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A STUDY OF ORNITHINE AMINOTRANSFERASE AND INTRACELLULAR ORNITHINE METABOLISM

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DECLARATION

This work has not been accepted for any other degree and is not concurrently being submitted for any other degree.

We certify that the work submitted was carried out by the author. Due acknowledgement has been made of any assistance received.

Signed .....

(Candidate)

Signed .....

(Director of Studies)

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ABSTRACT

A Study of Ornithine Aminotransferase and Intracellular Ornithine Metabolism - J. M. Leah

The mitochondrial matrix enzyme, ornithine aminotransferase, has been purified from rat and human sources. The enzyme was found to undergo self-association upon concentration, in agreement with the findings of previous workers. Native molecular weight studies in the absence and presence of detergent suggest that the hydrophobicity and aggregation of ornithine aminotransferase may spare a high concentration intramitochondrial ornithine pool from metabolism by this enzyme. Characterization of rat and human ornithine aminotransferase has shown them to be very similar. Polyclonal antibodies have been raised to the enzyme from both sources and immunodiffusion analysis indicates a close structural similarity. This similarity has been exploited to develop an immunoadsorbent capable of purifying human ornithine aminotransferase using affinity purified rabbit antibodies. This technique has been successfully applied to human liver, rat kidney and rat blood platelet extracts. Results have shown that blood platelets may be a suitable tissue source of ornithine aminotransferase for future investigation of patients with Gyrate Atrophy, an inherited deficiency of this enzyme. A competitive enzyme-linked immunosorbent assay has been developed to detect low levels of ornithine aminotransferase and both rat and human tissues have been assayed. Estimations were in agreement with activity measurements and the monospecificity of the immunoassay has been checked by Western blotting.

Two analytical systems have been assessed for quantitative amino acid analysis of physiological fluids from patients with inborn errors of metabolism, in particular, samples from Gyrate Atrophy patients. Attempts have been made to investigate intracellular amino acid levels and the phenomenon of membrane-associated amino acids, in rat liver extracts. Ornithine was found to be undetectable in mitochondria. This could indicate very low levels in the mitochondrial matrix, or might be an artifact due to rapid diffusion out of organelles.

ABBREVIATIONS

The following abbreviations are used in the text.

%CV	percentage coefficient of variation
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunsorbent assay
FCS	foetal calf serum
x g	relative centrifugal force, g (average)
HHH	hyperornithinemia-hyperammonemia-homocitrullinuria
HRP	horseradish peroxidase
HPLC	high performance liquid chromatography
IgG	Immunoglobulin G
Km	Michaelis constant
2-ME	2-mercaptoethanol
MOM	mitochondrial outer membrane
MIM	mitochondrial inner membrane
OAT	ornithine aminotransferase
OCT	ornithine carbamoyl transferase
OPA	o-phthalaldehyde
PBS	phosphate buffered saline
r.p.m.	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.	standard error of the mean
TEMED	N,N,N',N' - tetramethylenediamine
Tris	Tris (hydroxymethyl)-aminomethane

ENZYME COMMISSION (EC) NUMBERS

ARGINASE	EC 3.5.3.1
CARBAMOYL PHOSPHATE SYNTHETASE I	EC 6.3.4.16
CARBAMOYL PHOSPHATE SYNTHETASE II	EC 6.3.5.5
α-CHYMOTRYPSIN	EC 3.4.21.1
ORNITHINE AMINOTRANSFERASE	EC 2.6.1.13
ORNITHINE DECARBOXYLASE	EC 4.1.1.17
ORNITHINE CARBAMOYL TRANSFERASE	EC 2.1.3.3
GLYCINE TRANSAMIDINASE	EC 2.1.4.1
PROLINE OXIDASE	unassigned
PYRROLINE-5-CARBOXYLATE SYNTHASE	unassigned
PYRROLINE-5-CARBOXYLATE REDUCTASE	EC 1.5.1.2
PYRROLINE-5-CARBOXYLATE DEHYDROGENASE	EC 1.5.1.12
S-ADENOSYLMETHIONINE:GUANIDINOACETATE N-METHYLTRANSFERASE	unassigned

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1 GENERAL INTRODUCTION

1.1 The urea cycle: A brief overview

The main function of the urea cycle is to remove ammonia produced in protein and amino acid catabolism in ureotelic animals. Ammonia is toxic to the nervous system and is thus detoxified by incorporation into urea. Carbamoyl phosphate is formed in mitochondria from ammonia, ATP and bicarbonate, which then condenses with ornithine to form citrulline. Citrulline then leaves the mitochondrion and condenses with aspartate to form argininosuccinate and is then metabolized to arginine with the release of fumarate. Arginine is finally split to form ornithine and urea. Ornithine is then recycled into mitochondria (figure 1.1). The overall cycle involves enzyme reactions in both the cytosol and mitochondria which are controlled by the concentration of substrates, activators and inhibitors. Transport of substrates across the mitochondrial membrane is also an important factor of urea cycle control. Most of the body's urea synthesis takes place in the liver. The brain lacks ornithine carbamoyltransferase (OCT) and cannot therefore form urea from ammonia, but can do so from citrulline. The kidney has a very low arginase activity and is thus likely to be the major net source of arginine in the body.

1.2 Ornithine and the urea cycle

1.2.1 Role of ornithine in the urea cycle

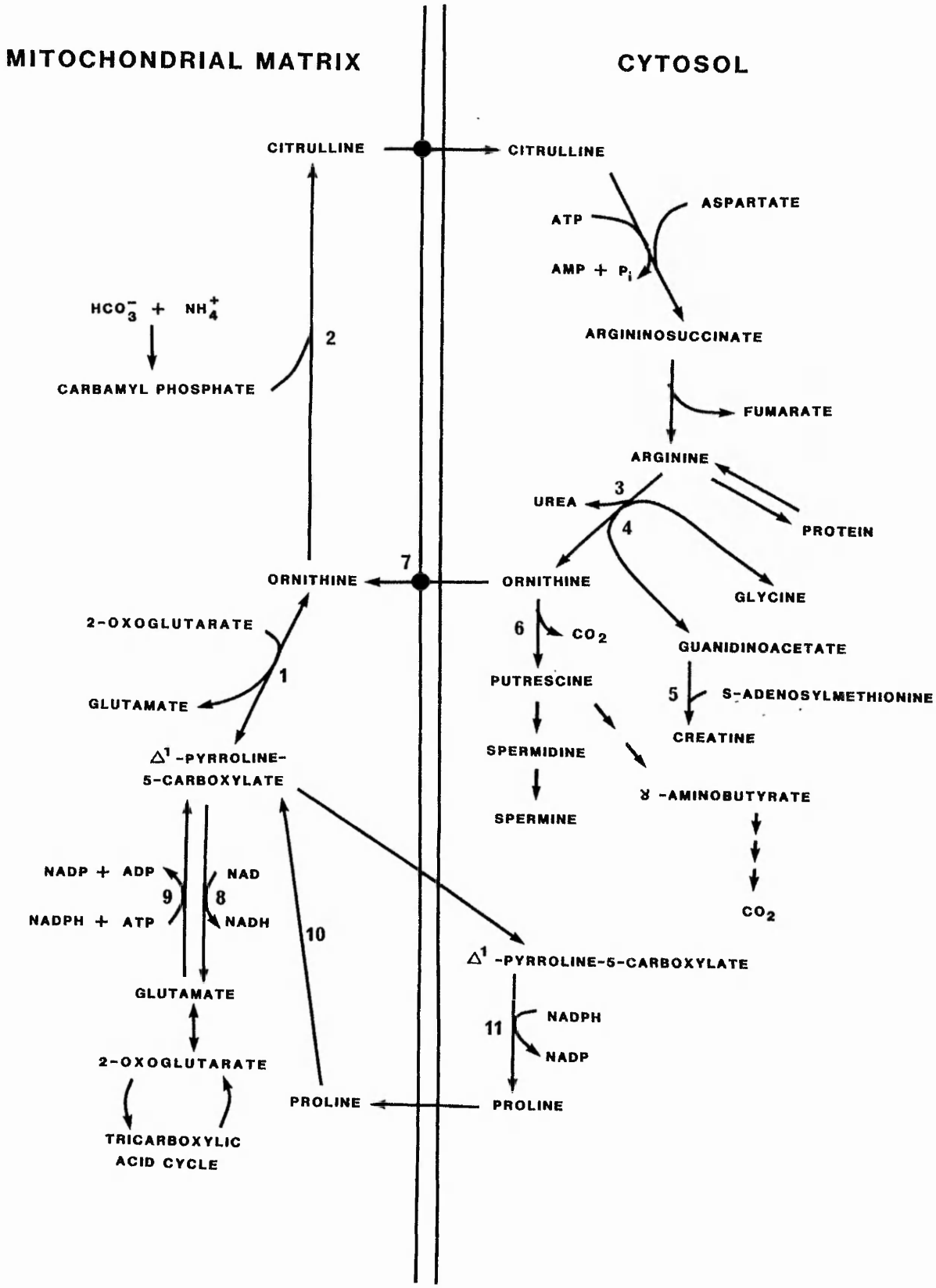
In most cases, the concentration of ornithine acts as a catalyst for urea cycle activity. The formation of citrulline in the mitochondrial matrix is effected by the transfer of the carbamoyl group of carbamoyl phosphate to the δ-amino nitrogen of ornithine. The reaction is catalysed by OCT which has a pH optimum of between 8-9 and is pH sensitive. Consequently, this suggests that zwitterionic ornithine is the actual substrate at physiological pH (Snodgrass, 1968). OCT is regulated at

FIGURE 1.1 Ornithine metabolism and compartmentation:

Numbers refer to the following enzymes and proteins:

1. ornithine aminotransferase; 2. ornithine carbamoyltransferase; 3. arginase; 4. glycine transamidinase; 5. S-adenosylmethionine: guanidinoacetate N-methyltransferase; 6. ornithine decarboxylase; 7. ornithine transport protein; 8. Δ'-pyrroline-5-carboxylate dehydrogenase; 9. Δ'-pyrroline-5-carboxylate synthase; 10. proline oxidase; 11. Δ'-pyrroline-5-carboxylate reductase.

N.B. subcellular localization of glycine transamidinase not established.


MITOCHONDRIAL MATRIX
CYTOSOL
CITRULLINE
CITRULLINE
ATP
ASPARTATE
AMP + Pi
ARGININOSUCCINATE
HCO3− + NH4+
2
CARBAMYL PHOSPHATE
FUMARATE
ARGININE
3
UREA
PROTEIN
4
7
ORNITHINE
ORNITHINE
GLYCINE
2-OXOGLUTARATE
6
CO2
GUANIDINOACETATE
1
PUTRESCINE
5
S-ADENOSYLMETHIONINE
GLUTAMATE
CREATINE
Δ1-PYRROLINE-
5-CARBOXYLATE
SPERMIDINE
γ-AMINOBUTYRATE
SPERMINE
NADP + ADP
NAD
9
8
NADPH + ATP
NADH
CO2
10
Δ1-PYRROLINE-5-CARBOXYLATE
GLUTAMATE
11
NADPH
NADP
2-OXOGLUTARATE
PROLINE
PROLINE
TRICARBOXYLIC
ACID CYCLE

the substrate and gene level although long term regulation is dependent upon dietary protein (Nuzum, 1971).

McGivan et al, (1977) has shown that matrix ornithine concentrations are dictated by entry of ornithine into the mitochondria, the action of OCT and that of OAT. Matrix ornithine and carbamoyl phosphate have not been satisfactorily measured by direct means although ornithine concentration is thought to be low relative to carbamoyl phosphate (Raijman, 1976). Meijer (1979) has suggested that ornithine may stimulate synthesis of carbamoyl phosphate by an unknown mechanism while carbamoyl phosphate itself may inhibit OCT (Stewart et al, 1980).

Urea cycle ornithine, produced from arginine catabolism, is catalysed by arginase in the cytosol. Two or more forms of arginase are thought to exist (Reddi et al , 1975; Herzfeld et al , 1976). The liver enzyme is notable for its high activity (Aebi, 1976) and low substrate affinity. A second form has been found in the kidney, small intestine, pancreas and mammary gland (Kaysen et al, 1973; Reddi et al, 1975; Herzfeld et al, 1976) and thought to be ultimately responsible for proline synthesis. Inherited arginase deficiency in children results in hyperargininemia, mental retardation and neurological abnormalities (Cederbaum et al, 1977). The activity of kidney arginase and glycine transamidinase in these patients results in near normal ornithine levels (see figure 1.1) providing alternative pathways to ornithine formation.

1.2.2 Intracellular ornithine transport

For the urea cycle to function, ornithine has to penetrate the inner and outer mitochondrial membrane. The outer membrane is permeable to amino acids, the inner membrane being impermeable (Kligenberg, 1970). A specific carrier protein mediates ornithine transport into the liver mitochondria but

there is some disagreement about the energy requirements needed. Gamble and Lehninger (1973) found that respiratory energy supplied by succinate or glutamate and membrane permeable anions (acetate, bicarbonate, HPO_4^{2-} , $H_2PO_4^-$) was necessary for mitochondria to accumulate cationic ornithine and that non-respiring mitochondria were impermeable to both cationic and zwitterionic ornithine. However, intra- and extramitochondrial ornithine concentrations were found to be dependent upon the pH gradient across the mitochondrial membrane and that the respective ornithine concentrations inside and outside the mitochondrion were virtually identical when the ornithine aminotransferase reaction was blocked by amino-oxyacetate (McGivan et al., 1977). This suggests that cationic ornithine is exchanged with H^+ electroneutrally and independently of respiratory energy. An ornithine/citrulline antiporter system has been proposed by Bradford and McGivan (1980) which suggests a close coupling of the transmitochondrial transport of these two metabolites and this supports previous work which indicates a link between them (Bryla and Harris, 1976).

It is therefore likely that the effect of these transport processes makes ornithine limiting for urea cycle activity by regulating citrulline synthesis and/or transamination reactions.

1.3 Synthesis of putrescine from ornithine

Metabolism of ornithine to putrescine occurs in the cytosol and is irreversibly catalysed by the pyridoxal phosphate dependent enzyme, ornithine decarboxylase. The precise functions of putrescine and other polyamines are not clear, but are thought to be important in cell replication and stability of membranes (Raina and Janne, 1975). The condensation of putrescine with decarboxy-s-adenosylmethionine forms spermidine and spermine in successive steps. Ornithine decarboxylase

activity provides the rate limiting step in polyamine synthesis and not the levels of ornithine. One of the first events which occurs after stimulation of resting cells to proliferating cells is a rapid and high increase of ornithine decarboxylase synthesis (Nissley et al., 1976), enzyme degradation being as rapid as synthesis.

Putrescine can be converted to γ-aminobutyrate either by deamination or metabolism of n-acetylated intermediates (Baxter, 1976; Seiler and Al-Therib, 1974). Degradation of this neurotransmitter by transamination and oxidation produces succinate and finally carbon dioxide. This permits oxidation of ornithine to carbon dioxide and allows the OAT reaction to be bypassed.

1.4 Creatine synthesis

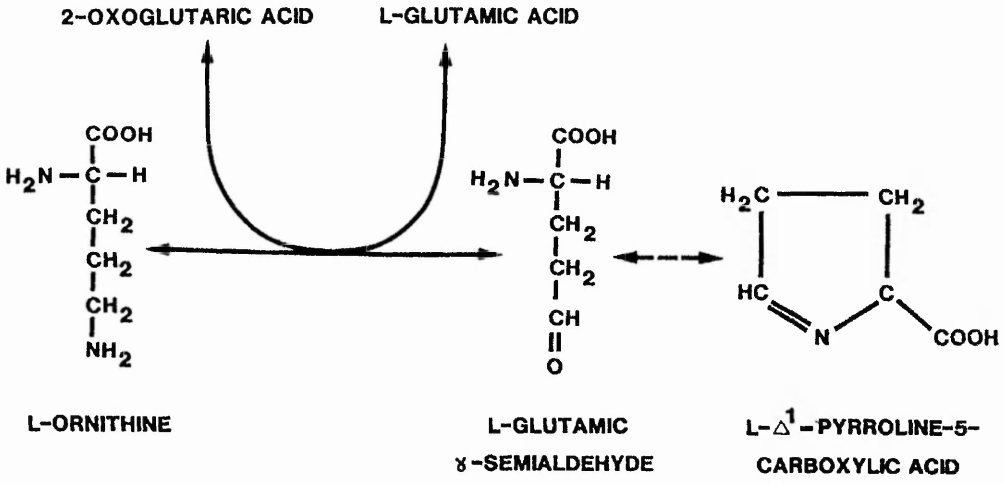
A second reaction which irreversibly metabolizes arginine to ornithine results in the production of guanidinoacetate. This reaction is catalyzed by glycine transamidinase and can be strongly inhibited by ornithine (Sipila, 1980). Guanidinoacetate is then N-methylated to form creatine predominantly in the liver, kidney and pancreas. Creatine is then thought to be transported to muscle and nerve and there phosphorylated to creatine phosphate to provide a reservoir of high energy phosphagen.

1.5 Ornithine aminotransferase (OAT)

1.5.1 The OAT reaction

Ornithine aminotransferase is a mitochondrial matrix enzyme, dependent upon pyridoxal phosphate as cofactor, which catalyzes the conversion of ornithine and 2-oxoglutarate to Δ'-pyrroline-5-carboxylate and glutamate (figure 1.2).

Figure 1.2 The ornithine aminotransferase reaction



Glutamate semialdehyde spontaneously cyclizes to form Δ'-pyrroline-5-carboxylate. This reaction is reversible (Matsuzawa, 1974) but at equilibrium lies far towards ornithine catabolism because of the spontaneous conversion of glutamate semialdehyde (Strecker, 1965). The equilibrium constant for the reaction has been estimated at about 70 (Valle and Simell, 1983).

The complete reaction involves two half reactions (Peraino, 1972) as follows:

1. ornithine + pyridoxal-OAT ----> Δ'-pyrroline-5-carboxylate + pyridoxamine-OAT

2. pyridoxamine-OAT + 2-oxoglutarate ----> glutamate + pyridoxal-OAT

Historically, ornithine aminotransferase activity was first reported by Quastel and Witty (1951) in pigeon homogenates. The

spontaneous cyclization of glutamate semialdehyde was first reported by Fincham (1951) in extracts of Neurospora crassa.

