Natl. Acad. Sci. U.S.A. 74 1488-1491.

Garlick P.J., Millward D.J., James W.P.T. and Waterlow J.C. (1975) Biochim. Biophys. Acta 414 71-84.

Garren L.D., Howell R.R., Tomkins G.M. and Grocco R.M. (1964) Proc. Natl. Acad. Sci. U.S.A. 52 1121-1129.

Garrison J.C. and Haynes R.C. (1973) J. Biol. Chem. 248 5333-5433.

Gebhardt R., Belleman P. and Mecke D. (1978) Exp. Cell Res. 112 431-441.

Gelehrter T.D., Emanuel J.R. and Spencer C.J. (1972) J. Biol. Chem. 247 6197-6203.

Gerich J.E. (1976) Metabolism 25 1437-1441.

Gerich J., Charles M. and Grodsky G. (1976) Annu. Rev. Physiol. 38 353-388.

Gerschenson L.E., Andersson M., Molson J. and Okigaki T. (1970) Science 170 859-861.

Gevers W. and Krebs H.A. (1966) Biochem. J. 98 720-735.

Gilula M.B. and Epstein M.L. (1976) in Calcium in Biological Systems (Soc. Exp. Biol. Symposium XXX) 257-272 (Ed. Duncan C.J.)

Gingell D., Garrod D.R. and Palmer J.F. (1970) in Calcium and Cell Function 59-64 (Ed. Cuthbert A.W.), Macmillan, London.

Glowinski J. and Iverson L.L. (1966) J. Neurochem. 13 655-659.

Goldberg A.L. and Dice J.F. (1974) Annu. Rev. Biochem. 43 835-869.

Goldberg A.L. and St. John A.C. (1976) Annu. Rev. Biochem. 45 747-803.

Gordon A.H. (1973) in Lysosomes in Biology and Pathology 3 89-137. (Ed. Dingle J.T.) North Holland, Amsterdam.

Granner D.K., Chase L., Auerbach G. and Tomkins G.M. (1968) Science 162 1018-1020.

Granner D.K., Hayashi S., Thompson E.B. and Tomkins G.M. (1968) J. Mol. Biol. 35 291-301.

Granner D.K., Lee A. and Thompson E.B. (1977) J. Biol. Chem. 252 3891-3897.

Grant A.G. and Black E.G. (1974) Eur. J. Biochem. 47 397-401.

Grant A.G. and Hoffenberg R. (1977) Brit. J. Nutr. 38 255-260.

Green D.E. (1941) in Mechanisms of Biological Oxidation, MacMillan Co., New York.

Greengard O., Smith M.A. and Acs G. (1963) J. Biol. Chem. 238 1548-1551.

Grimm J. and Manchester K.L. (1976) Biochim. Biophys. Acta 444 223-230.

Grossman A. and Boctor A. (1972) Proc. Natl. Acad. Sci. U.S.A. 69 1161-1164.

Grossman A. and Mavrides C. (1967) J. Biol. Chem. 242 1398-1405.

Guidotti G.G., Borghetti A.F. and Gazzola G.C. (1978) Biochim. Biophys. Acta 515 329-366.

Guidotti G.G., Gazzola G.C., Borghetti A.F. and Franchi-Gazzola R. (1975) Biochim. Biophys. Acta 406 264-279.

Gunn J.M. (1978) Exp. Cell Res. 117 448-450.

Günther T. and Göecke C. (1966) Z. Naturf. 21 b 1171-1175.

Hager C.B. and Kenney F.T. (1968) J. Biol. Chem. 243 3296-3300.

Hamm H-H. and Seubert W. (1977) Z. Naturforsch. 32 777-780.

Hansen J.N. (1976) Analyt. Biochem. 76 37-44.

Hardeland R. (1970) Life Sci. 9 901-905.

Hardeland R. (1972) J. Interdisciplinary Cycle Res. 3 109-114.

Harland W.A. and Orr J.S. (1975) Thyroid Hormone Metabolism, Academic Press, London.

Harris R.A. (1975) Arch. Biochem. Biophys. 169 168-180.

Harrison M.F. (1953) Nature 171 611

Hassler J.W. (1963) Activated Carbon, Rev. Ed., Chemical Publishing, New York.

Haung Y.L. and Ebner K.E. (1969) Biochim. Biophys. Acta 191 161-163.

Hayakawa T., Perkins J.P., Walsh D.A. and Krebs, E.G. (1973) Biochem. 12 567-573.

Hayashi S., Granner D.K. and Tomkins G.M. (1967) J. Biol. Chem. 242 3998-4006.

Hemmerle R., (1917) Ann. Chim. Phys. 7 226-230.

Hems D.A., Whitton P.D. and Taylor E.A. (1972) Biochem. J. 129 529-538.

Hems R., Lund P. and Krebs H.A.(1975) Biochem. J. 150 47-50.

Hems R., Ross B.D., Berry M.N. and Krebs H.A. (1966) Biochem. J. 101 284-292.

Herbert J.D., Coulson R.A. and Hernandez T. (1966) Comp. Biochem. Physiol. 17 583-590.

Herbert V., Kam-Seng L. and Gottlieb C.W. (1968) Adv. Tracer Methodology 4 273-290.

Hershko A., Mamont P., Shields R. and Tomkins G.M. (1971) Nature 232 206-211.

Hershko A. and Tomkins G.M. (1971) J. Biol. Chem. 246 710-714.

Higgins J.A. and Barrnett R.J. (1970) J. Cell Sci. 6 29-37.

Hoch F.L. (1962) Physiol. Rev. 42 605-673.

Höffken B., Kodding R., Von Zur Muhlen A., Hehrmann T., Juppner H. and Hesch R-D. (1978) Biochim. Biophys. Acta 539 114-124.

Holt P.G. and Oliver I.T. (1969) FEBS Lett. 5 89-91.

Holten D. and Kenney F.T. (1967) J. Biol. Chem. 242 4372-4377.

Hopgood M.F., Clark M.G. and Ballard F.J. (1977) Proc. Aust. Biochem. Soc. 10 30.

Hoshino J., Kuhne U., Filjak B. and Kriger H. (1975) FEBS Lett. 56 62-65.

Howard R.B., Christensen A.K., Gibbs F.A. and Pesch L.A. (1967) J. Cell Biol. 35 675-684.

Howard R.B. and Green C. (1965) Biochem. J. 96 33P.

Howard R.B., Lee J.C. and Pesch L.A. (1973) J. Cell Biol. 57 642-658.

Howard R.B. and Pesch L.A. (1968) J. Biol. Chem. 243 3105-3109.

Hue S.L., Bontemps F. and and Hers H.G. (1975) Biochem. J. 152 105-114.

Ichihara A. (1976) in Control Mechanism in Cancer 317-327 (Eds. Criss W.E., Ono T. and Sabine J.R.) Raven Press, New York.

Igo R.P., Mahoney C.P. and Limbeck G.A. (1968) Biochim. Biophys. Acta 151 88-98.

Ingebretsen W.R.Jr. and Wagle S. (1972) Biochem. Biophys. Res. Commun. 47 403-410.

Inoue H. and Pitot H.C. (1970) Adv. Enzyme Regul. 8 289-296.

IUPAC-IUB Commission on Biochemical Nomenclature (1971) J. Biol. Chem. 246 6127-6128.

Ivanov V.I. and Karpeisky M.Ya. (1969) Adv. Enzymol. 32 21-53.

Iwasaki Y., Lamar C., Danenberg K. and Pitot H.C. (1973) Eur. J. Biochem. 34 347-357.

Iwasaki Y. and Pitot H.C. (1971) Life Sci. 10 1071-1079.

Iynedjian P.B. and Hanson R.W. (1977) J. Biol. Chem. 252 655-662.

Jacob S.T. and Bhargava P.M. (1962) Exp. Cell Res. 27 453-467.

Jacoby G.A. and La Du B.N. (1963) J. Biol. Chem. 239 419-424.

Jeejeebhoy K.N., Bruce-Robertson A., Ho J., Kida S. and Muller-Ebhard U. (1976) Can. J. Biochem. 54 74-78.

Jeejeebhoy K.N., Ho J., Greenberg G.R., Phillips M.J., Bruce-Robertson A. and Sodtke U. (1975) Biochem. J. 146 141-155.

Jeejeebhoy K.N. and Phillips M.J. (1976) Gastroenterology 71 1086-1096.

Jenkins W.T. and Sizer I.W. (1960) J. Biol. Chem. 235 620-624.

Jensen E.V. and Desombre F.R. (1973) Science 182 126-130.

Johnson M.E.M., Das N.M., Butcher F.R. and Fain J.N. (1972) J. Biol. Chem. 247 3229-3235.

Johnson R.W. and Grossman A. (1974) Biochem. Biophys. Res. Commun. 59 1242-1249.

Johnson R.W., Robertson L.E. and Kenney F.T. (1973) J. Biol. Chem. 248 4521-4527.

Jones D.P. and Mason H.S. (1978) Biochem. Biophys. Res. Commun. 80 477-483.

Jørgensen A.J.F. and Majumdar A.P.N. (1976) Biochem. Med. 16 37-46.

Jost J.P., Khairallah E.A. and Pitot H.C. (1968) J. Biol. Chem. 243 3057-3058.

Kaltenbach J.P. (1952) Fed. Proc. 11 237-241.

Kaminskas E. (1975) Exp. Cell Res. 94 7-14.

Kaplan J.H. and Pitot H.C. (1970) in Mammalian Protein Metabolism IV 387-443 (Ed. Munro, H.N.), Academic Press, New York.

Kaufman S. (1958) J. Biol. Chem. 230 931-935.

Kaufman S. (1971) Adv. Enzymol. 35 245-319.

Kenney F.T. (1962a) J. Biol. Chem. 237 1610-1614.

Kenney F.T. (1962b) J. Biol. Chem. 237 3495-3498.

Kenney F.T. (1967) Science 156 525-528.

Kenney F.T. (1970) in Mammalian Protein Metabolism IV 131-172 (Ed. Munro H.N.), Academic Press, New York.

Kenney F.T. and Albritton C. (1965) Proc. Natl. Acad. Sci. (U.S.A.) 54 1693-1697.

Kenney F.T. and Flora R.M. (1961) J. Biol. Chem. 236 2699-2702.

Kindwall E.P. and Weiner N. (1966) J. Neurochem. 13 1523-1531.

King R.J.B. (1976) Essays in Biochemistry 12 41-76.

Kletzien R.F., Pariza M.W., Becker J.E. and Potter V.R. (1975) Nature 256 46-47.

Kletzien R.F., Pariza M.W., Becker J.E. and Potter V.R. (1976) J. Biol. Chem. 251 3014-3020.

Klowen H.M. and Appelman A.W.M. (1967) Biochem. J. 102 878-882.

Knox W.E. (1951) Brit. J. Exptl. Pathol. 32 462-465.

Knox W.E. and Edwards S.W. (1955) J. Biol. Chem. 216 489-498.

Knox W.E. and Le May-Knox M. (1951) Biochem. J. 49 686-693.

Knox W.E. and Mehler A.H. (1951) Science 113 237-240.

Knox W.E. and Pitt B.M. (1957) J. Biol. Chem. 225 675-688.

Kominami E., Kobayashi K., Kominami S. and Katanuma N. (1972) J. Biol. Chem. 247 6848-6855.

Kopin I.J. and Weise V.K. (1966) in Mech. Release Biogenic Amines (Proc. Int. Wenner-Gren. Cent. Symp., Stockholm) 229-246. Ed. Von Euler, U.S., Pergamon Press, London, U.K.

Krebs H.A. (1963) Adv. Enz. Regul. 1 385-399.