1.5.2 Assay of OAT

The most common method of OAT assay relies on the quantitative formation of a dihydroquinazolinium derivative produced when the chromogen, o-aminobenzaldehyde is reacted with Δ'-pyrroline-5-carboxylate (Vogel and Davis, 1953). The yellow supernatant is measured spectrophotometrically at 440 nm using a molar extinction coefficient of 2.71 (Strecker, 1965; Herzfeld and Knox, 1968) although a coefficient of 2.59 has also been reported (Mezl and Knox, 1976).

O-aminobenzaldehyde also reacts with other Δ'-pyrroline compounds such as Δ'-pyrroline-2-carboxylate (Meister, 1954; Valle et al., 1970; Adams and Frank, 1980) and this needs to be taken into consideration when choosing a control. The sensitivity of this assay has been increased by using HPLC to separate and detect the dihydroquinazolinium compound.

A second method of OAT assay involves the separation of radiolabelled Δ'-pyrroline-5-carboxylate from its labelled precursor, ornithine, by ion-exchange chromatography (Phang et al., 1973). Whilst the method has the advantage of increased sensitivity over the spectrophotometric assay, the commercially available radiolabelled ornithine is thought to contain contaminants which may inhibit OAT activity. Several radiochemical assays for OAT have since been developed involving determination of labelled ornithine (Ohura et al., 1983; Janssen et al., 1981); glutamate (Gopalakrishni and Nagarajan, 1982) and 2-oxoglutarate (Wong et al., 1981).

A sensitive enzyme immunoassay for OAT has been reported (Kato et al., 1977) in which 0.03 femtomoles of OAT protein could be detected. The assay involves use of the Fab (Fragment antigen binding) fragment

of rabbit polyclonal antibody coupled to β-galactosidase together with IgG adsorbed onto silicone pieces.

1.5.3 Regulation of OAT

1.5.3.1 Developmental factors

OAT activity in rat liver and kidney only becomes significant after 2 postnatal weeks. Levels of OAT then increase by about 15-fold to adult levels by 30 days of age (Herzfeld and Knox, 1968; Raiha and Kekomaki, 1968). However, in the small intestine, OAT activity is higher at 3 postnatal weeks than in the adult rat (Herzfeld and Raper, 1976).

1.5.3.2 Nutritional factors

Induction of OAT in rat by oral intubation of casein hydrolysate (Pitot and Peraino, 1964) and free amino acids (Peraino et al., 1965) has been reported. Furthermore, OAT activity is influenced by protein intake as is expected of an enzyme involved in protein catabolism. Increasing protein levels from 20% to 70% in the diet resulted in an increase in OAT after 4 days, to 6 times the basal levels in rat liver (Valle, 1983). Decreasing dietary protein to 5% causes a 2-fold reduction in OAT activity. Simultaneous feeding of glucose prevents protein-induced OAT increases (Sanada et al., 1970; Peraino and Pitot, 1964; Pestana, 1969). However, OAT levels in the kidney and small intestine are not influenced by dietary protein (Sanada et al., 1970).

1.5.3.3 Hormonal factors

The developmental formation of OAT in rat is enhanced in the kidney and to a lesser extent in the liver, by oestrogen (Herzfeld and Knox, 1968) and adult OAT levels remain unaffected. As a result, OAT levels in the adult female rat kidney are approximately twice as high as those in male rats and this difference is abolished after ovariectomy. Herzfeld and Greengard (1969) reported

that OAT in rat liver and kidney is under the control of antagonistic hormonal factors during development. Glucocorticoid administration after 4 postnatal days raises the liver OAT levels to adult levels within 24 hours. Oestrogen or adrenalectomy inhibits OAT increment during the third postnatal week and can inhibit the effects of glucocorticoids. In rat kidney, oestrogen elevates OAT levels but glucocorticoids have no influence during development.

In adult rat liver, OAT synthesis is increased 3-fold after glucagon administration (Lyons and Pitot, 1976). Glucagon induction of OAT is inhibited by glucose administration as a result of rapid inhibition of OAT synthesis. Glucagon induction of OAT has been demonstrated *in vitro* in rat hepatocytes; however the inhibition of glucagon induction by corticosteroids readily demonstrable *in vivo* was absent in cell culture (Spence *et al.*, 1980). Merrill and Pitot (1985) have shown that OAT synthesis can be induced by glucagon, cAMP and simple carbohydrates (glucose or fructose) in rat hepatocytes grown in cell culture. However, an increased rate of OAT synthesis cannot be maintained under culture conditions, unlike the *in vivo* situation (Pitot and Peraino, 1964; Peraino and Pitot, 1964). It has therefore been concluded that maintainance of an induced rate of OAT synthesis requires extrahepatic influences not present in culture media. The effect of glucocorticoids upon postnatal OAT synthesis is thought to be potentiated by thyroxine (Vandewater and Henning, 1985). The effects of tri-iodothyronine (T3) and oestradiol on OAT activity in the kidney of rats given pyridoxine-deficient and control diets have also been investigated (Ikeda and Okada, 1986). T3 can induce OAT synthesis in control rats but not in pyridoxine-deficient rats. However, oestradiol can induce OAT synthesis in both control and deficient rats. It has been concluded that pyridoxal phosphate may modulate OAT activity in rat kidney through the action of oestradiol.

Recent studies (Patnaik et al., 1987) have shown that ovariectomy decreases, and oestradiol increases the level of OAT in kidney cortex except in the case of old rats (85 weeks old) probably as a consequence of loss of ovarian function. Rat liver OAT levels were unresponsive to both of these treatments.

At the molecular level, Mueckler et al., (1983) have shown that OAT is regulated at the translational and pretranslational level. Under conditions of glucagon induction of rat liver OAT, synthesis increased by a factor of 10 to 12-fold whilst the level of functional mRNA increased by only 2-fold. It was therefore concluded that increased OAT synthesis was largely as a result of increased translational efficiency.

1.5.4 Purification and properties of OAT

One of the first attempts at partial purification of OAT was from Neurospora crassa (Vogel and Kopac, 1960). Pyridoxal phosphate cofactor dependence was demonstrated, a pH optimum of 7.4 and Michaelis constants of 2.0 mM and 1.7 mM for L-ornithine and 2-oxoglutarate respectively were reported.

A 7-fold purification of OAT was achieved from rat liver by isolating mitochondria followed by ammonium sulphate precipitation (Peraino and Pitot, 1963). A pH optimum of 7.4 and Michaelis constants of 7.2 mM and 1.1 mM for L-ornithine and 2-oxoglutarate were reported. Katunuma et al., (1964) achieved a 400-fold purification of rat liver OAT and reported a native molecular weight of 115,000, a pH optimum of 8.2 and Michaelis constants of 6.0 mM, 0.85 mM and 11.3 µM for L-ornithine, 2-oxoglutarate and pyridoxal phosphate respectively. Competitive inhibition by canalline and oxaloacetate was also demonstrated. Strecker (1965) reported a 7-fold purification of rat liver OAT and showed substrate inhibition with both substrates.

A 2000-fold purification and crystallization of rat liver OAT has been achieved (Matsuzawa et al., 1968). The purification method included 60°C heat treatment of extracts, demonstrating the thermostable nature of the enzyme. The native molecular weight of OAT was estimated at between 160,000 and 180,000 and pyridoxal phosphate was estimated at 2 molecules per molecule of enzyme. Peraino et al., (1969) also crystallized rat liver OAT and estimated a native molecular weight of 132,000 and a dissociated molecular weight of 33,000 indicating that native OAT is tetrameric. The isoelectric point (5.38) was also estimated and at least 4 thiol groups and no disulphide bonds were present in the native OAT molecule.

Rat liver OAT has been shown to be inhibited by branched chain amino acids (L-valine > L-isoleucine > L-leucine). The inhibitory action was shown to be largely due to the branched structure of the hydrophobic residue (Matsuzawa, 1974).

Pig kidney OAT has been purified (Jenkins and Tsai, 1970). Substrate inhibition, thermal stability, pH optimum (8.0) and native molecular weight (248,000) were reported, indicating similar characteristics to the rat liver enzyme.

Rat kidney OAT has been crystallized and compared to OAT from rat liver and small intestine, (Sanada et al., 1970). Michaelis constants of about 1 mM were obtained for both substrates used with all 3 sources of OAT, and for pyridoxal phosphate, a value of about 1 μM was obtained for rat liver and kidney OAT. Immunological comparison of OAT by immunodiffusion showed that OAT from the 3 rat sources were identical with respect to a polyclonal antiserum. It was concluded that OAT purified from different rat tissues was the same protein. Yip and Collins (1971) also compared rat liver and kidney OAT and estimated Michaelis constants for rat kidney OAT of 6 mM and 1 mM respectively for L-ornithine and 2-oxoglutarate, and a pH optimum of between 7.6 and

7.8. The true pH optimum for the overall reaction is 8.15 (Peraino, 1972) which results from a compromise between the optima obtained for the 2 half reactions (see section 1.5.1). Cofactor binding was investigated. It was found that excess pyridoxal phosphate can partially inactivate the enzyme possibly by binding to lysine residues involved in maintaining full catalytic activity.

The wide range of native molecular weight estimations obtained by different workers for rat liver OAT was noted by Morris et al., (1974). It was found that the molecular weight of OAT is concentration-dependent, forming a tetramer at about 10 mg/ml protein and forming aggregates of higher molecular weight at higher concentrations. The aggregation phenomenon was further investigated (Boernke et al., 1981). It was found that OAT aggregates in a two-stage process as its concentration increases. Monomers are first thought to associate into trimers, with association of trimers into higher order aggregates. Aggregation raises the Michaelis constants for both substrates and is thought to be of importance in sparing ornithine for use in the urea cycle. The fact that OAT has been estimated as a tetramer by many investigators suggests that this form of OAT may be the most stable or physiologically most favourable oligomer. However, recent studies using X-ray crystallography of OAT suggest that the native enzyme is hexameric (Markovic-Housley et al., 1987) although this was not thought to represent the oligomeric state of OAT in solution.

Volpe et al. (1974) have suggested that rat liver and kidney OAT differ with respect to temperature optimum, rate of heat inactivation and Michaelis constants. However, it is possible that inhibitors were present in the purified preparation. Lyons and Pitot (1976) have shown that rat liver and kidney OAT are immunologically identical, in agreement with Sanada et al. (1970).

Kekomaki et al. (1969) estimated Michaelis constants of 2.0 mM for both substrates in homogenates of human liver. Only one method of human liver OAT purification and characterization has been reported (Ohura et al., 1982). Crystallization involved extensive purification procedures including heat treatment and chromatographies on DEAE-cellulose, Octyl-Sepharose CL-4B and Sephadex G-200. The molecular weight of OAT was estimated at 177,000 for the oligomer and 44,000 for the monomer, indicating tetrameric OAT in its native state. Other parameters such as pH optimum and Michaelis constants were very similar to those reported for the rat enzyme. Comparison of the amino acid composition of rat and human liver OAT also showed similarities. However, the 2 proteins could be distinguished by immunodiffusion analysis.

Other biochemical investigations on human OAT have been reported in connection with clinical studies on Gyrate Atrophy of the choroid and retina (see section 1.6.2).

1.5.5 Metabolic role of OAT

OAT is involved in several different metabolic processes since it links the urea cycle with proline metabolism and the tricarboxylic acid cycle. The functional roles of OAT can be divided into 3 main processes.

1.5.5.1 Ornithine and arginine catabolism

The catabolism of arginine to Δ'-pyrroline-5-carboxylate via ornithine is in balance with the dietary intake of arginine derived from protein catabolism, arginine needed for protein synthesis, ornithine metabolism to putrescine and losses of both arginine and ornithine in urine, stools and skin secretion (summarized below, Valle et al., 1983).

In normal individuals protein accretion is negligible since there is no net change in body protein. Arginine metabolism for protein

production is only significant in patients recovering from protein depletion or in growing children. Furthermore, in the normal state, losses of ornithine and arginine in urine, stools and skin are negligible and only significant in patients with high ornithine levels resulting in hyperornithinuria. Ornithine catabolism to putrescine is also thought to be relatively small, estimated at 0.5 mmoles per day in adults (Mudd and Poole, 1975).

1.5.5.2 Proline synthesis

OAT catalyzes the catabolism of ornithine to Δ'-pyrroline-5-carboxylate, the precursor of proline (see figure 1.1). Alternatively the precursor can be synthesized from glutamate in a reaction catalyzed by Δ'-pyrroline-5-carboxylate synthase. Lodato et al. (1981) have shown that Δ'-pyrroline-5-carboxylate synthase is inhibited at physiological ornithine concentrations. It is therefore likely that at high ornithine concentrations, the predominant pathway for proline synthesis is via the OAT route. This has been shown to be the case in rat mammary gland (Mezl and Knox, 1977; Glass and Knox, 1973) where OAT, Δ'-pyrroline-5-carboxylate reductase and arginase activities increase proportionally in response to hormonal stimulation, and arginine serves as the major precursor of proline synthesis via ornithine. The relative contribution of the Δ'-pyrroline-5-carboxylate synthase pathway is not yet known.

1.5.5.3 Ornithine and arginine synthesis

Whilst the direction of the OAT reaction is usually towards Δ'-pyrroline-5-carboxylate synthesis, the unfavourable reverse reaction provides the only known pathway of de novo ornithine synthesis in man. Ornithine synthesis via acetylated metabolites is known to occur in micro-organisms but not in mammalian tissues (Smith et al., 1967). However, it has been shown by several workers that net ornithine synthesis can occur in mammals

(Meister, 1954; Matsuzawa, 1974; Ross et al., 1978). It is therefore possible that the reverse reaction catalyzed by OAT could assist in replenishing low urea cycle intermediates and synthesis of arginine under conditions of arginine depletion. Moser et al. (1967) have shown that patients with argininosuccinate lyase deficiency can excrete argininosuccinate in excess of the expected output for dietary arginine input and this may be explained by the reverse OAT reaction. The reverse OAT reaction is known to have a lower affinity for glutamate (Matsuzawa et al., 1974); however the precise factors which favour the reverse reaction are unknown.

1.5.6 Intracellular synthesis and processing of OAT

Like nearly all the mitochondrial matrix proteins, OAT is encoded in the nucleus. The monomers of OAT are synthesized on cytoplasmic ribosomes as larger precursor polypeptides (Mueckler, 1982) with a molecular weight of 49,000, mature OAT being estimated at 43,000 in this report. Other matrix proteins have also been shown to be synthesized in the cytoplasm as larger molecular weight precursors, OCT for instance (Conboy et al., 1979). The signal peptide directs the OAT precursor to the mitochondrial matrix, the additional peptide being removed as the precursor enters the mitochondrion. The mature OAT molecule then has to assemble into an oligomer and is then thought to combine with pyridoxal phosphate, since aspartate aminotransferase has been shown to enter the matrix as an apoenzyme (Sharma and Gehring, 1986).

The precursor has been shown to bind to a receptor on the outer mitochondrial membrane, which has been partially purified by Ono and Tuboi (1985). Furthermore, no receptor capable of binding precursor OAT could be found on the inner mitochondrial membrane. It is therefore possible that mature OAT is imported through the inner membrane and could be present in the space between the two membranes.

Studies on the degradation of OAT show that proteases specific for pyridoxal phosphate dependent enzymes may degrade OAT (Kominami and Katunuma, 1976) although contradictory evidence has been reported regarding specificity (Grisolia et al., 1980). Kominami and Katunuma found that OAT activity and OAT protein decreased proportionally and that no inactive pool of OAT existed during degradation.

Cloning of DNA complementary to OAT mRNA in rat liver has been accomplished (Himeno et al., 1982) and the molecular cloning of human OAT mRNA has also been achieved (Inana et al., 1986). Both have shown that levels of mRNA coding for OAT are very low in the cytoplasm. This is supported by Mueckler et al. (1983) who have shown that glucagon induction of rat liver OAT produces a small increase in mRNA with respect to the rate of OAT synthesis. Having produced these clones, investigations into Gyrate Atrophy at the gene level should soon be reported. Simmaco et al. (1986) have used the DNA clone to elucidate the primary structure of the active site of the OAT molecule and located the site of cofactor binding (lys 292).

1.5.7 Tissue studies on OAT

OAT is found in most mammalian tissues but is predominantly found in the kidney, liver and small intestine (Sanada et al., 1970). Lower levels are found in pancreas, adrenal, lung, brain, heart and spleen (Herzfeld and Knox, 1968). Tissues which exhibit a sex difference of OAT abundance are kidney, adrenal, and hepatomas where female OAT levels are about 10-fold higher. OAT activity has been measured by Shih and Schulman (1970) in cells cultured from human skin biopsies and amniotic fluid to investigate a patient with hyperornithinemia (see section 1.6). Immunofluorescent localization of OAT in liver has been reported by Brennan et al. (1970) who found that OAT is randomly distributed throughout the liver tissue. More recently, Kasahara et al. (1986)

have reported immunohistochemical localization of OAT in several rat tissues. Strong immunoreactivity was found in cerebral neurons, hepatocytes, epithelial cells of renal tubuli, gut mucous membranes and ocular tissues (the epithelial cells of the choroid plexus, ciliary body and retina).

Muscle fibres obtained at biopsy from normal human individuals have been reported to contain no OAT activity (Askanas et al., 1980). However, OAT activity has been reported in hair roots, providing a non-invasive method of measuring human OAT levels (Janssen et al., 1981).

1.6 Hyperornithinemia

1.6.1 Introduction

Plasma ornithine concentrations after overnight fasting in normal individuals range from 40-120 μM with a mean value of between 60-80 μM (Valle and Simmell, 1983). Two inborn errors of metabolism can result in significantly elevated plasma ornithine: Gyrate Atrophy of the choroid and retina, and the Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) syndrome. In Gyrate Atrophy patients, plasma ornithine concentrations range from 400-1400 μM by late childhood, but plasma ammonia remains normal. In the HHH syndrome, the plasma ornithine concentration ranges from 380-630 μM. In this disorder, plasma ammonia is elevated, particularly after a protein load. The exact age of onset of hyperornithinemia is not known in either of these diseases. Shih et al., (1969) found normal plasma ornithine in one HHH syndrome patient upon neonatal screening.

Valle and Simell (1983) have described a case of moderate hyperornithinemia originally reported by Bickel et al. (1968) in 2 siblings. Both had mental retardation, kidney and liver abnormalities and a 60-80% reduction in liver OAT activity (possibly due to an idiopathic liver disease). By late childhood both siblings had normal

ocular examinations and normal plasma ornithine. The defect of this syndrome remains unknown.

Associated with hyperornithinemia in these two disease states is the urinary excretion of the δ-lactam of ornithine, 3-aminopiperid-2-one (see section 1.7.3). The δ-lactam causes a faint brown spot with ninhydrin and allows differentiation of inborn hyperornithinemias from others upon initial screening.

Mild increases in plasma ornithine can be caused by isoniazid therapy, a drug which causes increases in γ-aminobutyric acid in the brain (Perry and Hansen, 1978). It is also possible to obtain raised plasma estimations by standing blood at room temperature for lengthy periods, as erythrocyte arginase can convert arginine to ornithine.

1.6.2 Gyrate atrophy of the choroid and retina

1.6.2.1 History of the disease

Gyrate atrophy was first recognized as a distinct disease state and subsequently named by Fuchs in 1896. The genetic aspects of the disorder were first reviewed by Usher (1935). Since then numerous cases and several reviews (Kurstjens, 1965; Takki, 1974; Takki and Simell, 1976; Francois, 1979) have been reported on the clinical aspects of Gyrate Atrophy. The association of elevated plasma and urine ornithine with Gyrate Atrophy was first recognized by Simell and Takki (1973).

1.6.2.2 Genetics

Inheritance of Gyrate Atrophy is autosomal recessive, males and females being equally effected (Takki, 1974). Obligate heterozygotes have partially reduced OAT levels. The incidence of Gyrate Atrophy in Finland has been estimated at 1:50,000 with a heterozygote frequency of 1:110. The nationality of patients (over 91 confirmed cases) has included Finnish, Spanish, Italian, Dutch, English, Welsh and Japanese.

Variability of the clinical severity of Gyrate Atrophy (Kaiser-Kupfer et al., 1980) and the variability of OAT activity suggests that mutations causing Gyrate Atrophy are heterogeneous. The search for genetic evidence of heterogeneity has been attempted (Valle et al., 1979; Shih et al., 1981). Fusion of fibroblasts from two unrelated patients produced heterokaryons which were subsequently tested for restored OAT activity. No complementations were found which suggests that different mutations are occurring on the same structural gene or that all mutations are identical, which is unlikely.

1.6.2.3 Clinical aspects of Gyrate Atrophy

The main clinical feature of Gyrate Atrophy is the gradual loss of vision leading to blindness usually by the fourth decade of life (Takki, 1974; Takki and Simell, 1976; Francois, 1979). The first symptoms to appear before the age of 10 are myopia and decreased night vision. Patients then develop reduced peripheral vision and then subcapsular cataracts. At about puberty, well defined circular areas of chorioretinal degeneration are present in the midperiphery of the ocular fundus. The lesions start as yellow dots which gradually enlarge. By the second decade the degeneration of the retina increases (Francois, 1979). Eventually the lesions fuse together leaving only a few choroid vessels running between atrophic areas and pigmentation increases. Takki (1974) has indicated that the pigment epithelium may be the initial site of damage since this has been found to be abnormal around the periphery of the atrophic areas.

Ultrastructural abnormalities in mitochondria and skeletal muscle fibres have been described in many patients although they are asymptomatic in these respects. Elongation, branching and segmentation of mitochondria in Gyrate Atrophy patients has been described (McCulloch et al., 1978). Almost all patients have structural

abnormalities of the type 2 fibres of skeletal muscle (McCulloch and Marliss, 1975; McCulloch et al., 1978; Sipila et al., 1979; Kennaway et al., 1980; Kaiser-Kupfer et al., 1981) which form narrow tubular aggregates seen under electron microscopy. Furthermore, many patients have abnormal electromyograms with short duration, low amplitude action potentials (Sipila et al., 1979). Electroencephalography shows a moderate slowing in some patients although they are of normal intelligence (Kaiser-Kupfer et al., 1981).

1.6.2.4 Biochemical features of Gyrate Atrophy

Raised ornithine concentrations in plasma, cerebrospinal fluid and aqueous humour together with overflow ornithinuria were the main biochemical abnormalities associated with Gyrate Atrophy which enabled the primary defect to be discovered (Simell and Takki, 1973; Takki and Simell, 1976). Mild decreases in plasma lysine, glutamate and glutamine with slightly depressed cerebrospinal fluid lysine have been reported in these patients (Takki and Simell, 1976; Valle et al., 1980). Urinary excretion of ornithine, its δ-lactam, arginine and lysine are increased and clearance values have been reported (Valle and Simell, 1983; Table 1.1).

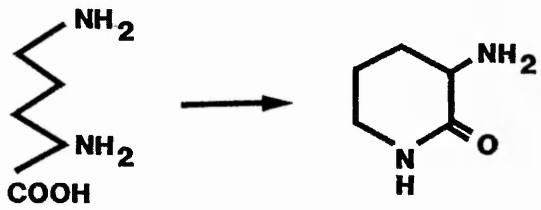
Table 1.1 Urinary excretion of basic amino acids in Gyrate Atrophy patients (Valle and Simell, 1983)

		Excretion (µmoles/day)			
	n	ornithine	arginine	lysine	δ-lactam
PATIENTS	9	3130	180 (50-3800)	1300 (400-2300)	1090 (520-3100)
CONTROLS	14	18 ± 23	10 ± 6	155 ± 114	____

mean (range)
mean ± standard deviation

The δ-lactam of ornithine (figure 1.3) was first detected in the urine of a patient with HHH syndrome but was originally thought to be the methyl ester of ornithine (Gordon et al., 1977). It was subsequently shown that the δ-lactam of ornithine is formed spontaneously from the methyl ester (Oberholzer and Briddon, 1978; Fell and Pollitt, 1978) in both Gyrate Atrophy and HHH Syndrome.

Figure 1.3 Chemical structure of ornithine and its δ-lactam (3-aminopiperid-2-one)



L-ORNITHINE **3-AMINOPIPERID-2-ONE**

Valle and Simell (1983) have suggested that the δ-lactam may be formed in the kidney since plasma levels have been reported as unmeasurable (Valle et al., 1980). The mechanism of formation and role of this compound are unknown.

1.6.2.5 Gyrate Atrophy and OAT deficiency

OAT deficiency has been reported in skin fibroblasts (Trijbels et al., 1977; Kennaway et al., 1977; O'Donnell et al., 1977; O'Donnell et al., 1978; Shih et al., 1978; Kennaway et al., 1980), in phytohaemagglutinin-stimulated lymphocytes (Valle et al., 1977), skeletal muscle primary cultures (Askanas et al., 1980), liver biopsy material (Sipila et al., 1981) and hair roots (Janssen et al., 1981). Out of 32 fibroblast cell lines from Gyrate Atrophy patients, OAT levels have ranged from 0-5.7% of mean control levels (Valle and Simell, 1983).

Sipila et al. (1981) have proposed the existence of a mutant liver enzyme, having measured the residual OAT activity in Gyrate Atrophy liver biopsy homogenates. A Michaelis constant of 200 mM for ornithine was reported (50-fold higher than their estimation in normal homogenates).

No inhibitor of OAT has been described in studies where normal tissues extracts have been mixed with Gyrate Atrophy tissue extracts (Shih et al., 1978; Valle et al., 1977; Sipila et al., 1981). OAT activity of Gyrate Atrophy patients has been shown to increase in the presence of very high concentrations of pyridoxal phosphate in the assay mixture (Kennaway et al., 1980; Shih et al., 1978). The validity of the assay at this cofactor concentration has not been proven however. It has also been shown that most patients respond to pyridoxine administration with a partial reduction of plasma ornithine concentration. Gyrate Atrophy fibroblasts have been shown to be virtually unable to synthesize proline or glutamate.

OAT activity in heterozygotes has been assayed in 13 fibroblast cell lines (O'Donnell et al., 1978; Kennaway et al., 1980; Shih et al., 1978; Valle et al., 1979). OAT activity has ranged from 32 to 61% of normal mean OAT levels.

1.6.2.6 Ornithine metabolism in Gyrate Atrophy

Up to 75% of arginine input in Gyrate Atrophy patients cannot be accounted for, since total excretion of the carbon skeletons of arginine, ornithine, 3-aminopiperid-2-one, citrulline and argininosuccinate is much less than arginine intake (Valle et al., 1980). This could be partly explained by residual OAT activity, gastrointestinal losses, underestimated putrescine synthesis or an unknown alternative pathway. While putrescine can be converted to both other polyamines and carbon dioxide via γ-aminobutyrate, ornithine decarboxylase is thought to be

too highly regulated by cell proliferation to account for significant ornithine catabolism (Williams-Ashman et al., 1973).

Accumulation of ornithine in patients has only been measured in plasma, cerebrospinal fluid and aqueous humour which represents only about 10% of the total ornithine pool (Scriver and Rosenberg, 1973). The major pools of amino acids are located in the intracellular fluid of skeletal muscle and liver. In normal individuals, intracellular muscle ornithine concentration has been estimated at about 5-fold higher than in plasma (Bergstrom et al., 1974). However, the validity of intracellular amino acid pool measurement has recently been questioned (Wheatley et al., 1986). Assuming the estimations are correct, the total body pool of ornithine would be 100-150 mmoles (Valle and Simell, 1983). It would therefore only take a minor redistribution of intracellular ornithine to dramatically alter plasma ornithine concentrations. Redistribution may partly explain the increased clearance of lysine and other metabolites in Gyrate Atrophy patients.

1.6.2.7 Possible biochemical causes of Gyrate Atrophy

OAT activity is abundant in both neural retina tissue and pigment epithelium (Baich and Tarzlaff, 1980; Hayasaka et al., 1980). The retinal pigment epithelium forms the outer cell layer of the retina and nutrients are transported through the epithelium to photo-receptor cells. Valle et al. (1981) showed that pigment epithelium, neuroretina and choriocapillaries were involved in retinal degeneration in a cat with Gyrate Atrophy. It has also been shown that injection of ornithine into the vitreous humour results in degeneration of the pigment epithelium in rats and monkeys (Kuwabara et al., 1981).

A deficiency of creatine and creatine phosphate has been proposed as a cause of chorioretinal atrophy and muscle abnormalities (Sipila,

1980; Sipila et al., 1979). High ornithine concentrations may inhibit glycine transamidinase and thus reduce creatine synthesis. Sensitivity of this enzyme to ornithine has been demonstrated in vitro (Ratner and Rochovansky, 1956; Sipila et al., 1980). Also, serum creatine and creatinine and their urinary clearance are reduced in Gyrate Atrophy patients (Sipila et al., 1980). These reports together with observations of histological improvements in muscle of Gyrate Atrophy patients given creatine (Sipila et al., 1981) support this hypothesis.

Another proposal for the pathophysiology of Gyrate Atrophy is the impaired synthesis of Δ'-pyrroline-5-carboxylate as a result of OAT deficiency, since the inhibition of Δ'-pyrroline-5-carboxylate by ornithine is known to occur at physiological ornithine concentrations (Lodato et al., 1981). The effect of reduced Δ'-pyrroline-5-carboxylate may be detrimental due to decreased proline synthesis or may affect the regulatory role of Δ'-pyrroline-5-carboxylate on the intracellular redox potential and hexose monophosphate shunt (Phang et al., 1979; Yeh et al., 1981). Cells which have access to extracellular proline or possess proline oxidase activity would theoretically be unaffected by Gyrate Atrophy. However, retinal pigment epithelium and neural retinal tissue lack proline oxidase (Matsuzawa and Ishigura, 1980) and it is likely that extracellular proline availability to the retina is poor since proline cannot easily cross the blood-brain barrier (Oldendorf, 1971) and is virtually absent from cerebrospinal fluid (Scriver and Rosenberg, 1973).

1.6.2.8 Treatment of Gyrate Atrophy

Three main therapeutic methods have been undertaken to improve Gyrate Atrophy symptoms. These are pyridoxine therapy to enhance any residual OAT activity, a reduced arginine diet to correct ornithine accumulation or by increasing ornithine excretion, and creatine therapy.

Administration of 500-1000 mg/day pyridoxine hydrochloride has resulted in about a 50% reduction in plasma ornithine in several cases, with plasma lysine returning to normal (Berson et al., 1981; Weleber et al., 1978; Weleber et al., 1981). Weleber et al. (1981) found that the effective dose could be reduced to 15-20 mg/day. Deterioration of vision has been halted in these patients.

Restricting dietary arginine intake has been achieved by reducing protein intake to about 0.2 g/Kg body weight/day and adding essential nutrients (Berson et al., 1981; Valle et al., 1980; Valle et al., 1981). On this diet, ornithine levels have been reduced to normal levels for long periods with improvements in plasma lysine, glutamate and glutamine levels. Whilst the diet is very restrictive, chorioretinal atrophy has been halted.

Administration of lysine (Yatsiv et al., 1978; Valle et al., 1980) or γ-aminoisobutyric acid (Valle et al., 1980; Valle et al., 1981) interferes with dibasic amino acid transport and increases renal loss of ornithine. However, these compounds become ineffective at ornithine concentrations below 300 µM. Long term studies of this treatment have not been reported.

Sipila et al. (1981) have treated a patient with creatine administration of 1.5 g/day for one year which resulted in improvements in muscle abnormalities but failed to completely halt chorioretinal degeneration.

1.6.3 The Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) Syndrome

1.6.3.1 History of the disease

This syndrome was originally reported by Shih et al. (1969) and is characterized by elevated plasma ornithine, postprandial hyperammonemia, and homocitrullinuria (Wright and Pollitt, 1973; Fell et al., 1974; Gatfield et al., 1975) reported

in 5 male and 3 female patients. Gatfield et al. (1975) have reported the syndrome in 6 related individuals which suggests autosomal recessive inheritance.

1.6.3.2 Clinical symptoms

Symptoms appear at childhood, with refusal to eat and periodic vomiting, lethargy and coma when given a high protein load. Growth is retarded and mental retardation has varied from severe to lower normal. Ophthalmologically, all patients have appeared normal except one who developed abnormalities during an acute hyperammonemic period (Gatfield et al., 1975). Mild hepatomegaly has been described in one case and examination of liver mitochondria showed structural abnormalities. Such abnormalities have been reported in mitochondria of cultured fibroblasts of HHH syndrome patients (Metoki et al., 1984).

1.6.3.3 Biochemical studies

On a low protein diet, plasma ornithine ranges from 380-630 μM in these patients, plasma lysine is slightly decreased and plasma glutamate and alanine are slightly elevated. Loading responses to citrulline, lysine and homocitrulline produce no significantly elevated plasma levels of these respective amino acids. Elevated ornithinuria is variable (73-8160 μmoles/g creatinine). Homocitrulline excretion is highly elevated with a mean value reported at 148 ± 119 μmoles/g creatinine (mean ± S.D.) compared to 51 ± 38 in controls.

The δ-lactam of ornithine has been detected in the urine of these patients, ranging from 130-1050 μmoles/g creatinine whereas normal individuals contain very little or no δ-lactam (Oberholzer and Briddon, 1978; Gordon et al., 1977; Fell and Pollitt, 1978). Elevated orotic acid excretion is thought to result from accumulation of

carbamoyl phosphate which appears to be synthesized normally in these patients (Gatfield et al., 1975).

Mitochondrial carbamoyl phosphate synthetase I was found to be 13% of normal values in leukocytes and 20% of normal values in liver biopsy materials, whereas cytoplasmic carbamoyl phosphate synthetase II was elevated (Gatfield et al., 1975). OAT activity is normal, but ornithine decarboxylase activity has been reported as low (20-30% of normal values) in the fibroblasts of one patient (Shih and Mandell, 1974).

1.6.3.4 Possible causes of HHH Syndrome

The importance of decreased carbamoyl phosphate synthetase I activity is unknown, but is unlikely to be the primary cause of the syndrome. Patients with carbamoyl phosphate synthetase I deficiency do not accumulate ornithine or excrete orotic acid and homocitrulline (Valle and Simell, 1983). Similarly, deficiency of N-acetylglutamate, an allosteric activator of carbamoyl phosphate synthetase I has been reported (Bachmann et al., 1981). Hyperammonemia, but no elevated plasma ornithine or homocitrulline excretion, was noted.

Fell et al. (1974) proposed a defect of ornithine transport across the mitochondrial membrane as a possible explanation of this syndrome. Transport of ornithine into mitochondria is mediated by one or possibly two specific carriers, at least one of which may be energy-dependent (Gamble and Lehninger, 1973; McGivan et al., 1977; Bradford and McGivan, 1980). The consequence of reduced ornithine intake into mitochondria would be reduced urea cycle activity, reducing the rate of ammonia detoxification. Since mitochondrial OAT is responsible for the majority of ornithine catabolism, ornithine would accumulate in the cytosol and extracellular fluids. Fell et al. (1974) increased the plasma concentration of ornithine in their patient by adding 6 g/day of

ornithine to the diet. This had the effect of reducing plasma ammonia which supports the transport defect theory since increased cytosolic ornithine would increase the rate of ornithine transport into the mitochondria and increase urea cycle activity. The defective transport theory is supported by reports of defective mitochondrial transport in fibroblast cultures from these patients (Shih et al., 1980; Hommes et al., 1982).

The hypothesis of Fell et al. (1974) suggests that normal lysine uptake increases the ratio of lysine:ornithine in the mitochondrial matrix and results in OCT-catalyzed conversion of lysine to homocitrulline which would account for the rise in homocitrulline clearance in one patient, following a lysine load.

1.6.3.5 Treatment of HHH Syndrome

The addition of ornithine or lysine to the diet has been shown to reduce plasma ammonia with a resultant rise in plasma ornithine. The long term consequences of this diet are unknown and may result in severe ornithine accumulation and symptoms associated with Gyrate Atrophy. A low protein diet (1.2 g/Kg body weight/day) decreases plasma ornithine concentrations and prevents postprandial hyperammonemia (Shih et al., 1969; Wright and Pollitt, 1973; Fell et al., 1974).

1.7 Present studies

Studies undertaken by previous workers have not as yet determined the precise intracellular level at which the metabolic defect of OAT deficiency occurs. Indeed, genetic evidence suggests that this disease may be the result of several different genetic mutations. Ohura et al. (1984) have reported immunoassay of OAT protein in cultured human skin fibroblasts from Gyrate Atrophy patients and have suggested that no inactive form of OAT protein is being produced.