Krebs H.A. (1964) Proc. Roy. Soc. London B 159 545-564.

Krebs H.A. (1976) Discussion with Seglen P.O., in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Eds. Tager J.M., Soling H-D. and Williamson J.R.) p 260, North-Holland, Amsterdam.

Krebs H.A., Cornell, N.W., Lund P. and Hems R. (1974) in Regulation of Hepatic Metabolism (Eds. Lundquist F. and Tugstrub N.) pp 726-750, Munksgaard, Copenhagen.

Krebs H.A. and Henseleit K. (1932), Hoppe-Seyler's Z. Physiol. Chem. 210 33-66.

Krebs H.A., Notton B.M. and Hems R. (1966) Biochem. J. 101 607-617.

Krebs H.A., Freedland R.A., Hems R. and Stubbs M. (1969) Biochem. J. 112 117-124.

Krone W., Huttner W.B., Seitz H.J. and Tarnowski W. (1974) FEBS Lett. 46 158-161.

Kuczenski R.T. and Mandell A.J. (1972) J. Biol. Chem. 248 2261-2265.

Kvetnansky R., Weise V.K. and Kopin I.J. (1970) Endocrinol. 87 744-749.

Laborit H. and Thuret F. (1977) Res. Commun. Chem. Pathology Pharmacol. 17 77-86.

Labrie F. and Korner A. (1968a) J. Biol. Chem. 243 1116-1119.

Labrie F. and Korner A. (1968b) J. Biol. Chem. 243 1120-1122.

Labrie F. and Korner A. (1969) Arch. Biochem. Biophys. 129 75-78.

La Du B.N. and Greenberg D.M. (1951) J. Biol. Chem. 190 245-250.

Laishes B.A. and Williams G.M. (1976a) In Vitro 12 521-532.

La Martiniere C.A. and Feigelson M. (1977) J. Biol. Chem. 252 3234-3239.

Lambiotte M., Susor W.A. and Cahn R.D. (1972) Biochimie 54 1179-1187.

Lang N. and Sekeris C.E. (1964) Life Sci. 3 391-395.

Lardy H.A., Foster D.O., Young J.W., Schrago E. and Ray P.D. (1965) J. Cellular Comp. Physiol. 66 39-52.

Lawrence A.M. (1969) Annu. Rev. Med. 20 207-222.

Laws J.O. and Stickland L.H. (1961) Exp. Cell Res. 24 240-254.

Le Cam A. and Freychet P. (1976) Biochem. Biophys. Res. Commun. 72 893-901.

Le Cam A. and Freychet P. (1977a) Mol. Cell. Endocrinol. 9 205-214.

Le Cam A. and Freychet P. (1977b) J. Biol. Chem. 252 148-156.

Le Cam A. and Freychet P. (1978a) Diabetologia 15 117-123.

Le Cam A. and Freychet P. (1978b) Endocrinol. 102 379-385.

Lee K-L., Darke P.L. and Kenney F.T. (1977) J. Biol. Chem. 252 4958-4961.

Lee K-L. and Kenney F.T. (1971) J. Biol. Chem. 246 7595-7601.

Lee K-L. and Nichol J.M. (1974) J. Biol. Chem. 249 6024-6026.

Lee K-L., Reel J.R. and Kenney F.T. (1970) J. Biol. Chem. 245 5806-5812.

Lee S.Y., Mendecki J. and Brawerman G. (1971) Proc. Natl. Acad. Sci. (U.S.A.) 1331-1335.

Lees G.J. (1977) Life Sci. 20 1749-1762.

Leffert H.L. and Paul D. (1972) J. Cell Biol. 52 559-568.

Leichtling B.H., Koontz J., Su J-L., Wagner K., Roper M.D., Wimalasena J. and Wicks W.D. (1978) Arch. Biochem. Biophys. 185 525-534.

Lentz P.E. and Di Luzio N.R. (1971) Exp. Cell Res. 67 17-26.

Lerner A.B. (1949) J. Biol. Chem. 181 281-294.

Lerner A.B. (1953) Adv. Enzymol. 14 73-128.

Levin E.Y. and Kaufman S. (1961) J. Biol. Chem. 236 2043-2049.

Levitan I.B. and Webb T.E. (1970) J. Mol. Biol. 339-348.

Levitt M., Spector S., Sjoerdsma A., and Udenfriend S. (1965) J. Pharmacol. Exp. Ther. 148 1-8.

Lin E.C.C., Civen M. and Knox W.E. (1958a) J. Biol. Chem. 233 1183-11

Lin E.C.C. and Knox W.E. (1957) Biochim. Biophys. Acta 26 85-88.

Lin E.C.C., Pitt B.M., Civen M. and Knox W.E. (1958b) J. Biol. Chem. 233 668-673.

Lineweaver H. and Burk D.J. (1934) J. Amer. Chem. Soc. 56 658-666.

Lipson L.G., Capuzzi D.M. and Margolis S. (1972) J. Cell Sci. 10 167-179.

Litwack G. (1957) J. Biol. Chem. 228 823-834.

Litwack G. (1970) in Biochemical Action of Hormones I, Academic Press, New York.

Litwack G., Al-Nejjar Z.H., Sears M.L. and Ostenheimer G.W. (1964) Nature 201 1028-1030.

Litwack G. and Squires J.M. (1968) Anal. Biochem. 24 438-442.

Long C.N.H., Smith O.K. and Fry E.G. (1960) in 'Metabolic Effects of Adrenal Hormones' p4 (Eds. Wolstenholme, G.E.W. and O'Connor M.) Churchill, London.

Lovenberg W., Jequier E. and Sjoerdsma A. (1967) Science 155 217-219.

Lovenberg W., Weissbach H. and Udenfriend S. (1962) J. Biol. Chem. 237 89-93.

Lowry O.H. and Passonneau J.V. (1972) in A Flexible System of Enzymic Analysis pp 21-42. Academic Press, New York.