In an attempt to determine the precise intracellular 'location' of the metabolic defect of Gyrate Atrophy this study reports the development of a number of immunological techniques which, combined with OAT activity measurements, may enable the defect to be located. Since the OAT protein may be being produced in an inactive form, an immunoassay (an ELISA) has been developed using polyclonal antibodies raised to OAT from human and rat tissue. Since this assay relies only on the presence of OAT protein and can also detect low levels of OAT (sub-femtomole level) it provides the ideal tool to study an inactive OAT enzyme. Such techniques could enable the following conditions to be demonstrated: the presence of inactive OAT protein, the presence of precursor OAT protein in the absence of mature OAT protein (using Western blotting techniques based upon the assumption that the precursor can be separated from mature OAT and will react with anti-OAT antibodies) or the absence of OAT protein consistent with absence of OAT activity, in other words, a pre-translational defect.

Reports on the biochemical analysis of OAT from rat tissue have indicated that the enzyme subunits undergo self-association with increasing enzyme concentration to produce higher molecular weight aggregates. This study reports further investigations of this phenomenon in order to assess the effect of detergents on this process and to propose a possible physiological role for aggregation based upon the hydrophobicity of OAT and its decreased affinity for ornithine substrate when aggregated.

The latter sections of this study deal with the problems which have been encountered by previous workers when attempting to measure intracellular amino acid levels. This work complements the review of Wheatley *et al.* (1987) which has highlighted the difficulty of amino acid measurement in organelles and has questioned the validity of such measurements reported previously. In this study, the association of

amino acids with cell membranes has been provisionally studied and results suggest that more rigorous investigations may enable levels of 'bound' amino acid pools to be measured although the validity of such measurements remains doubtful.

2 PURIFICATION OF ORNITHINE AMINOTRANSFERASE

2.1 Introduction

The purpose of OAT purification from human and rat tissue was to produce a pure enzyme extract necessary for reliable characterization (since contaminants may affect kinetic data) and to allow future production of polyclonal antibodies which are monospecific for OAT (section 4). Polyclonal antibodies raised to OAT will be used to develop an immunoadsorbent capable of rapid isolation of OAT from crude extracts (section 5) and to develop a competitive ELISA capable of sensitive detection of OAT protein (section 6).

Purification of OAT from rat kidney, a tissue with the highest abundance of this enzyme, has been reported by Sanada et al. (1970). This method appears to be relatively simple compared to that of human liver OAT (Ohura et al., 1982) which includes 3 chromatographic procedures. Other reports of OAT, largely from rat liver tissue, have been reviewed elsewhere (section 1.5.4). In this section, the purification procedures used to obtain pure OAT from rat kidney, rat liver and human liver are described and assessed. Observations made during purification of OAT such as the poor solubility of OAT in water and the binding of human OAT to the hydrophobic interaction resin, Octyl-Sepharose CL-4B are discussed. The property of OAT to self-aggregate with increasing concentration (Morris et al., 1974; Boernke et al., 1981) is used to explain the possible cause of these observations.

The validity of the spectrophotometric assay for OAT described by Herzfeld and Knox, (1968) has been assessed and employed as the standard assay. Other assays have been described (section 1.5.2).

2.2 Materials And Methods

2.2.1 Assays

2.2.1.1 Estimation of protein

For protein determination in crude extracts the method of Lowry et al. (1951) was used, with modifications. The assay mixture contained a final concentration of 0.17 %(w/v) SDS in order to solubilize all proteins for assay. Stock reagent solutions comprised of 4.0 %(w/v) NaOH, 2.0 %(w/v) Na_2CO_3 and 0.02 %(w/v) sodium tartrate (solution A) and 0.5 %(w/v) $Cu_2SO_4.5H_2O$ (solution B) in distilled water. To 100 µl of protein sample was added 100 µl of 2 % (w/v) SDS. Stock solutions were then mixed in a ratio of 12.25 A: 0.25 B and 1.0 ml of this solution added to each solubilized protein sample. Samples were vortex mixed and incubated for 20 minutes at room temperature. To each sample, 100 µl of Folin-Ciocalteau reagent (diluted 1:1 with distilled water) was added. Samples were vortexed and incubated for a further 20 minutes prior to measurement of absorbance at 750 nm. Bovine serum albumin (50 - 750 µg/ml) was used to construct a standard curve (figure 2.1).

Semi-pure protein extracts were measured directly by the method of Warburg and Christian (1941) based on absorbance measurement at 280 nm and 260 nm which corrects for nucleic acid and nucleotide content.

Pure protein was measured at 280 nm having determined the extinction coefficient (E_{1cm}) for the protein of interest.

2.2.1.2 Estimation of OAT activity

Enzyme activity was assayed by the method of Herzfeld and Knox (1968) with modifications. The assay is based on reaction of the enzyme product, Δ'-pyrroline-5-carboxylate with a chromogen, o-aminobenzaldehyde producing an orange dihydroquinazolinium compound (Vogel and Davis, 1953).

The incubation mixture, which was made up to a total volume of 2.0 ml with distilled water, contained the following components: 0.5 ml of 0.2 M potassium phosphate (pH 8.0); 0.2 ml of 350 mM L-ornithine; 0.2 ml of 50 mM 2-oxoglutaric acid; 0.1 ml of 1.1 mM pyridoxal phosphate; 0.1 ml of 0.1 mM o-aminobenzaldehyde (dissolved in 40 %(w/v) ethanol) and 0.02 - 0.4 ml sample.

Reagents were vortex mixed and pre-incubated at 37°C prior to initiating the reaction by addition of sample and mixing. The reaction was stopped after 20 minutes by addition of 1.0 ml of 10 %(w/v) trichloroacetic acid and vortex mixing. Samples were assayed in duplicate and control tubes not containing 2-oxoglutarate included in each case. Tubes were left at room temperature for 20 minutes to allow complete colour development prior to removal of precipitated protein by centrifugation at 1000 x g for 10 minutes. The absorbance of the supernatant was measured at 440 nm. A molar extinction coefficient of 2.71×10^3 was used to calculate the amount of product, Δ'-pyrroline-5-carboxylate, produced.

One unit of enzyme activity was defined as one μmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C unless otherwise stated in the text.

2.2.1.3 Estimation of ammonium sulphate

Ammonium sulphate concentration was determined using Nessler reagent by the method of Vogel (1961). The reagent (an alkaline solution of mercuric iodide in potassium iodide), when added to a dilute ammonium salt solution, liberates ammonia which reacts to form an orange-brown product. The reaction may be represented as follows:

$$2K_2(HgI_4) + 2NH_3 \longrightarrow NH_2Hg_2I_3 + 4KI + NH_4I$$

The reagent was prepared as follows: 10 g of mercuric iodide and 7 g of potassium iodide were dissolved in 10 ml of distilled water. A cooled solution of 16 g of sodium hydroxide dissolved in 70 ml of distilled water was added to the first solution. The mixture was stirred and diluted to 100 ml. The reagent was allowed to stand for 72 hours to allow any precipitate to settle.

For assay, 10 ml of diluted sample was vortex mixed with 0.2 ml of Nessler reagent. After incubation for 10 minutes absorbance was read at 410 nm against a blank containing 10 ml of distilled water and 0.2 ml of reagent. A calibration curve (figure 2.2) was constructed using ammonium sulphate standards (2 - 20 mg/1).

The assay was used to determine % saturation of ammonium sulphate in protein extracts and subsequently to adjust values using the following formula:

$$\text{Ammonium sulphate concentration (g/1)} = \frac{533\ (S_2 - S_1)}{100 - 0.3\ S_2}$$

where S_1 = Initial % saturation

S_2 = Final % saturation

Interference to the assay by sample protein was negligible due to large sample dilution factors prior to assay. Nessler reagent was also used qualitatively to ensure complete dialysis of extracts.

2.2.2 Purification of rat OAT from liver and kidney

Purification of rat OAT from both sources was based on the method of Sanada et al. (1970). Female Sprague-Dawley rats weighing 200-250 g were killed by cervical dislocation. Livers and kidneys were removed, trimmed of fat, washed in saline and frozen at -20°C prior to purification. Respective tissues were thawed, minced and homogenized in 50 mg/1 pyridoxal phosphate using a Waring blender at top speed for 30 seconds to produce a 20 %(w/v) homogenate. All steps were performed below 4°C.

The homogenate was sonicated at 10 Kilocycles per second for 2 minutes using an M.S.E. Soniprep 150 sonicator in aliquots of 30 ml to solubilize mitochondrial OAT. The sonicated extract was centrifuged at 18,000 x g for 20 minutes using an M.S.E. 21 centrifuge. The pellet was discarded and the supernatant adjusted to pH 6.0 after the addition of potassium phosphate and 2-oxoglutarate to produce final concentrations of 50 mM and 5 mM respectively.

The solution was heated to 60^{o}C in a 75^{o}C water bath in 15 ml aliquots with constant stirring. The aliquots were rapidly cooled in ice immediately upon reaching 60^{o}C. Heat denatured protein was removed by centrifugation at 12,000 x g for 15 minutes.

Solid ammonium sulphate was added slowly to the supernatant to produce 50% saturation (31.3 g/100 ml) with constant stirring. After 20 minutes the mixture was centrifuged at 12,000 x g for 15 minutes and the supernatant discarded.

The pellet was resuspended in 0.2 M potassium phosphate buffer (pH 8.0) using 180 µl per g of original tissue. The suspension was centrifuged at 12,000 x g for 10 minutes leaving the enzyme in the pellet comprising of a translucent yellow band contaminated with a small amount of grey material. This pellet was resuspended in distilled water using 55 μl per g of original tissue. This suspension was centrifuged at 100,000 x g for 30 minutes using a Beckman ultracentrifuge. The yellow supernatant contained the rat kidney enzyme in a virtually pure form.

The rat liver enzyme required additional purification by gel filtration using Sephadex 'Superfine' G-200 (Pharmacia Ltd.). A column (1.6 cm x 85 cm) was packed and equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 2 mM 2-oxoglutarate. The sample was concentrated by collodion bag (Sartorius Instruments) and applied

to the column. Fractions were collected in 2.5 ml aliquots eluted at a flow rate of 10 ml per hour. Active fractions were pooled.

The purified OAT from both rat kidney and liver respectively were concentrated by collodion bag prior to dialysis against distilled water. The two OAT preparations were crystallized by the method of Matsuzawa et al. (1968). Solid ammonium sulphate was added to the concentrates with constant stirring until a faint turbidity was observed. The mixtures were left at $0^{o}C$ for 48 hours, after which the precipitates were collected by centrifugation at 12,000 x g for 10 minutes. The pellet was resuspended in distilled water and dialyzed against 3 x 5000 ml of distilled water before being freeze-dried for long term storage or resuspended in 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and kept at $0^{o}C$.

2.2.3 Purification of human OAT from liver

Human liver OAT purification was based on the method of Ohura et al. (1982).

2.2.3.1 Materials

Human liver was obtained at autopsy, minced and frozen at -20°C in 200 g portions. DEAE-cellulose was obtained from Sigma Chemical Co. Octyl-Sepharose CL-4B and Sephadex 'Superfine' G-200 were obtained from Pharmacia Ltd. PM-10 ultrafiltration membranes were obtained from Amicon.

2.2.3.2 Methods

Human liver was processed in 200 g portions and final extracts pooled prior to DEAE-cellulose chromatography. All steps were carried out at or below 4°C. Minced tissue was thawed and homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 5 mM 2-oxoglutarate and 20 µM pyridoxal phosphate. A 20 %(w/v) homogenate was produced using a Waring blender at top speed for 30 seconds.

The homogenate was centrifuged at 12,000 x g for 20 minutes using an M.S.E. 21 centrifuge. The recovered supernatant was heated to 60°C in a 75°C water bath in aliquots of 30 ml and immediately cooled in ice. The heat treated extract was allowed to cool for 20 minutes and then centrifuged at 12,000 x g for 10 minutes to remove precipitated protein.

Solid ammonium sulphate was added slowly to the recovered supernatant to produce 50% saturation (31.3 g/100 ml) with constant stirring. After 20 minutes the mixture was centrifuged at 12,000 x g for 15 minutes and the supernatant discarded.

The pellets were frozen at -20°C until all liver tissue was processed. Pellets were thawed, dissolved in the minimum of 50 mM potassium phosphate buffer (pH 8.0) containing 2 mM 2-oxoglutarate and

pooled prior to dialysis against 3 x 5000 ml of distilled water. All chromatographic procedures were carried out in a cold room at 4°C.

The dialysate was applied to a DEAE-cellulose column (5.3 cm x 20 cm) equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 2 mM 2-oxoglutarate. This buffer was used to wash off unbound protein at a flow rate of 80 ml per hour and fractions were collected in 10 ml aliquots. Once the absorbance at 280 nm of collected fractions had decreased to the initial value, bound protein (which included OAT) was eluted from the column using a linear gradient of 0 - 0.5 M sodium chloride contained in the column buffer. The salt gradient was formed using a two cylinder gradient former in a total elution volume of 500 ml. The gradient was estimated by titration of fractions against silver nitrate.

Fractions containing OAT activity were pooled and concentrated by ammonium sulphate precipitation at 50% saturation (31.3 g/100 ml) and the pellet recovered after centrifugation at 12,000 x g for 10 minutes. The pellet was dissolved in the minimum of 50 mM potassium phosphate buffer (pH 8.0) containing the 2 mM 2-oxoglutarate. The ammonium sulphate concentration of the solution was adjusted to 25% saturation (14.4 g/100 ml) by addition of solid ammonium sulphate using Nessler reagent (see section 2.3.1.3) to determine the initial % saturation in the resuspended pellet.

The solution was applied to a column of Octyl-Sepharose CL-4B (2.6 cm x 10 cm) equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 25% saturation ammonium sulphate and 2 mM 2-oxoglutarate. Unbound protein was washed off the column at a flow rate of 20 ml per hour and collected in 4 ml fractions. When the absorbance at 280 nm of the eluted fractions reached the initial value, a second batch of protein was eluted from the column using the same buffer containing 15% saturation ammonium sulphate. Protein which had OAT activity was

eluted from the column with buffer containing 10% saturation ammonium sulphate.

Fractions containing OAT activity were pooled and concentrated on a PM-10 ultrafiltration membrane. The concentrate was applied to a Sephadex G-200 column (1.6 cm x 82 cm) equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 0.15 M sodium chloride and 2 mM 2-oxoglutarate. Fractions were collected at a flow rate of 9 ml per hour in 3.0 ml aliquots.

The peak fraction with the highest specific activity was dialyzed against 3 x 5000 ml of distilled water prior to lyophilization. This fraction contained pure OAT and was used for enzyme characterization (section 3) and antibody production (section 4).

The remaining fractions containing OAT activity were pooled and concentrated on a PM-10 membrane and re-applied to the Sephadex column. The eluted fractions containing the pure enzyme were dialyzed and lyophilized as described previously.

The purification procedure is summarized in figure 2.3.

2.3 RESULTS

2.3.1 The OAT assay

The OAT assay produced a linear reaction rate over the incubation period of 20 minutes (figure 2.4a). Beyond 30 minutes the production of product began to slow. A linear time course for the reaction was obtained for both crude homogenates and pure OAT preparations. Controls without 2-oxoglutarate produced absorbances of about twice the values of controls without L-ornithine, 0.9 and 0.4 absorbance units respectively (figure 2.4b), after a 20 minute incubation. This difference was not observed with pure OAT preparations, absorbances being reduced to those obtained with the 'no ornithine' controls.

2.3.2 Purification of rat kidney and liver OAT

A purification factor of 1029 was required to purify OAT from rat kidney (Table 2.1). The crude homogenate had a specific activity of 0.034 units/mg protein compared to 35 units/mg protein obtained for the pure OAT.

Purification of OAT from rat liver (Table 2.2) required an additional purification step to the method used for rat kidney, Sephadex G-200 chromatography (figure 2.5). This step achieved a 2.6-fold purification of the extract. Most of the contaminants present at this stage eluted after OAT protein. A purification factor of 2078 was required for purification, about twice that needed for rat kidney OAT. This was consistent with the specific activity of the rat liver homogenate estimated at 0.016 units/mg protein (about 50% of rat kidney). A specific activity of 34.5 units/mg protein was obtained for the purified liver OAT.

2.3.3 Purification of human liver OAT

A purification factor of 4357 was required to purify OAT from human liver (Table 2.3). The

specific activity (0.00787 units/mg protein) of the human liver homogenate was 4.3 times lower than that of rat kidney. Ion-exchange chromatography of human liver extract using DEAE-cellulose, a weak anion exchanger (figure 2.6), achieved a purification factor of 6.24. OAT bound to the exchanger allowing a large quantity of contaminating protein to be eluted. The protein peak containing OAT activity was eluted with a salt gradient at between 0.1 M and 0.25 M sodium chloride.

Hydrophobic interaction chromatography was then performed using Octyl-Sepharose CL-4B (figure 2.7). Elution of a large peak of unbound protein occurred in the presence of 25% saturation ammonium sulphate which increases hydrophobic interactions. A second peak of protein which bound to the resin was eluted by lowering the ammonium sulphate saturation to 15%. Finally, a third peak which contained OAT activity was eluted by lowering the ammonium sulphate saturation to 10%. This step was most effective, producing a purification factor of 13.5.

A third chromatographic step, Sephadex G-200 chromatography, was used to obtain a peak fraction of pure OAT (figure 2.8). This fraction, which contained 14.5% of the total activity in the homogenate, had a specific activity of 34.3 units/mg protein, comparable to that of pure rat OAT. Other fractions containing OAT activity were pooled, concentrated and re-applied to the column to produce a second batch of pure OAT increasing the yield by 8% to a total of 22.5%.

The purity of OAT obtained from rat and human sources was assessed by SDS-PAGE (see section 3.3.1).

TABLE 2.1

PURIFICATION OF RAT KIDNEY OAT[a]

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	% YIELD	SPECIFIC ACTIVITY (units/mg)	PURIFICATION FACTOR
HOMOGENATE	660	29,436	100	0.034	1
SONIC EXTRACT	586	18,465	85.2	0.046	1.35
60°C SUPERNATANT	560	4690	79.8	0.168	4.94
FINAL SUPERNATANT	7.5	47	46.8	9.85	290
COLLODION BAG CONCENTRATE	3.0	42.6	45	10.45	307
CRYSTALLIZED OAT:DIALYSATE	3.5	11	39	35	1029

[a]134g rat kidney tissue.

1 unit = 1 μmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

TABLE 2.2

PURIFICATION OF RAT LIVER OAT[a]

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	% YIELD	SPECIFIC ACTIVITY (units/mg)	PURIFICATION FACTOR
HOMOGENATE	1070	71,690	100	0.0165	1
SONIC EXTRACT	895	38,709	81	0.0248	1.5
60^{o} SUPERNATANT	700	14,210	62	0.052	3.12
FINAL SUPERNATANT	10.3	72.1	35.8	5.82	356
COLLODION BAG CONCENTRATE	3.2	68	34.2	5.95	361
SEPHADEX G-200 CONCENTRATE	2.0	21.8	28.7	15.58	944
CRYSTALLIZED OAT:DIALYSATE	3.2	8.38	24.3	34.3	2078

[a]192g rat liver tissue.

1 unit = 1 µmole of Δ'-pyrroline-5-carboxylate formed per minute at 37^{o}C.

TABLE 2.3

PURIFICATION OF HUMAN LIVER OAT[a]

FRACTION	VOLUME (ml)	TOTAL PROTEIN (g)	% YIELD	SPECIFIC ACTIVITY (units/mg)	PURIFICATION FACTOR
HOMOGENATE	4630	125.93	100	0.00787	1
60°C SUPERNATANT	3615	70.85	99	0.0139	1.76
AMMONIUM SULPHATE[b]	180	14.3	96.5	0.066	8.5
DEAE-CELLULOSE[c]	220	1.82	76	0.414	53
OCTYL-SEPHAROSE[c]	28	0.12	68	5.6	714
G-200 SEPHADEX[d]	3	0.0042	14.5	34.3	4357
G-200 SEPHADEX[e]	4	0.0023	8	34.1	4333

[a] 954g of human liver

[b] 50% saturation, resuspended pellet

[c] pooled fractions

[d] peak fraction

[e] values obtained after repeated chromatography (see text)

1 unit = 1 µmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

FIGURE 2.1 Standard curve for the Lowry protein assay

Typical protein standard curve obtained for bovine serum albumin by the method of Lowry et al. (1951).

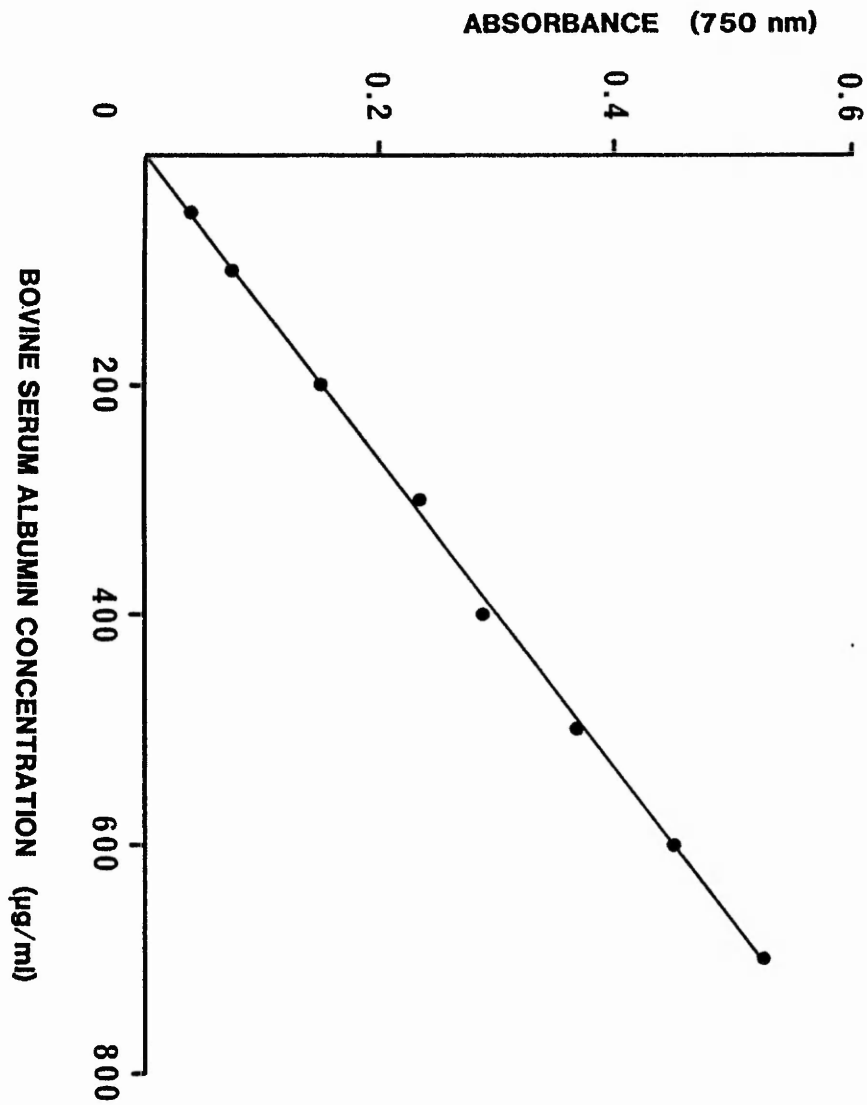

ABSORBANCE (750 nm)
0.2
0.4
0.6
0
200
400
600
800
BOVINE SERUM ALBUMIN CONCENTRATION (µg/ml)

FIGURE 2.2 Standard curve for the Nessler ammonium sulphate assay

Typical standard curve obtained for ammonium sulphate estimations by the Nessler assay (Vogel, 1961).

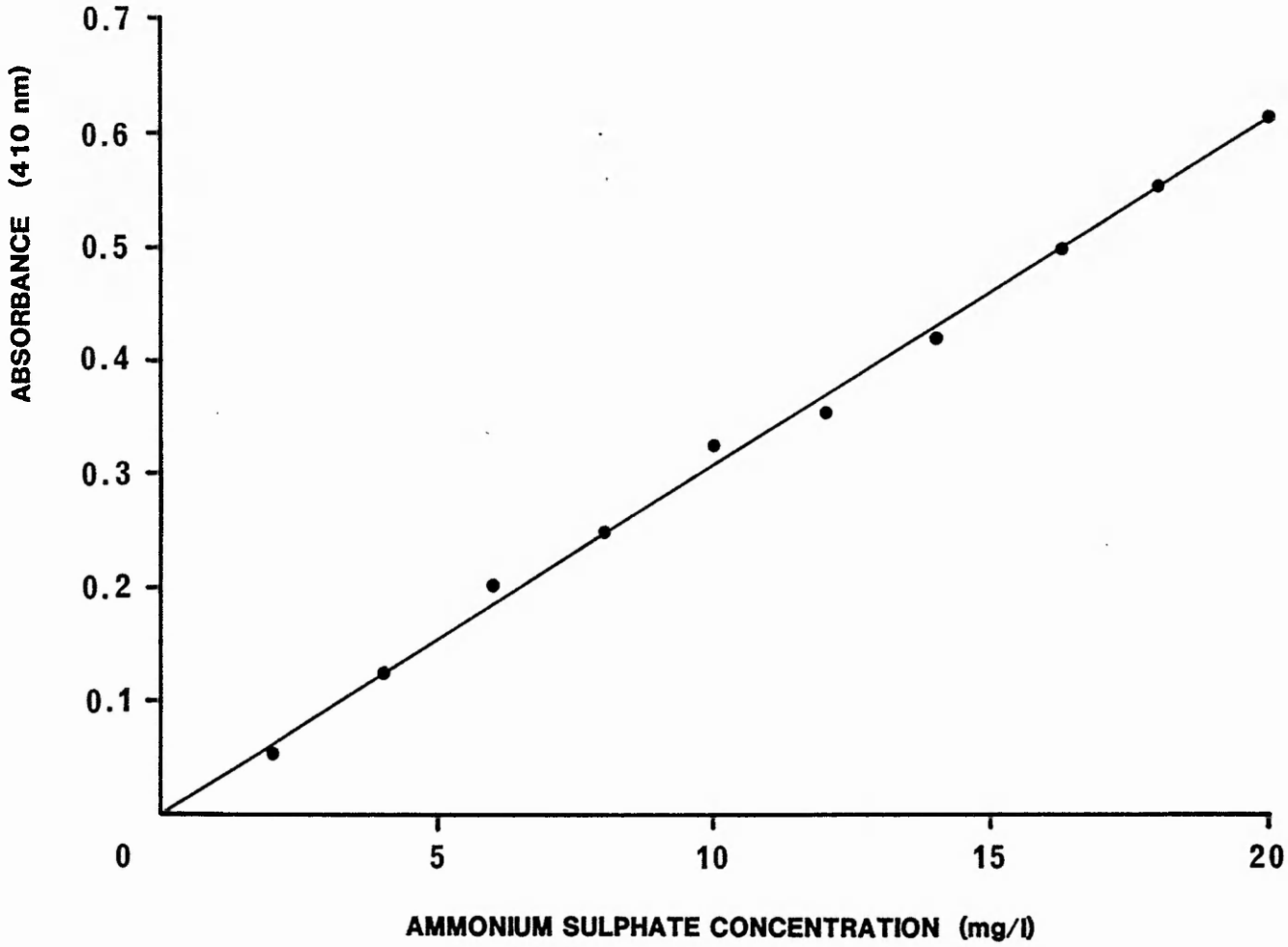

ABSORBANCE (410 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
AMMONIUM SULPHATE CONCENTRATION (mg/l)

FIGURE 2.3 Purification chart for human liver OAT

Purification chart summarizing the protocol used for OAT purified from human liver based upon the method of Ohura et al. (1982).

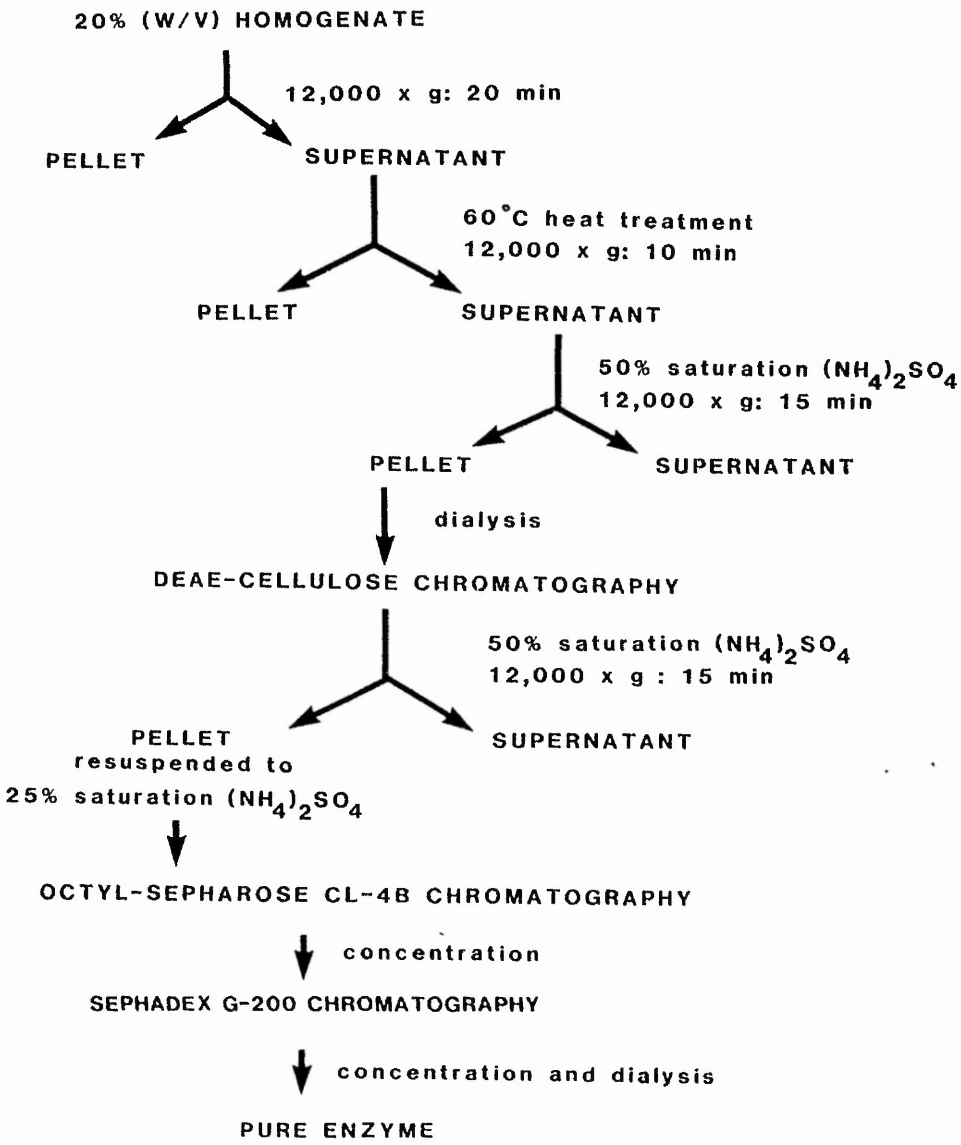

20% (W/V) HOMOGENATE
12,000 x g: 20 min
PELLET
SUPERNATANT
60°C heat treatment
12,000 x g: 10 min
PELLET
SUPERNATANT
50% saturation $(NH_4)_2SO_4$
12,000 x g: 15 min
PELLET
SUPERNATANT
dialysis
DEAE-CELLULOSE CHROMATOGRAPHY
50% saturation $(NH_4)_2SO_4$
12,000 x g : 15 min
PELLET
resuspended to
25% saturation $(NH_4)_2SO_4$
SUPERNATANT
OCTYL-SEPHAROSE CL-4B CHROMATOGRAPHY
concentration
SEPHADEX G-200 CHROMATOGRAPHY
concentration and dialysis
PURE ENZYME

FIGURE 2.4 Assessment of the OAT assay

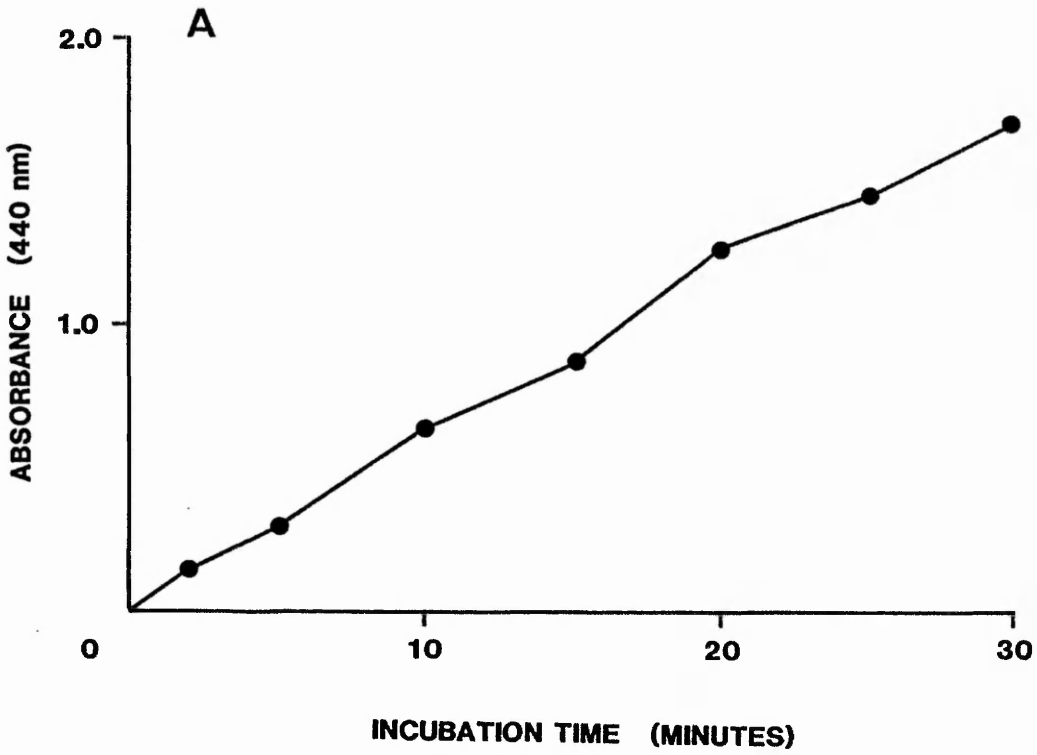
A. Time course for the OAT assay (section 2.3.1.2) demonstrating linearity of reaction rate over the standard incubation time (20 minutes) and a slowing of the rate of product formation after 30 minutes.

B. Product formation in control tubes:

Without 2-oxoglutarate ●—●

Without L-ornithine ▲—▲

The assay mixture contained 0.1 ml of 20 %(w/v) rat kidney homogenate in 50 mM potassium phosphate buffer (pH 8.0). Absorbances are the mean of duplicate assays.