Lowry O.H., Roseborough N.J., Farr A.L. and Randall R. (1951) J. Biol. Chem. 193 265-275.

McGeer P.L., Bagchi S.P. and McGeer E.G. (1965) Life Sci. 4 1859-1867.

McGivan J.D., Bradford N.M. and Mendes-Mourão J. (1977) FEBS Lett. 80 380-384.

McGown E., Richardson A.G., Henderson L.M. and Swan P.B. (1973) J. Nutr. 103 109-116.

McNamara D.J. and Webb T.E. (1973) Biochim. Biophys. Acta 313 356-362.

McNamara D.J. and Webb T.E. (1974) Arch. Biochem. Biophys. 163 777-783.

MacDonald R.A. (1961) Arch. Int. Med. 107 335-339.

Maenpaa P.H., Raivio K.O. and Kekomaki M.P. (1968) Science 161 1253-1254.

Malamud D. (1971) in The Cell Cycle and Cancer p137 (Ed. Baserga R.), Marcel Dekker Inc., New York.

Malette L.E., Exton J.H. and Park C.R. (1969) J. Biol. Chem. 244 5724-5728.

Maloof F. and Soodak M. (1963) Pharmacol. Rev. 15 43-95.

Manchester K.L. (1960) in Discussion in: Christensen H.N., CIBA Foundation Study Group 6 p56.

Mandel P. and Aunis D. (1974) in CIBA Foundation Symposium 22 67-79 (Eds. Wolstenholme, G.E.W. and Fitzsimons D.W.).

Mans R.J. and Novelli G.D. (1961) Arch. Biochem. Biophys. 94 48-53.

Mantell C.L. (1951) Adsorption, 2nd Ed., McGraw-Hill, New York.

Mapes J.P. and Harris R.A. (1975) FEBS Lett. 51 80-83.

Markert C.L. and Whitt G.S. (1968) Experientia 24 977-991.

Marston F.A.O. and Pogson C.I. (1977) FEBS Lett. 83 277-280.

Mehler A.H. and Knox W.E. (1950) J. Biol. Chem. 187 431-438.

Mendes-Mourão J., McGivan J.D. and Chapell J.B. (1975) Biochem. J. 146 457-464.

Metzler, D.E.,Ikawa M. and Snell E.E. (1954) J. Amer. Chem. Soc. 76 648-653.

Michalopoulos G. and Pitot H.C. (1975) Exp. Cell Res. 94 70-78.

Michalopoulos G., Sattler G.L. and Pitot H.C. (1978) Cancer Res. 38 1550-1555.

Milgrom E., Atger M. and Baulieu E.E. (1973) Biochem. 12 5198-5205.

Miller J.E. and Litwack G. (1971) J. Biol. Chem. 246 2375-2383.

Miller J.V. and Thompson E.B. (1972) Anal. Biochem. 47 487-494.

Miller M.R., McClure D. and Shiman R. (1976) J. Biol. Chem. 251 3677-3684.

Miller M.R. and Shiman R. (1976) Biochem. Biophys. Res. Commun. 68 740-745.

Milstein S. and Kaufman S. (1975a) J. Biol. Chem. 25 4776-4781.

Milstein S. and Kaufman S. (1975b) J. Biol. Chem. 25 4782-4785.

Molinoff P.B. and Axelrod J. (1971) Annu. Rev. Biochem. 40 465-500.

Morhenn V., Kram R., Hershko A. and Tomkins G.M. (1974) Cell 1 91-94.

Morrison M. and Allen P.Z. (1966) Science 152 1626-1628.

Mortimore G.E. and Mondon C.E. (1970) J. Biol. Chem. 245 2375-2383.

Müller M., Schreiber M., Kartenbeck J. and Schreiber G. (1972) Cancer Res. 32 2568-2576.

Munro H.N. (1970) in Mammalian Protein Metabolism IV 299-386 (Ed. Munro H.N.) Academic Press, New York.

Munthe-Kas A.C. and Seglen P.O. (1974) FEBS Lett. 45 282-285.

Murty C.N., Verney E. and Sidransky H. (1977) Biochim. Biophys. Acta 474 117-128.

Murty C.N. and Sidransky H. (1972) Biochim. Biophys. Acta 262 328-335.

Nagatsu T., Levitt M. and Udenfriend S. (1964) J. Biol. Chem. 239 2910-2917.

Nagatsu T., Mizutani K., Nagatsu I., Matsura S. and Sugimoto T. (1972) Biochem. Pharmacol. 21 1945-1953.

Nakano M. (1967) J. Biol. Chem. 242 73-81.

Neely A.N., Cox J.R., Fortney J.A., Schworer C.M. and Mortimore G.E. (1977) J. Biol. Chem. 252 6948-6954.

Neidhart F.C. and Magasanick B. (1956) Nature 178 801-802.

Nilsson, Å., Sundler R. and Åkesson B. (1974) FEBS Lett. 45 282-285.

Noall M.W., Riggs T.R., Walker L.M. and Christensen, H.N. (1957) Science 126 1002-1005.

Noguchi T., Diesterhaft M. and Granner D. (1978) J. Biol. Chem. 253 1332-1335.

Noguchi T., Minatogawa Y. and Kido R. (1975) J. Neurochem. 25 579-582.

Odashima S., Sturgess J.M. and Rothstein A. (1976) Cell and Tissue Res. 169 167-178.

Oehme P. (1968) in Pyridoxal Catalysis: "Enzyme and Model Systems" (Eds. Snell E.E. et al.,) Wiley (Interscience) New York.

Ohisalo J.J. (1977) Medical Biol. 55 234-240.

Ohneda A., Parada E., Eisentraut A.M. and Unger R.H.(1968) J. Clin. Invest. 47 2305-2322.

Ohtsuka H. (1974) J. Biochem (Tokyo) 75 53-58.

Oliver I.T., Edwards A.M. and Pitot H.C. (1978) Eur. J. Biochem. 221-227.