A
2.0
1.0
ABSORBANCE (440 nm)
0
10
20
30
INCUBATION TIME (MINUTES)

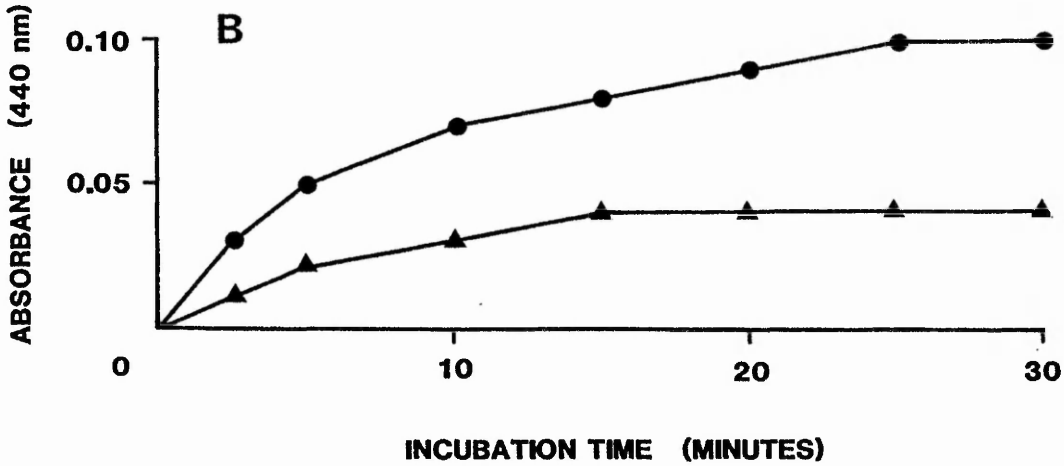

B
0.10
0.05
ABSORBANCE (440 nm)
0
10
20
30
INCUBATION TIME (MINUTES)

FIGURE 2.5 Sephadex G-200 chromatography of rat liver extract

Protein elution was monitored at 280 nm and OAT activity expressed as follows:

1 unit = 1 µmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

Protein: •—•

OAT activity: ●—●

Flow rate: 10 ml/hour

Column dimensions: 1.6 cm x 85 cm

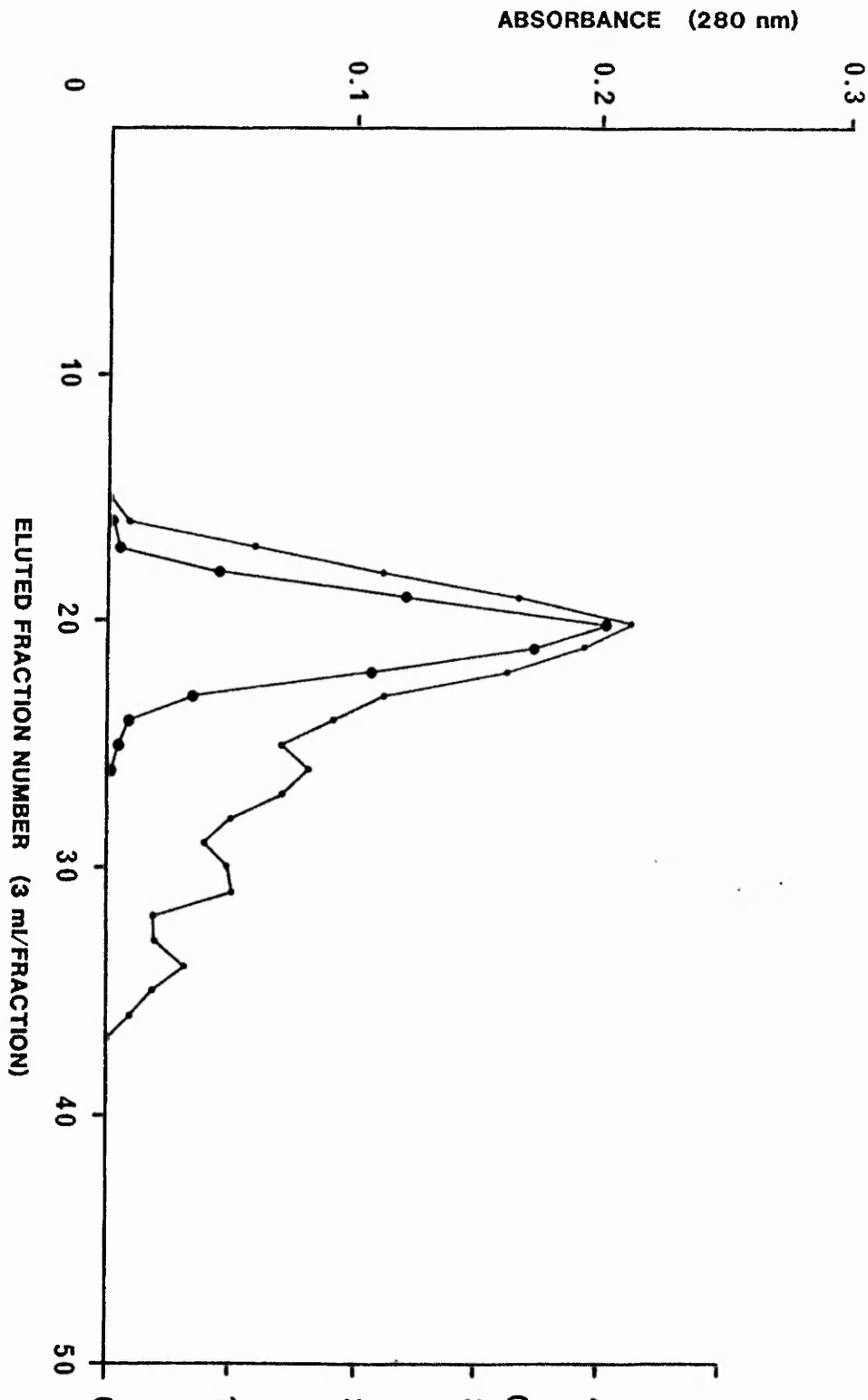

ABSORBANCE (280 nm)
0
0.1
0.2
0.3
ELUTED FRACTION NUMBER (3 ml/FRACTION)
10
20
30
40
50
ACTIVITY (UNITS/FRACTION)
0
100
200
300
400
500

FIGURE 2.6 DEAE-Cellulose chromatography of human liver extract

Protein elution was monitored at 280 nm and OAT activity expressed as follows:

1 unit = 1 µmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

Sodium chloride concentration was estimated by titration against silver nitrate.

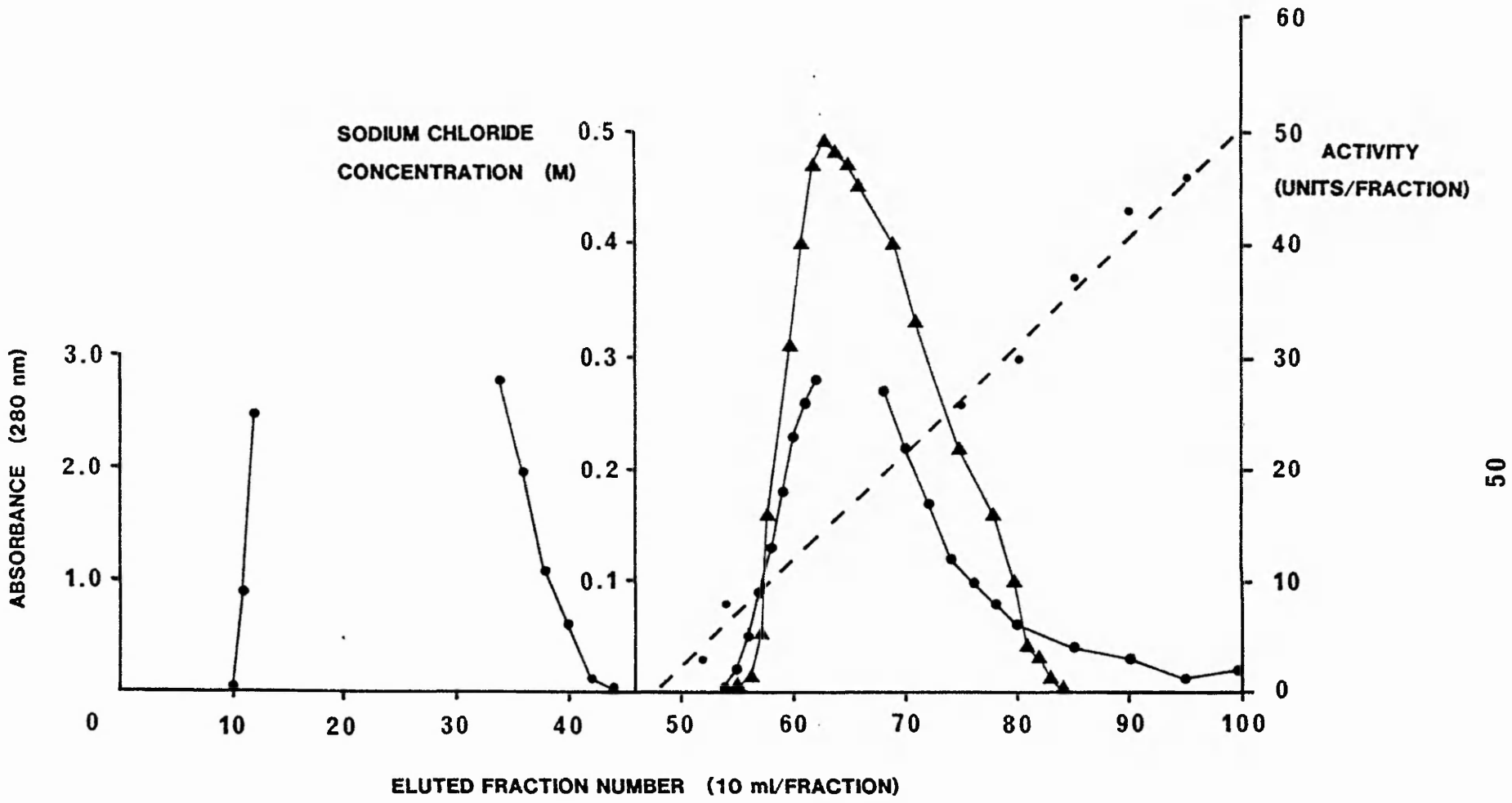
Protein: ●—●

Sodium Chloride: •—•

OAT activity: ▲—▲

Flow rate: 80 ml/hour

Column dimensions: 5.3 cm x 20 cm


SODIUM CHLORIDE
CONCENTRATION (M)
ACTIVITY
(UNITS/FRACTION)
ABSORBANCE (280 nm)
ELUTED FRACTION NUMBER (10 ml/FRACTION)
0.5
0.4
0.3
0.2
0.1
60
50
40
30
20
10
0
3.0
2.0
1.0
0
10
20
30
40
50
60
70
80
90
100

50

FIGURE 2.7 Octyl-Sepharose CL-4B chromatography of human liver extract

Protein elution was monitored at 280 nm and OAT activity expressed as follows:

1 unit = 1 μmole of Δ'-pyrroline-5-carboxylate formed per minute at $37^{o}C$.

Arrows indicate the point of buffer changeover initiated at the top of the column.

Protein: •—•

OAT activity: ●—●

Flow rate: 20 ml/hour

Column dimensions: 2.6 cm x 10 cm

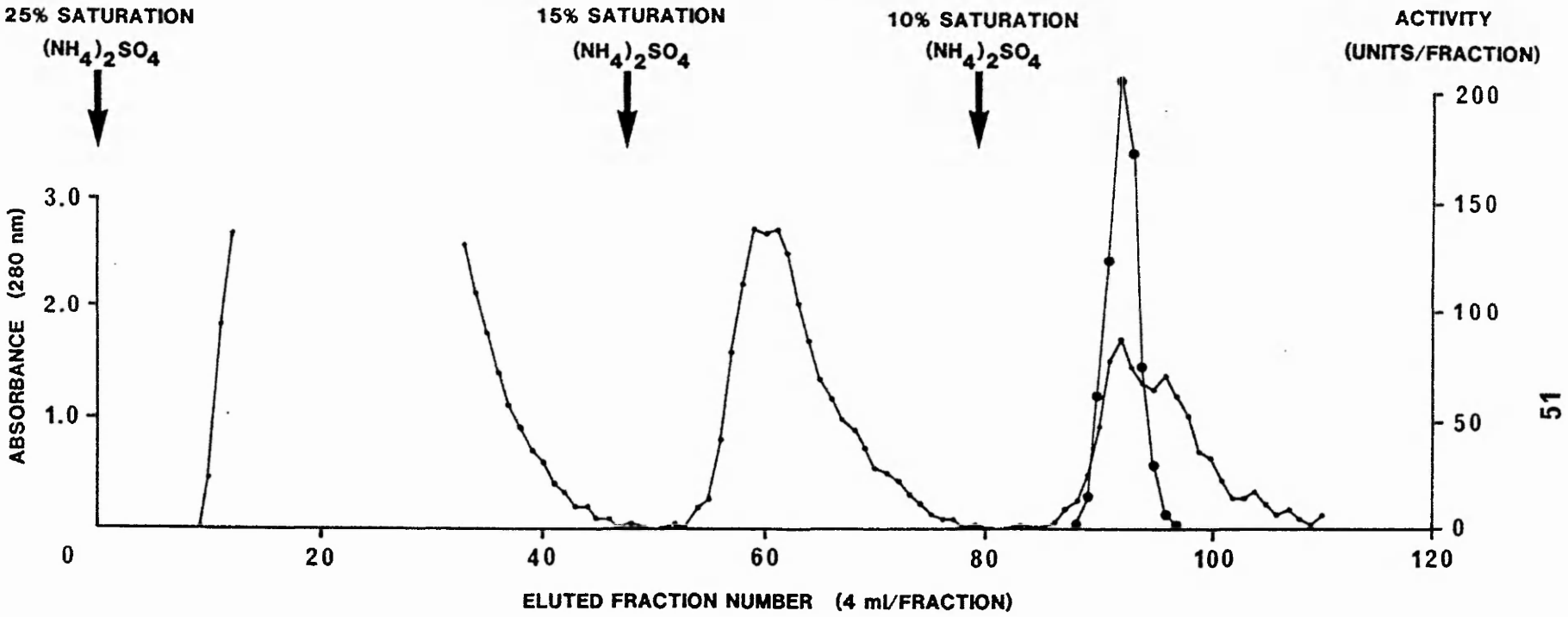

25% SATURATION
$(NH_4)_2SO_4$
15% SATURATION
$(NH_4)_2SO_4$
10% SATURATION
$(NH_4)_2SO_4$
ACTIVITY
(UNITS/FRACTION)
ABSORBANCE (280 nm)
3.0
2.0
1.0
0
20
40
60
80
100
120
200
150
100
50
0
ELUTED FRACTION NUMBER (4 ml/FRACTION)

FIGURE 2.8 Sephadex G-200 chromatography of human liver extract

Protein elution was monitored at 280 nm and OAT activity expressed as follows:

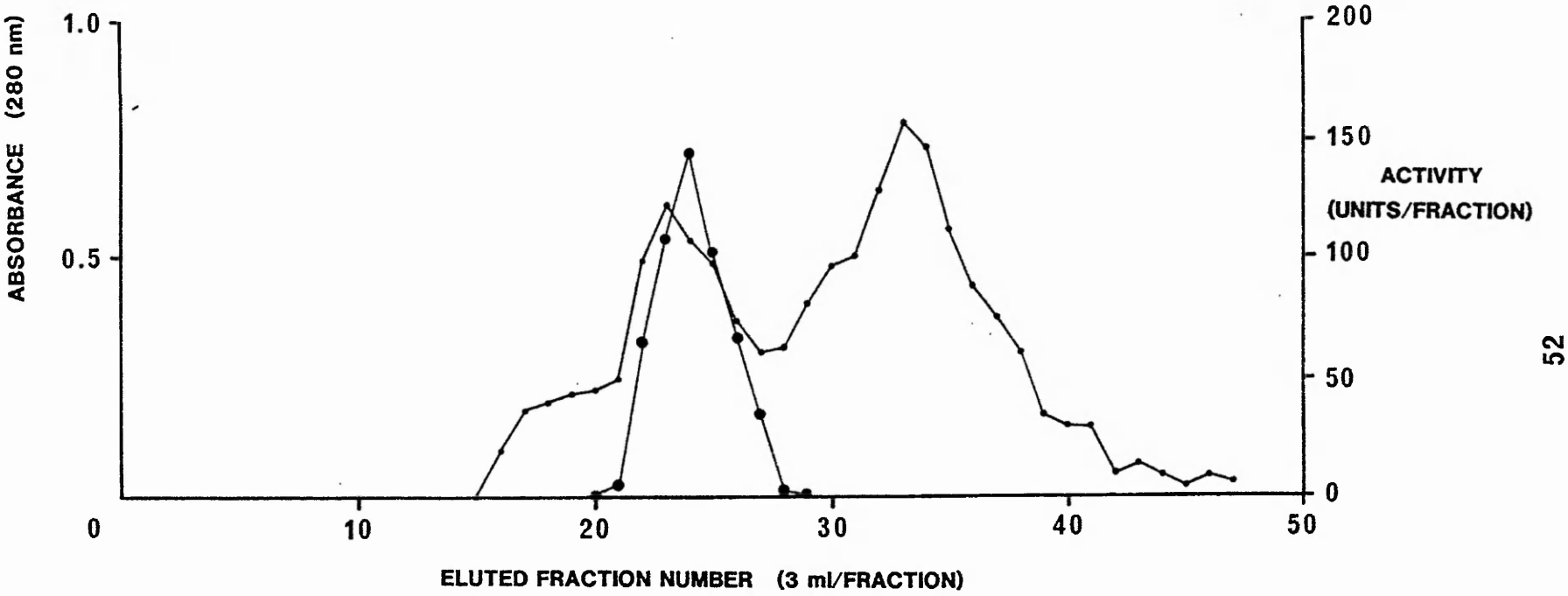
1 unit = 1 μmole Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

Protein: •—•

OAT activity: ●—●

Flow rate: 9 ml/hour

Column dimensions: 1.6 cm x 82 cm


ABSORBANCE (280 nm)
1.0
0.5
0
10
20
30
40
50
200
150
100
50
0
ACTIVITY
(UNITS/FRACTION)
ELUTED FRACTION NUMBER (3 ml/FRACTION)

2.4 Discussion

2.4.1 Assessment of the OAT assay

The spectrophotometric method of OAT assay has been shown to be valid since a linear reaction rate was obtained over the 20 minute incubation period, in agreement with Herzfeld and Knox (1968). A control containing no 2-oxoglutarate was found to be the most reliable since there may be endogenous keto acids present in tissue extracts which probably accounts for the increased absorbance of this control compared to the 'no-ornithine' control. This observation is supported by the fact that pure OAT samples produce lower control absorbances similar to 'no-ornithine' controls. Furthermore, since 2-oxoglutarate is present in the buffers used in OAT purification, this control acts as an indicator as to whether the sample has been sufficiently diluted or dialyzed prior to assay, to produce a valid measurement. The chromogen, o-aminobenzaldehyde has been shown to react with other pyrroline carboxylate compounds (see section 1.5.2), but the controls should take account of this. The low control absorbances indicate that negligible levels of endogenous pyrroline carboxylate compounds are present in tissue extracts assayed in this study.

The pH of the incubation mixture was adjusted from 7.4, originally used by Herzfeld and Knox (1968) to pH 8.0, the optimum for OAT activity (see section 3.3.5).