O'Malley B.W. and Means A.R. (1974) Science 183 610-620.

Ontko J.A. (1972) J. Biol. Chem. 247 1788-1800.

Oshima T. and Tamiyama N. (1961) Biochem. J. 78 116-119.

Oshiro Y., Gerschenson L.E. and Di Paolo J.A. (1972) Cancer Res. 32 877-879.

Otter W.D. and Tait G. (1972) Anatom. Record 173 325-332.

Oxender D.L. (1974) in Biomembranes (Ed. Manson L.A.) 5 25-79, Plenum, New York.

Oxender D.L. and Christensen H.N. (1963) J. Biol. Chem. 238 3686-3699.

Oxender D.L., Lee M. and Cecchini G (1976) in Membranes and Neoplasia: New Approaches and Strategies (Ed. Marchesi V.T.) 41-47, Alan R. Liss, New York.

Pariza M.W., Butcher F.R., Becker J.E. and Potter V.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74 234-237.

Pariza M., Butcher F.R., Kletzien R.F., Becker J.E. and Potter V.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73 4511-4515.

Paul J. (1970) in Cell and Tissue Culture, 4th Edition, Livinston, Edinburgh.

Peraino C. (1967) J. Biol. Chem. 242 3860-3867.

Peraino C., Blake R.L. and Pitot H.C. (1965) J. Biol. Chem. 240 3039-3053.

Peraino C. and Harper A.E. (1963) J. Nutr. 80 270-278.

Perissel B., Charbonne F., Chessebeuf M. and Malet P. (1976) Cell and Tissue Res. 171 157-174.

Perske W.F., Parks R.E. and Walker D.L. (1957) Science 125 1290-1293.

Philipson L., Wall R., Glickman G. and Darnell J.E. (1971) Proc. Natl. Acad. Sci. (U.S.A.) 68 2806-2812.

Pickart L. and Thaler M.M. (1973) Nature New Biol. 243 85-87.

Pilkis S.J., Claus T.H., Johnson R.A. and Park C.A. (1975) J. Biol. Chem. 250 6328-6336.

Piras M.M. and Knox W.E. (1967) J. Biol. Chem. 242 2952-2965.

Pisano J.C., Filkins J.P. and Di Luzio N.R. (1968) Proc. Soc. Exp. Biol. Med. 128 917-922.

Pitot H.C. and Peraino C. (1964) J. Biol. Chem. 239 1783-1788.

Pitot H.C. and Yatvin M.B. (1973) Physiol Rev. 53 228-325.

Pogson C.I. and Smith S.A. (1975) Biochem. J. 152 401-408.

Pohorecky L.A., Zigmond M., Karten H. and Wurtman, R.J. (1969) J. Pharmacol. Exp. Ther. 165 190-195.

Pomerantz S.H. and Chuang L. (1970) Endocrinol. 87 302-310.

Post J. and Hoffman J. (1964) Exp. Cell Res. 36 111-117.

Poste G. (1971) Exp. Cell Res. 65 359-367.

Potter G.D., Tong W. and Chaikoff I.L. (1959) J. Biol. Chem. 234 350-354.

Potter V.R. (1972) Cancer Res. 32 1998-

Prydz H. and Jonsen J. (1964) Scand. J. Clin. Lab. Invest. 16 300-306.

Puvion E., Moyne G. and Bernhard W. (1976) J. de Microscopie et de Biol. Cell. 25 17-32.

Quistorff B., Bondesen S. and Grunnet N. (1973) Biochim. Biophys. Acta 320 503-516.

Rappaport C. and Howze G.B. (1966) Proc. Soc. Exp. Biol. Med. 121 1010-1016.

Ravdin R.G. and Crandall D.I. (1951) J. Biol. Chem. 189 137-

Reel J.R. and Kenney F.T. (1968) Proc. Natl. Acad. Sci. U.S.A. 61 200-206.

Reel J.R., Lee K-L. and Kenney F.T. (1970) J. Biol. Chem. 245 5800-5805.

Renson J., Weissbach H. and Udenfriend S. (1962) J. Biol. Chem. 237 2261-2264.

Reynolds R.D. (1978) Arch. Biochem. Biophys. 186 324-334.

Ricca G.A., Liu D.S.H., Coniglio J.J. and Richardson A. (1978) J. Cell Physiol. 97 137-146.

Riggs T.R., Sanders R.B. and Weindling H.K. (1963) Endocrinol. 73 789-792.

Risser W.L. and Gelehrter T.D. (1973) J. Biol. Chem. 248 1248-1254.

Robison G.A., Butcher R.W. and Sutherland E.W. (1971) in Cyclic AMP, Academic Press, New York.

Rodbell M. (1967) in Adipose Tissue 471-482.

Rodriguez J.M. and Pitot H.C. (1975) Biochem. Biophys. Res. Commun. 65 510-518.

Rodriguez J.M. and Pitot H.C. (1976) Arch. Biochem. Biophys. 177 185-195.

Roewekamp W. and Sekeris C.E. (1977) FEBS Lett. 73 225-228.

Rognstad R. and Clark D.G. (1974) Arch. Biochem. Biophys. 161 638-646.

Roper M.D. and Wicks W.D. (1978) Proc. Natl. Acad. Sci. (U.S.A.) 75 140-144.

Rosen F. and Milholland R.J. (1963) J. Biol. Chem. 238 3730-3735.

Rosen F., Roberts N.R., Rudnick L.E. and Nichol L.E. (1958) Science 127 287-

Rosenberg J.S. and Litwack G. (1970) J. Biol. Chem. 245 5677-5684.

Roser B. (1968) J. Reticuloendothelial Soc. 5 455-471.

Ross B.D., Hems R. and Krebs H.A. (1967) Biochem. J. 102 942-951.

Rubin G.S. and Rosen O.M. (1975) Annu. Rev. Biochem. 44 831-837.

Ryan W.L. and Orr W. (1966) Arch. Biochem. Biophys. 113 684-612.