2.4.2 OAT purification

Initial attempts at purification of OAT from rat liver using the method described by Peraino et al. (1969) proved unsuccessful. They reported sedimentation of homogenate particles after centrifugation at 1200 r.p.m. for 10 minutes which contained the majority of the OAT activity. In contrast to this, it was found that most of the activity remained in the supernatant, as

would be expected since this would contain the mitochondrial fraction. Furthermore, the precipitation of OAT with 50% saturation ammonium sulphate in the presence of Tris. buffer (pH 8.0) repeatedly resulted in about 40% of the total OAT activity remaining in the supernatant, whereas complete precipitation was achieved using phosphate buffer (pH 8.0).

The method of rat kidney OAT purification (Sanada et al., 1970) was successfully adopted. This method has proved to be a simple and effective means of OAT purification from this tissue. The most effective purification step in this method is thought to rely upon the poor solubility of OAT in water, a property first exploited by Matsuzawa et al., (1968) and Peraino et al., (1969) as a purification step. However, OAT is known to aggregate into higher order oligomers when concentrated (Morris et al., 1974; Boernke et al., 1981) and this may account for the poor solubility after precipitation of OAT by ammonium sulphate in phosphate buffer (pH 8.0). A final resuspension of OAT into distilled water, however, dissolves the protein which would appear to contradict the poor water solubility property of OAT. No work has been reported regarding the dissociation of aggregated OAT and it may be that OAT dissociates into lower order oligomers or even monomers which are readily soluble in an aqueous environment. In any case, the resuspension steps of this purification method results in a purification factor of 59 and is thus highly effective.

The method of Sanada et al., (1970) was also successfully used to purify rat liver OAT. The purification factor (114) for the resuspension step was even more dramatic indicating that OAT aggregation may occur just as readily in the presence of contaminating proteins as in a highly purified state.

Human liver OAT required extensive purification due to the problem of additional protein contaminants in human liver tissues and the

relatively low abundance of OAT compared to rat kidney tissue. Furthermore, a large amount of tissue (954g) had to be processed in batches of about 200g until a manageable volume of extract could be obtained, necessitating storage of semi-pure OAT. Fortunately, an early step of OAT purification from both rat and human tissues, is a 60^{o}C heat treatment which is likely to inactivate proteolytic enzymes which could destroy OAT activity. Additional problems are associated with successive chromatographic steps such as repeated overnight dialysis and concentration of OAT extract. The Nessler assay (see section 2.3.1.3) has proved useful as a qualitative check for complete dialysis of ammonium sulphate precipitated samples.

The most effective purification step involved hydrophobic interaction chromatography. Human liver OAT bound to Octyl-Sepharose CL-4B quite tightly since no active OAT protein was leached off the resin and remained bound to the resin when a batch of bound protein was eluted with buffer containing 15% saturation ammonium sulphate (see figure 2.7). This suggests that OAT is unusually hydrophobic for a mitochondrial matrix enzyme. Again, this property might be explained by the aggregation phenomenon of OAT. In an aggregated form, OAT may exhibit a more hydrophobic nature than the monomeric form. Coincidentally, attempts to bind pure rat kidney OAT to Octyl-Sepharose CL-4B were unsuccessful, under conditions identical to the human OAT procedure. However, at the concentration of OAT used in these experiments (4 mg/ml), it is likely that OAT would be in a monomeric form as reported previously (Morris et al., 1974). In this form, OAT may not be sufficiently hydrophobic to bind to the resin. Alternatively, rat OAT may be less hydrophobic than human OAT and this needs to be checked by ascertaining whether aggregated OAT (at a concentration above 10 mg/ml) binds to Octyl-Sepharose CL-4B as strongly as the human OAT extract.

The presence of increasing concentrations of ammonium sulphate increases the hydrophobicity of many proteins which do not exhibit the property of self-aggregation. This is the basis of 'salting-out' procedures used for ammonium sulphate precipitation and for promoting hydrophobic interaction with Octyl-Sepharose CL-4B.

In conclusion, purification of OAT from human liver is time consuming and complex compared to purification of rat kidney OAT. Hence, it would be advantageous if rat kidney OAT could be used to develop techniques applicable to analysis of human tissues. The development of a faster, less complex method of human OAT purification would also be useful since only one method has been reported (Ohura et al., 1982).

3 CHARACTERIZATION OF ORNITHINE AMINOTRANSFERASE

3.1 Introduction

The characterization of OAT has been performed in this section in order to compare human and rat OAT biochemically, to determine the effect of heat and pH upon OAT activity and to investigate the property of OAT to form increasingly higher molecular weight aggregates upon concentration of the enzyme. This data has important implications in the following sections, particularly in section 5 where the similarity of rat and human OAT has been exploited to develop a novel immunoadsorption procedure capable of isolating OAT from crude tissue extracts.

Biochemical investigations of the properties of OAT have been reviewed elsewhere (section 1.5.4). A notable feature of the enzyme is its thermostable nature, exploited during most OAT purification methods and the necessity of cofactor to maintain enzyme activity. A major biochemical property of OAT is the self-association of the enzyme monomers with increasing concentration (Morris et al., 1974; Boernke et al., 1981). This has resulted in a wide range of reported molecular weight estimations for oligomeric OAT. This study includes investigation of the self-aggregation phenomenon of OAT by estimating native molecular weight in the absence and presence of a detergent. In view of the hydrophobic properties of OAT discussed in section 2, the presence of detergent may prevent aggregation.

3.2 Materials And Methods

3.2.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using the method of Laemmli (1979) modified for use in vertical slab gel electrophoresis.

3.2.1.1 Solutions

The following solutions, made up in distilled water, were required:

Tris-glycine electrode buffer	15.5 g Tris base
(25 mM Tris, 192 mM glycine)	72.0 g glycine
	5.0 g SDS
	pH 8.3 in 5000 ml
Tris-SDS stock solution	30.3 g Tris base
(90.25 M Tris) pH 6.8	2 g SDS
	pH 6.8 in 1000 ml
Tris-SDS stock solution	90.8 g Tris base
(0.75 M Tris) pH 8.8	2 g SDS
	pH 8.8 in 1000 ml
Sample buffer	25 ml Tris-SDS stock, pH 6.8
(62.5 mM Tris)	2 g SDS
	10 ml glycerol
	5 ml 2-mercaptoethanol
	0.1 ml 1 %(w/v) bromophenol blue
	in 100 ml
Acylamide stock	30 g acrylamide
	0.8 g N'-N methylene-
	bisacrylamide in 100 ml
Ammonium persulphate	0.1 g in 10 ml

TEMED (N,N,N',N'-tetramethylenediamine)

3.2.1.2 Sample preparation

Samples were dissolved in sample buffer to produce a final protein concentration of 1-2 mg/ml. Prior to electrophoresis, samples were immersed in boiling water for 4 minutes to ensure that proteins were dissolved and fully denatured. For protein homogenates, 50-100 µg of protein was required whereas 5 µg was typically used for pure proteins.

3.2.1.3 Procedure

The gel mould apparatus used for vertical slab gel electrophoresis (L.K.B., Bromma, Sweden) was assembled according to the manufacturer's instructions after the glass plates had been cleaned with detergent, followed by distilled water and ethanol. Gel spacers were used to produce 1.5 mm thick gels. The resolving gel was prepared by mixing 19.8 ml of acrylamide stock solution, 30 ml Tris-SDS stock solution (pH 8.8) and 10.2 ml distilled water in a Buchner flask. This solution was degassed for 10 minutes. Immediately before the gels were to be poured, 1.5 ml of freshly made 1 %(w/v) ammonium persulphate and 15 µl TEMED were added. The solution was mixed avoiding introduction of air and then poured into the gel mould to a height of 12 cm. The gel solution was carefully overlayed with 2-butanol to exclude air and produce a flat top to the gel. Polymerisation was allowed to proceed for 2 hours at room temperature. Before applying the stacking gel, the 2-butanol was removed and the surface of the gel washed with 50 %(v/v) Tris-SDS stock solution (pH 8.8) and any excess buffer absorbed with filter paper.

The stacking gel solution was prepared by mixing 2 ml of acrylamide stock, 10 ml Tris-SDS stock solution (pH 6.8) and 8 ml distilled water. Following degassing for 10 minutes, 0.5 ml of freshly made 1 %(w/v) ammonium persulphate and 10 µl TEMED were added and the mixed solution poured on top of the resolving gel up to the top of the

gel mould which contained a comb for the formation of sample wells. Polymerisation was allowed to proceed for 2 hours at room temperature before sample application.

The gels prepared contained acrylamide concentrations of 10 %(w/v) and 3 %(w/v) in the resolving gel and stacking gel, respectively.

The formed sample wells were washed and overlayed with electrode buffer and samples applied to the base of the wells beneath the buffer using a microlitre syringe. The remaining electrophoresis apparatus was assembled and electrophoresis performed at a constant current of 30 mA per gel for 4 hours at 10^{o}C until the tracking dye was approximately 1 cm from the gel base.

Molecular weight protein standards (Sigma) were also electrophoresed adjacent to samples to allow estimation of sample protein molecular weights. The protein standards used were:

β-galactosidase (116,000); phosphorylase B (97,400); bovine albumin (66,000); egg albumin (45,000); carbonic anhydrase (29,000).

3.2.1.4 Fixing, staining and destaining of gels

Following electrophoresis, gels were carefully removed from the glass mould and fixed for 30 minutes in fixative solution containing 500 ml methanol, 500 ml distilled water and 100 ml glacial acetic acid. The gel was then stained for 2 hours in 1000 ml of stain containing 0.1 %(w/v) Coomassie Brilliant Blue G (Sigma) dissolved in fixative and filtered. Gels were destained in a solution containing 5 %(v/v) methanol and 7 % (v/v) glacial acetic acid in distilled water.

3.2.2 Native molecular weight estimations

3.2.2.1 Sephadex G-200 chromatography

Molecular weight determinations of native OAT protein were determined by gel filtration. A glass column (1.6 cm x 82 cm) was packed with pre-swollen Sephadex G-200 and

equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 0.15 M sodium chloride and 2 mM 2-oxoglutaric acid. All chromatographic procedures were performed at 4°C in a cold room. A flow rate of 9 ml per hour was used for elution. Protein standards (Pharmacia) were used to calibrate the column. Each protein standard was dissolved in 1 ml of column buffer at 10 mg/ml. Void volume was determined by measuring the elution volume of blue dextran at 5 mg/ml. Eluted fractions were collected in 2 ml aliquots and protein content monitored spectrophotometrically at 280 nm. For measurements made in the presence of detergent, the column was equilibrated with column buffer containing 0.1 %(w/v) Triton X-100 and recalibrated with protein standards dissolved in detergent column buffer.

3.2.2.2 Preparation of OAT samples for chromatography

Lyophilized human liver OAT, rat liver OAT and rat kidney OAT samples were each dissolved in column buffer to 10 mg/ml in the presence and absence of 0.1 %(w/v) Triton X-100 and incubated overnight on ice to allow dissociation to occur prior to chromatography. A sample of rat kidney OAT left at 10 mg/ml overnight was diluted to 5 mg/ml and sonicated at 10 kilocycles per second for 2 minutes prior to chromatography.

Additional rat kidney OAT samples were concentrated using a collodion bag, to 10 mg/ml from a freshly purified OAT preparation in the presence and absence of 0.1 %(w/v) Triton X-100. This was incubated on ice overnight prior to chromatography in order to check that samples concentrated to 10 mg/ml from a dilute preparation produced the same results as a lyophilized sample left to dissociate at 10 mg/ml.

3.2.3 Apo-enzyme formation

The cofactor of OAT, pyridoxal phosphate was removed to produce the apo-enzyme by the method of

Katunuma et al. (1964). Aliquots of pure OAT were dialyzed against 0.2 mM hydroxylamine for 2 hours at 4°C followed by dialysis against distilled water for 2 hours.

3.2.4 Estimation of apparent Km values

The OAT assay (section 2.2.1.2) was used to determine enzyme activities at varying substrate concentrations. Both ornithine and 2-oxoglutarate concentrations, separately, were varied between 1-128 mM. The concentration of the substrate not being varied was kept constant at 35 mM for ornithine and 5 mM for 2-oxoglutarate. For estimation of apparent Km for cofactor, varying concentrations of pyridoxal phosphate were incubated with apo-enzyme for 30 minutes to restore holoenzyme prior to assay. Cofactor concentration was varied between 0.25 µM and 16 µM for this determination and kept constant at 55 µM for substrate determinations.

3.2.5 Thermal stability experiments

Stock solutions of pure OAT dissolved in 50 mM potassium phosphate buffer (pH 8.0) were diluted in a series of the same buffer containing 20 µM pyridoxal phosphate, 5 mM 2-oxoglutarate and 35 mM ornithine plus combinations of these substrates and cofactor respectively. All tubes contained 10 µg of pure OAT protein in 5 ml aliquots of buffer at 4°C. Duplicate test and control samples were heated to 60°C in a 75°C water bath for one minute, with constant stirring. Samples were immediately cooled on ice to 4°C. Samples were then dialyzed against 50 mM potassium phosphate buffer (pH 8.0) to remove substrates and free cofactor, prior to assay. Further experiments, varying time of heat treatment and temperature, were undertaken.

3.3 Results

3.3.1 Subunit molecular weight of OAT (SDS-PAGE)

Estimation of the OAT monomer molecular weight was performed under dissociating conditions by SDS-PAGE (figure 3.1). Single bands were obtained for OAT purified from human and rat sources which corresponded to a molecular weight marker of 45,000. Both human and rat OAT bands appeared identical.

3.3.2 Native molecular weight estimations

Preliminary assay of OAT in the presence of concentrations of Triton X-100 (a non-ionic detergent) ranging from 0.01 %(w/v) to 2 %(w/v) showed no significant effect on enzyme activity. As a result, the molecular weight of OAT was measured in the absence and presence of 0.1 %(w/v) Triton X-100 in order to investigate the aggregation of OAT. Calibration of the Sephadex G-200 column produced almost identical elution volumes in the presence and absence of 0.1 %(w/v) Triton X-100. Consequently, molecular weight of OAT was measured using calibration data from molecular weight markers in the absence of detergent. A calibration curve was constructed (figure 3.2) and the following data obtained after 4 determinations in each case: The native molecular weight of rat liver and kidney OAT at 10 mg/ml in the absence of detergent produced a value of 282,000 $\pm$ 16,500 (mean $\pm$ S.D.). This estimation was reduced to 188,000 $\pm$ 13,000 in the presence of 0.1 %(w/v) Triton X-100. The molecular weight of human liver OAT was estimated to be 178,000 $\pm$ 12,500 both in the presence and absence of detergent. A value of 47,000 $\pm$ 2,800 was obtained for rat kidney OAT after sonication of a 5 mg/ml solution for 5 minutes at 10 kilocycles per second. Extension of the study to include an estimation of rat kidney samples, freshly prepared and concentrated to 10 mg/ml, produced a molecular weight of

278,000 ± 9,900 (mean ± S.D., n=2) in the absence of 0.1 %(w/v) Triton X-100 and a value of 181,000 ± 8,700 (n=2), in the presence of detergent.

3.3.3 Apparent Km values of OAT

Apparent Km values for ornithine, 2-oxoglutarate and pyridoxal phosphate were determined from Lineweaver-Burk plots constructed for rat kidney, rat liver and human liver OAT (figures 3.3-3.6). Values are summarized below (Table 3.1).

TABLE 3.1 Apparent Km values determined as described in the text and figure legends

Variable component	Apparent Km value (mM)		
	rat kidney	rat liver	human liver
ornithine	1.25	1.1	2.3
2-oxoglutarate	1.15	1.1	2.5
pyridoxal phosphate	0.004	0.002	0.001

Apparent Km values for human liver OAT appear to be about twice those of rat liver and kidney, for both substrates. No significant difference in values for OAT from the two rat sources was noted, although this requires statistical confirmation after repeated analysis. Human liver OAT had the lowest apparent Km for cofactor. Both substrates produced substrate inhibition of OAT in all cases. Ornithine and 2-oxoglutarate concentrations above 32 mM and 16 mM respectively, were inhibitory under the assay conditions used. No inhibitory effect was noted with pyridoxal phosphate concentrations up to 16 µM used for Km determination. Since this concentration range is relatively low, assays were performed at 50, 500 and 1000 µM pyridoxal phosphate with no decrease in activity.

3.3.4 Thermal stability of OAT

The effect of temperature upon rat kidney OAT in 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and 20 μM pyridoxal phosphate was to rapidly decrease activity at incubation times greater than 1 minute, at temperatures above 50°C (figure 3.7). At 40°C, 34% recovery was obtained after 8 minutes. The conditions used in purification procedures (60°C for 1 minute) were optimal for recovery of OAT activity. Rapid loss of OAT activity occurred at 70°C, although 48% activity was still retained after one minute.

Investigation of the protective effect of buffer components upon 60°C heat treatment for 1 minute (figure 3.8) showed that the presence of cofactor was the most protective component of the buffer (buffer C.). In the presence of 2-oxoglutarate and cofactor, recovery of OAT was very high (95% of rat OAT and 98% of human OAT). The presence of 2-oxoglutarate alone resulted in retention of at least 58% activity in all cases. However, addition of ornithine (buffer E.) reduced OAT recovery to less than 25% in human and rat enzyme. Addition of ornithine in the absence of cofactor resulted in complete loss of activity. This was also the case in the absence of 2-oxoglutarate with cofactor present.

3.3.5 pH-activity curves for OAT

The pH optimum for OAT from human and rat tissues was estimated at pH 8.0. Activity profiles (figure 3.9) for the 3 OAT samples were very similar over a range of pH values (7.0-9.0). Activity was reduced to less than 10% of the optimal value at pH 7.0, while at pH 9.0, rat OAT activity was about 60% of optimal activity.

FIGURE 3.1 SDS-PAGE of purified OAT extracts

SDS-PAGE of OAT purified from human liver (lane B), rat kidney (lane C) and rat kidney (lane D) in a 10% gel.
Molecular weight markers (lane A) were as follows:
carbonic anhydrase (28,000), egg albumin (45,000), bovine serum albumin (66,000), phosphorylase B (97,400) and beta-galactosidase (116,000).