Sanders R.B. and Riggs T.R. (1967) Endocrinol. 80 29-37.

Sarkar P.K. and Griffiths B. (1976) Biochem. Biophys. Res. Commun. 68 675-681.

Sattler C.A., Michalopoulos G., Sattler G.L. and Pitot H.C. (1978) Cancer Res. 38 1539-1549.

Savage C.R. and Bonney R.J. (1978) Exp. Cell Res. 114 307-315.

Scandalios J.G. (1974) Annu. Rev. Plant Physiol. 25 225-258.

Schafer J.A. and Williams A.E. (1977) in Amino Acid Transport and Uric Acid Transport 20-25 (Eds. Silbernagel G., Lang F. and Greger R.), Georg Thieme, Stuttgart.

Schepartz B. (1951) J. Biol. Chem. 193 293-298.

Schepartz B. (1969) Analyt. Biochem. 30 443-448.

Schepartz B. and Gurin S. (1949) J. Biol. Chem. 180 663-670.

Schimke R.T. (1964) J. Biol. Chem. 239 3808-

Schimke R.T. (1970) in Mammalian Protein Metabolism IV 177-228 (Ed. Munro H.N.).

Schimke R.T. (1973) Adv. Enzymol. 37 135-187.

Schimke R.T. and Doyle D. (1970) Annu. Rev. Biochem. 39 927-977.

Schimke R.T., Sweeney E.W. and Berlin C.M. (1964) Biochem. Biophys. Res. Commun. 15 214-219.

Schimke R.T., Sweeney E.W. and Berlin C.M. (1965) J. Biol. Chem. 240 322-321.

Schlenk F. and Fisher A. (1947) Arch. Biochem. 12 69-79.

Schneider W.C. and Potter V.R. (1943) J. Biol. Chem. 149 217-227.

Schrago E., Lardy H.A., Nordlie R.C. and Foster D.O. (1963) J. Biol. Chem. 238 3188-3192.

Schreiber G. and Schreiber M. (1972) J. Biol. Chem. 247 6340-6346.

Schreiber G. and Schreiber M. (1973) Sub-Cell. Biochem. 2 321-383.

Schutz G., Beato M. and Feigelson P. (1973) Proc. Natl. Acad. Sci.

U.S.A. 70 1218-1221.

Schutz G. and Feigelson P. (1972) Analyt. Biochem. 46 149-155.

Schutz G., Killewich L., Chen G. and Feigelson P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72 1017-1020.

Seglen P.O. (1971) Biochim. Biophys. Acta 230 319-326.

Seglen P.O. (1972) Biochim. Biophys. Acta 264 398-410.

Seglen P.O. (1973a) Exp. Cell Res. 76 25-30.

Seglen P.O. (1973b) Exp. Cell Res. 82 391-398.

Seglen P.O. (1973c) FEBS Lett. 30 25-28.

Seglen P.O. (1974) Biochim. Biophys. Acta 308 317-325.

Seglen P.O. (1975) Biochem. Biophys. Res. Commun. 66 44-52.

Seglen P.O. (1976a) Biochim. Biophys. Acta 442 391-404.

Seglen P.O. (1976b) Methods in Cell Biology 13 29-83.

Seglen P.O. (1977a) Biochim. Biophys. Acta 496 182-191.

Seglen P.O. (1977b) Exp. Cell Res. 107 207-218.

Seglen P.O. and Solheim A.E. (1978a) Eur. J. Biochem. 85 15-25.

Seglen P.O. and Solheim A.E. (1978b) Biochim. Biophys. Acta 520 630-641.

Seifter S. and Harper E. (1970) Meth. Enzymol. XIX 613-635.

Seubert W. and Huth W. (1965) Biochem. Z. 343 176-191.

Shaw C.R. (1969) Int. Rev. Cytol. 25 297-332.

Sidransky H., Sarma D.S.R., Borgiono M. and Verney E. (1968) J. Biol. Chem. 243 1123-1132.

Siess E.A. and Wieland O.H. (1975) Biochem. Biophys. Res. Commun. 64 323-330.

Siev M., Weinberg R. and Penman S. (1969) J. Cell Biol. 41 510-520.

Smith G.J., Pearce H. and Oliver I.T. (1975) Life Sci. 16 437-450.

Smith G.J., Pearce P.H. and Oliver I.T. (1976) Life Sci. 19 1763-1775.

Smith S.A. (1977) Ph.D Thesis, University of Kent, Canterbury,U.K.

Smith S.A., Elliott K.R.F. and Pogson C.I. (1978) Biochem. J. 176 817-825.

Smith S.A., Marston F.A.O., Dickson A.J. and Pogson C.I. (1979) Biochem. Pharmacol. in press.

Snell, E.E. (1963) in Chemical and Biological Aspects of Pyridoxal Catalysis (Eds. Snell E.E., Fasella P.M., Braunstein A. and Rossi-Fanelli A.), Pergamon Press, Oxford and New York.

Snell E.E. (1972) Tapakushitsu, Kakusan, Koso 17 229-239.

Soffer R.L., Hechtman P. and Savage M. (1973) J. Biol. Chem. 248 1224-1230.

Soll A.H., Kahn C.R. and Neville D.M. (1975) J. Biol. Chem. 250 4702-4707.

Spencer, C.J. and Gelehrter, T.D. (1974) J. Biol. Chem. 249 577-583.

Stadtman, E.R. and Chock P.B. (1978) Curr. Topics in Cell. Regul. 13 53-97.

Stanislawski-Birencwajg M., Uriel J. and Grabar P. (1967) Cancer Res. 27 1990-1999.

Stanley P.E. and Williams S.G. (1969) Analyt. Biochem. 29 381-392.

Stellwagen R.H., Kohli K.K. and Sailor R.D. (1977) J. Cell Physiol. 91 51-62.

Stinson R.A. and Gutfreund H. (1971) Biochem. J. 121 235-240.

Sudilovsky O., Pestana A., Hinderaker P.H. and Pitot H.C. (1972) Science 174 142-144.

Suhadolnik R.J. (1970) in Nucleoside Antibiotics pp 50-75, Wiley Interscience, New York.

Surks, M.I. and Oppenheimer J.H. (1971) J. Clin. Endocrinol. Metab. 33 612-618.

Swick, R.W. (1957) J. Biol. Chem. 231 751-764.

Taurog A., Potter G.D. and Chaikoff I.L. (1955) J. Biol. Chem. 213 119-134.

Taurog A., Potter G.D., Tong W. and Chaikoff I.L. (1956) Endocrinol. 58 121-131.

Tergis D., Fishman N. and Rivlin R.S. (1975) in Thyroid Hormone Metabolism 213-221 (Eds. Harland, W.A. and Orr, J.S.), Academic Press, London.

Tews J.K. and Harper A.E. (1969) Biochim. Biophys. Acta 183 601-610.

Tews J.K., Woodcock N.A. and Harper A.E. (1970) J. Biol. Chem. 245 3026-3032.

Thompson E.B., Granner D.K. and Tomkins G.M. (1970) J. Mol. Biol. 54 159-175.

Thompson E.B. and Gelehrter T.D. (1971) Proc. Natl. Acad. Sci. (U.S.A.) 68 2589-2593.

Thompson E.B. and Tomkins G.M. (1971) J. Cell Biol. 49 921-927.

Tokuyama K. (1959) J. Biochem. (Tokyo) 46 1379-1391.

Tomkins G.M., Levinson B.B., Baxter J.D. and Dethlefsen L. (1972) Nature New Biol. 239 9-14.

Tomkins G.M., Thompson E.B., Hayashi S., Gelehrter T., Granner D. and Peterofsky B. (1966) Cold Spring Harbour Symposium Quant. Biol. 31 349-360.

Truman J.T. and Klenow H. (1968) Mol. Pharmacol. 4 77- 84.

Tryfiates G.P. and Saus F.L. (1975) Cancer Biochem. Biophys. 1 63-68.

Tsai C.M., Best N. and Ebner K.E. (1966) Exp. Cell Res. 44 332-337.

Udenfriend S. (1963) Amer. J. Clin. Nutr. 12 287-290.

Udenfriend S. and Dairman W. (1970) Adv. Enz. Regul. 9 1445-1465.

Unger R. (1971) Diabetes 20 834-838.

Ureta T. (1978) Curr. Topics in Cell. Regul. 13 233-258.

Umezawa H. and Takaai A. (1977) Res. Monogr. Cell Tissue Physiol. 2 (Proteinases Mamm. Cells Tissues), 637-662.

Valeriote F., Auricchio F., Tomkins G.M. and Riley D.J. (1969) J. Biol. Chem. 244 3618-3624.

Van Der Meer R., De Haan E.J. and Tager J. M. (1976) in Uses of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Eds. Tager, J.M., Söling H-D, and Williamson J.R.), North-Holland, Amsterdam.

Van Lan V., Yamaguchi N., Garcia M.J., Ramey E.R. and Penhos J.C. (1974) Endocrinol. 94 671-675.

Van Venrooji, W.J.W.,Henshaw, E.C. and Hirsch C.A. (1972) Biochim. Biophys. Acta 259 127-137.

Varandani P.T., Raveed D. and Nafz M.A. (1978) Biochim. Biophys. Acta 538 343-353.

Vidrich A., Airhart J., Bruno M.K. and Khairallah E.A. (1977) Biochem. J. 162 257-266.

Voight J. and Sekeris C.E. (1978) Hoppe-Seylers Z. Physiol. Chem. 359 1363-1370.

Volpe P., Sawamura R., and Strecker H.J. (1969) J. Biol. Chem 244 719-726.

Waalkes T.P. and Udenfriend S. (1957) J. Lab. Clin. Med. 50 733-736.

Wade R.S, and Gusseck D.J. (1976) Biochem. J. 154 245-247.

Wagle S.R. (1976) Acta Diabetologia Latina 13 186-201.

Wagle S.R., Gaskins R.K., Jacoby A. and Ashmore J. (1966) Life Sci. 5 655-663.

Wagle S.R. and Ingebretsen W.R. (1973) Biochem. Biophys. Res. Commun. 52 125-129.

Wagle S.R. and Ingebretsen W.R. (1975) Methods in Enzymol. 35 579-584 (Ed. Lowenstein, J.M.).

Walker P.R. (1977a) Biochim. Biophys. Acta 496 255-263.

Walker P.R. (1977b) J. Cell. Physiol. 91 169-179.

Walker P.R. (1977c) Essays in Biochemistry 13 39-70.

Walker P.R. and Grindle M.J. (1977) J. Cell. Physiol. 91 181-191.

Walli A.K. and Schimassek H. (1976) in Uses of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Eds. Tager J.M., Söling H-D. and Williamson J.R.) pp 426-429, North Holland, Amsterdam.

Walter U., Luthe H., Gerhart F. and Söling H-D. (1975) Eur. J. Biochem. 59 395-403.

Ward W.F., Cox J.R. and Mortimore G.E. (1977) J. Biol. Chem. 252 6955-6961.

Ward W.F. and Mortimore G.E. (1978) J. Biol. Chem. 253 3581-3587.

Weber G. (1967) Gastroenterology 53 984-988.

Weber G., Banerfee G. and Bronstein S.B. (1962) Amer. J. Physiol. 202 137-144.

Weber G. and Singhal R.L. (1964) Biochem. Pharmacol. 13 1173-1187.

Weber G., Singhal R.L. and Srivastava S.K. (1965) Adv. Enz. Regul. 3 43-75.

Weiner N. (1970) Annu. Rev. Pharmacol. 10 273-290.

Weiner N. (1972) in Progr. Endocrinol., Proc. Int. Congr. Endocrinol. 3rd Colo.) 294-301 Ed. Gual C., Excerpta Med., Amsterdam.

Weinhouse S. and Millington H. (1948) J. Biol. Chem. 175 995-1002.

Weinstein A., Medes G. and Litwack G. (1967) Analyt. Biochem. 21 86-97.

Wicks W.D., Barnett C.A. and McKibbin J.B. (1974) Fed. Proc. 33 1105-1111.

Wicks W.D., Kenney F.T. and Lee K-L. (1969) J. Biol. Chem. 244 6008-6013.

Wicks W.D., Koontz J. and Wagner K. (1975) J. Cyclic Nucleotide Res. 1 49-58.

Wicks W.D., Lewis and McKibbin J.B. (1972b) Biochim. Biophys. Acta 264 177-185.

Wicks W.D., Van Wijk R. and McKibbin J.B. (1972a) Adv. Enzyme Regul. 11 117-135.

Wicks W.D. and Su J-L. (1978) J. Cyclic Nucleotide Res. 4 113-112.

Wieland T., and Pfleiderer G. (1963) Adv. Enzymol. 25 329-368.

Williams G.M., Bermudez E. and Scaramuzzino D. (1977) In Vitro 13 809-817.

Williams G.M. and Gunn J.M. (1974) Exp. Cell Res. 89 139-142.

Wincek T.J., Hupka A.L., and Sweat F.W. (1975) J. Biol. Chem. 250 8863-8873.

Wittman J.S. and Miller O.N. (1971) Amer. J. Clin. Nutr. 24 770-778.

Womack M. and Rose W.C. (1946) J. Biol. Chem. 166 429-434.

Woodside K.H. (1976) Biochim. Biophys. Acta 421 70-79.

Woodside K.H. and Mortimore G.D. (1972) J. Biol. Chem. 247 6474-6481.

Woodside K.H., Ward W.F. and Mortimore G.E. (1974) J. Biol. Chem. 249 5458-5463.

Wu S.Y., Chopra I.J. and Nakamura Y. (1976) J. Clin. Endocrinol. Metab. 43 682-685.

Wurtman R.J. (1965) New Engl. J. Med. 273 637-646.

Wurtman R.J. (1970) in Mammalian Protein Metabolism IV 445-479. (Ed. Munro H.N.), Academic Press, New York.

Wurtman R.J. (1974) Life Sci. 15 827-847.

Wurtman R.J. and Axelrod J. (1966) Science 150 1464-1465.

Wurtman R.J. and Larin F. (1968) Biochem. Pharmacol. 17 817-818.

Wurtman R.J., Rose C.M., Chou C., and Larin F.F. (1968) New Engl. J. Med. 279 171- 176.

Yatvin M.B. and Pitot H.C. (1970) J. Biol. Chem. 245 4673-4676.

Yuwiller A., Wetterberg L. and Geller E. (1970) Biochim. Biophys. Acta 208 428-433.

Zahlten R.N. and Stratman F.W. (1974) Arch. Biochem. Biophys. 163 600-608.

Zigmond M.J. and Wilson S.P. (1973) Biochem. Pharmacol. 22 2151-2163.

SOURCES OF CHEMICALS AND ENZYMES

1) CHEMICALS

These were obtained from the following suppliers:

Abbott Laboratories Ltd., Queenborough, Kent, U.K.

Nembutal (sodium pentobarbitone), as a solution ($60 mg.ml^{-1}$) in aq. 10%(v/v) ethanol - 20%(v/v) propylene glycol.

Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.

bovine serum albumin, fraction V (20% aq. solution)

Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

ADP

ATP

NADH

oxaloacetate

2-oxoglutarate

pyridoxal 5'-phosphate

pyruvate

triethanolamine hydrochloride

Evans Medical Ltd., Speke, Liverpool, U.K.

Heparin ($140 - 170 U.ml^{-1}$)

Gibco Bio-Cult Ltd., Paisley, Scotland, U.K.

Foetal calf serum

Horse serum

Norit Clydesdale Co., Glasgow, Scotland, U.K.

Norit GSX charcoal

Sigma (London) Chemical Co., Norbiton, Surrey, U.K.

acrylamide

L-alanine

ammonium persulphate

aminooxyacetate

L-arginine

Na-ascorbate

L-asparagine

choline chloride

cycloheximide

L-cysteine

dexamethasone

diethyldithiocarbimate

dithiothreitol

folic acid

glucagon

L-glutamine

reduced glutathione

L-glycine

hematin

L-histidine

hydrocortisone

i-inositol

L-isoleucine

L-leucine

L-lysine-HCl

2-mercaptoethanol

methaemoglobin

L-methionine

methylene-bisacrylamide

monoiodotyrosine

MTT

Ca-pantothenate

phenazine methosulphate

L-phenylalanine

phenylmethylsulphonylfluoride

L-proline

riboflavin

serine

TEMED

thiamin

L-threonine

triamcinolone

triton-X-100

L-tryptophan

tween 80 (polyoxyethylene sorbitan-mono oleate)

L-tyrosine

L-valine

ITP was made by treatment of ATP with HNO_2 according to the method of Kaplan N.O. (1957) Methods Enzymol. III 874-879.

2) ENZYMES

Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

collagenase [EC 3.4.24.3.]

glutamate-oxaloacetate transaminase [EC 2.6.1.1.]

lactate dehydrogenase [EC 1.1.1.27.]

malate dehydrogenase [EC 1.1.1.37.]

pyruvate kinase [EC 2.7.1.40.]

urease [EC 3.5.1.5.]

Sigma (London) Chemical Co., Norbiton, Surrey, U.K.

glucose oxidase [EC 1.1.3.4.]

peroxidase [EC 1.11.1.7.]

3) RADIOISOTOPES

Radiochemical Centre, Amersham, Bucks, U.K.

U-[^{14}C] acetate

U-[^{14}C] inulin

L-[4,5-^{3}H] leucine

L-[side chain 2,3-^{3}H] tyrosine