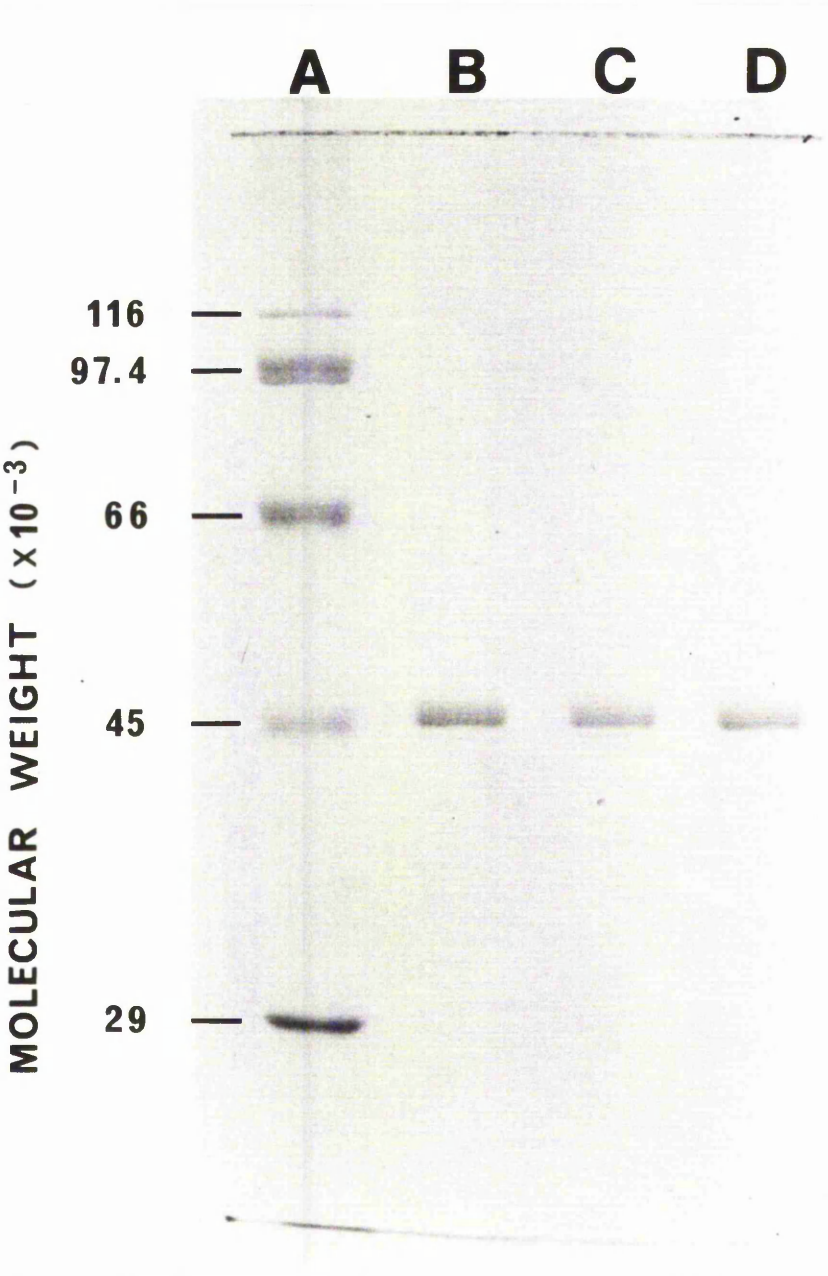

A
B
C
D
116
97.4
66
45
29
MOLECULAR WEIGHT (X10^{-3})

FIGURE 3.2 Estimation of the molecular weight of OAT by Sephadex G-200 chromatography

Kav = (Ve - Vo)/(Vt - Vo) where Ve = elution volume of protein (ml), Vo = void colume (45 ml) and Vt = total bed volume (135 ml).
Molecular weight markers are as follows:
1. ribonuclease (13,700); 2. chymotrypsinogen A (25,000); 3. ovalbumin (43,000); 4. bovine serum albumin (66,000); 5. aldolase (158,000); 6. catalase (232,000); 7. ferritin (440,000).
Mean values (n=4) were determined for the following samples:
A. sonicated rat kidney; B. human liver with and without detergent; C. rat liver and kidney with detergent present; D. rat liver and kidney without detergent present. Standard deviations are given in the text. (Regression line was fitted by the method of least squares).

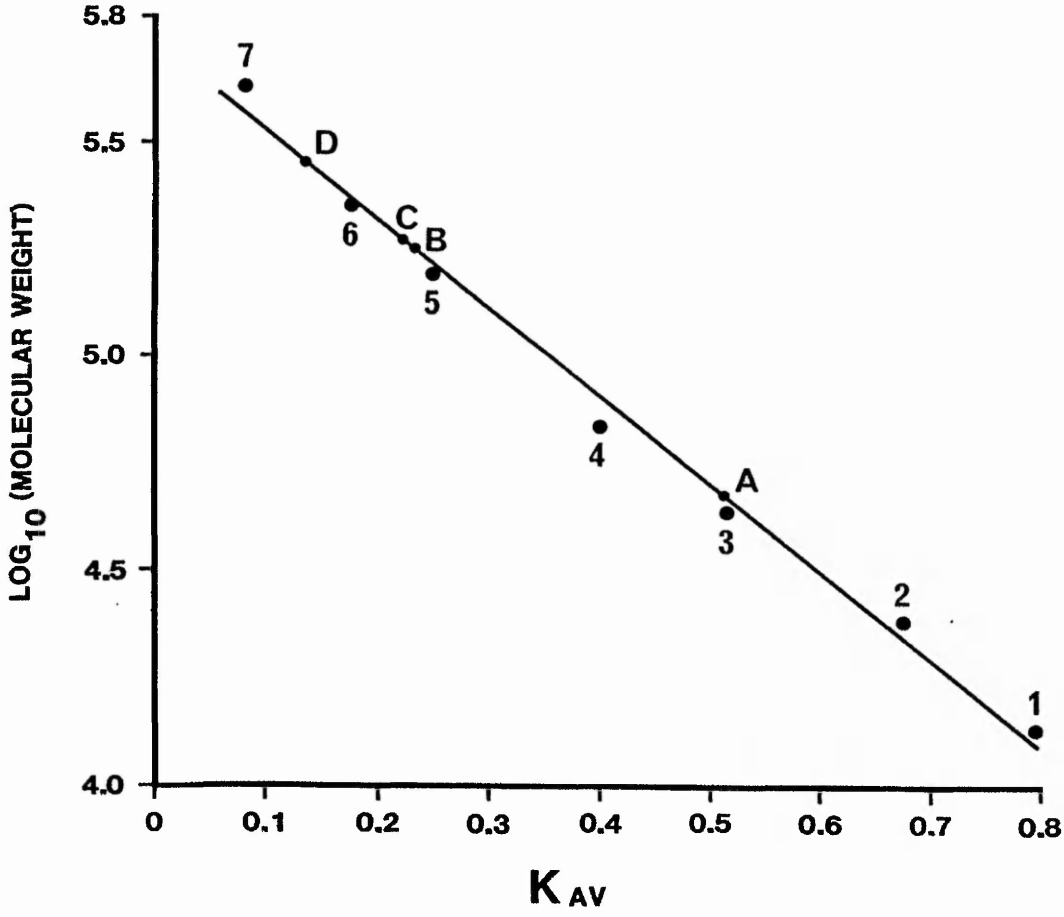

5.8
5.5
5.0
4.5
4.0
LOG10 (MOLECULAR WEIGHT)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
KAV
7
D
6
C
B
5
4
A
3
2
1

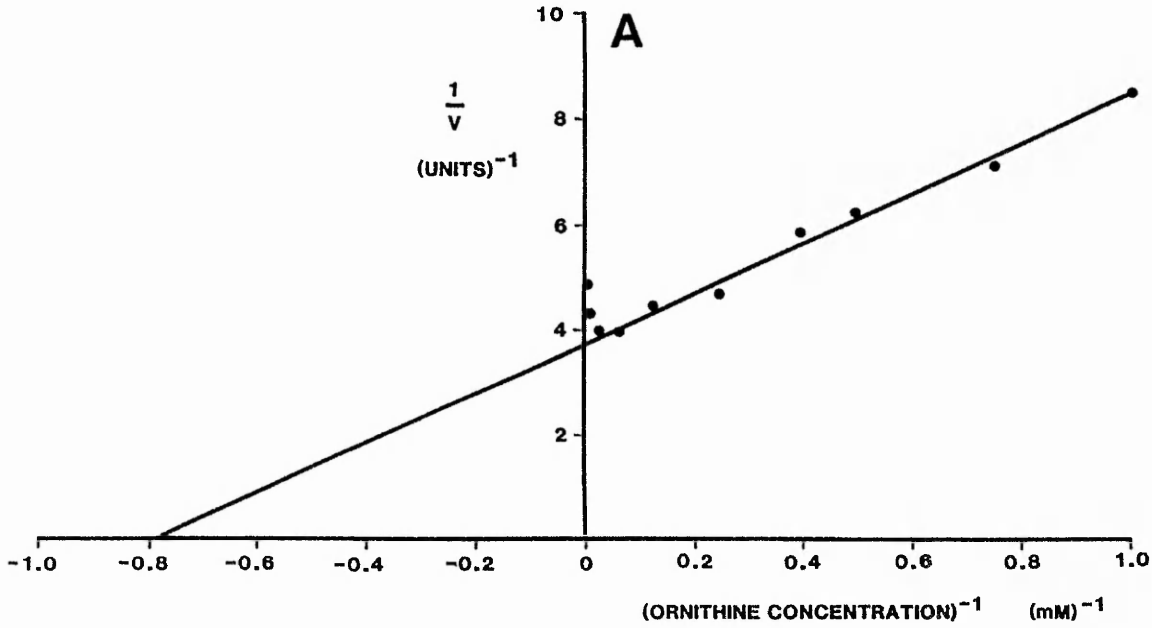
FIGURE 3.3 Determination of apparent Km for substrates using rat kidney OAT

Lineweaver-Burk plots were constructed to determine the apparent Km for both substrates (varied between 1 and 128 mM) from the intercept of the x-axis (equal to $-\frac{1}{Km}$).

A. Apparent Km (ornithine) = 1.25 mM

B. Apparent Km (2-oxoglutarate) = 1.15 mM

1 unit = 1 µmole Δ'-pyrroline-5-carboxylate formed per minute at $37^{\circ}C$.


A
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
(ORNITHINE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

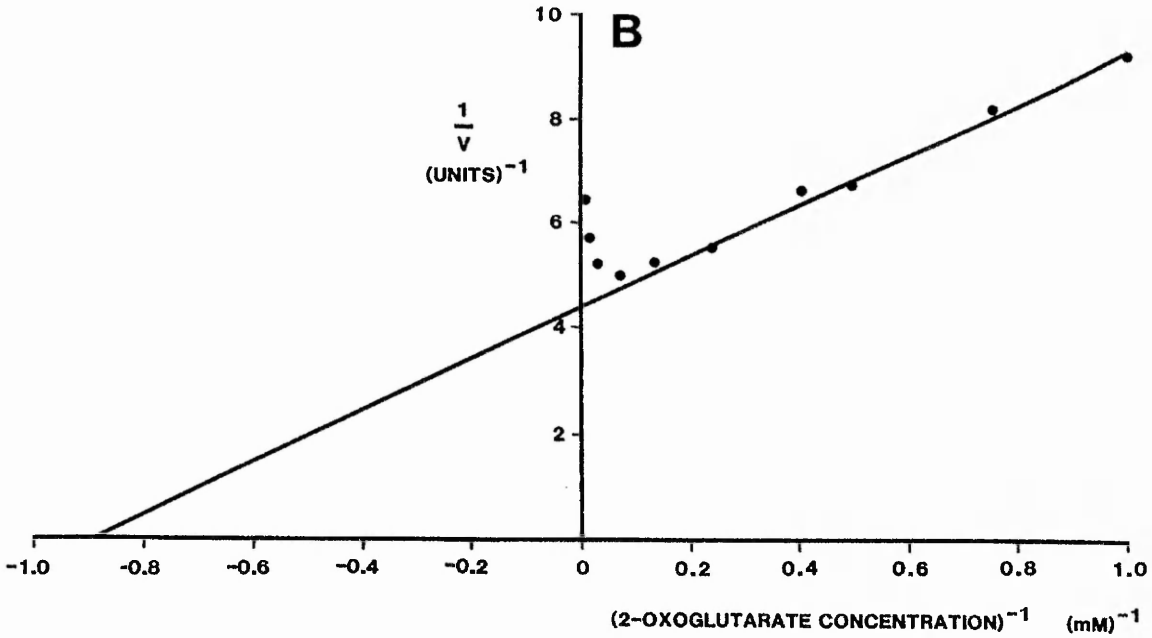

B
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
(2-OXOGLUTARATE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

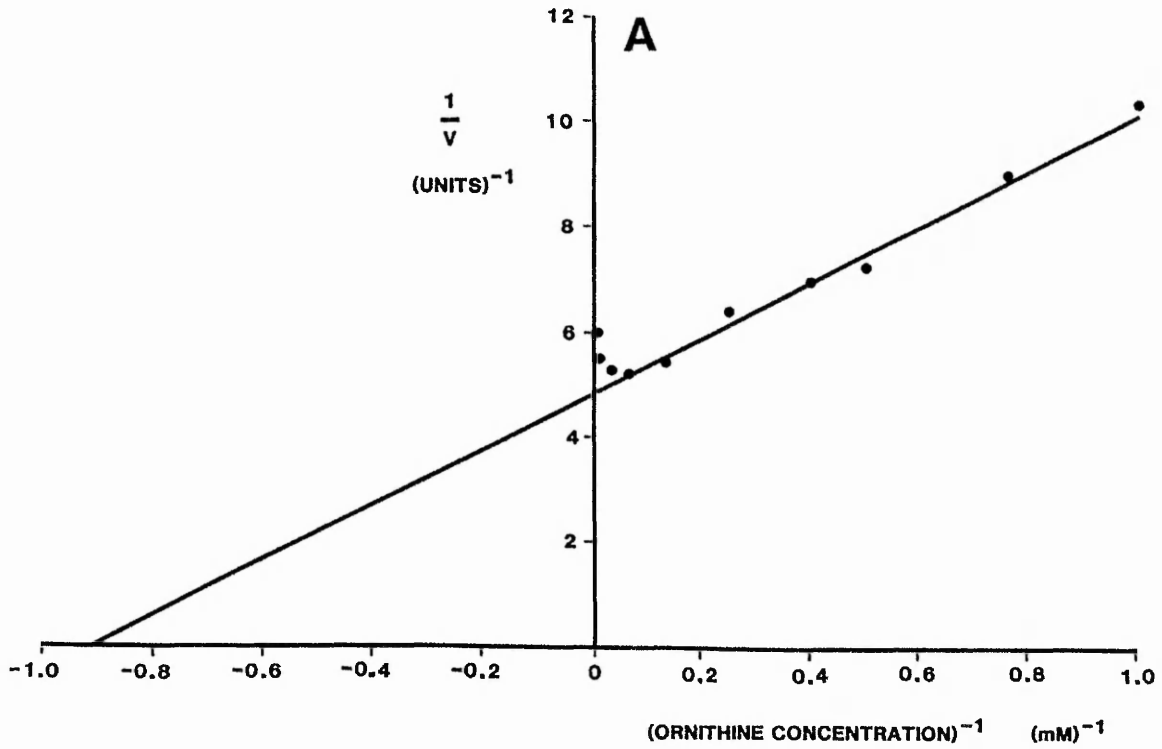
FIGURE 3.4 Determination of apparent Km for substrates using rat liver OAT

Lineweaver-Burk plots were constructed to determine the apparent Km for both substrates (varied between 1 and 128 mM) from the intercept of the x-axis (equal to $-\frac{1}{Km}$).

A. Apparent Km (ornithine) = 1.1 mM

B. Apparent Km (2-oxoglutarate) = 1.1 mM

1 unit = 1 µmole Δ^1-pyrroline-5-carboxylate formed per minute at $37^{\circ}C$.


A
12
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-1.0
-0.8
-0.6
-0.4
-0.2
0
0,2
0.4
0.6
0.8
1.0
(ORNITHINE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

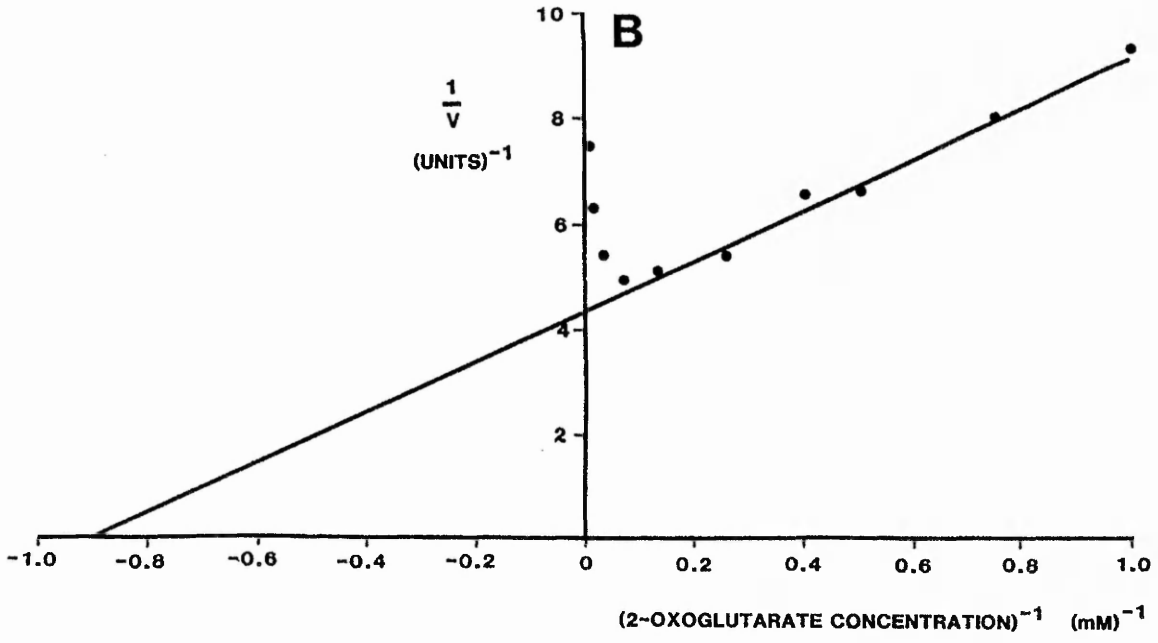

B
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
(2-OXOGLUTARATE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

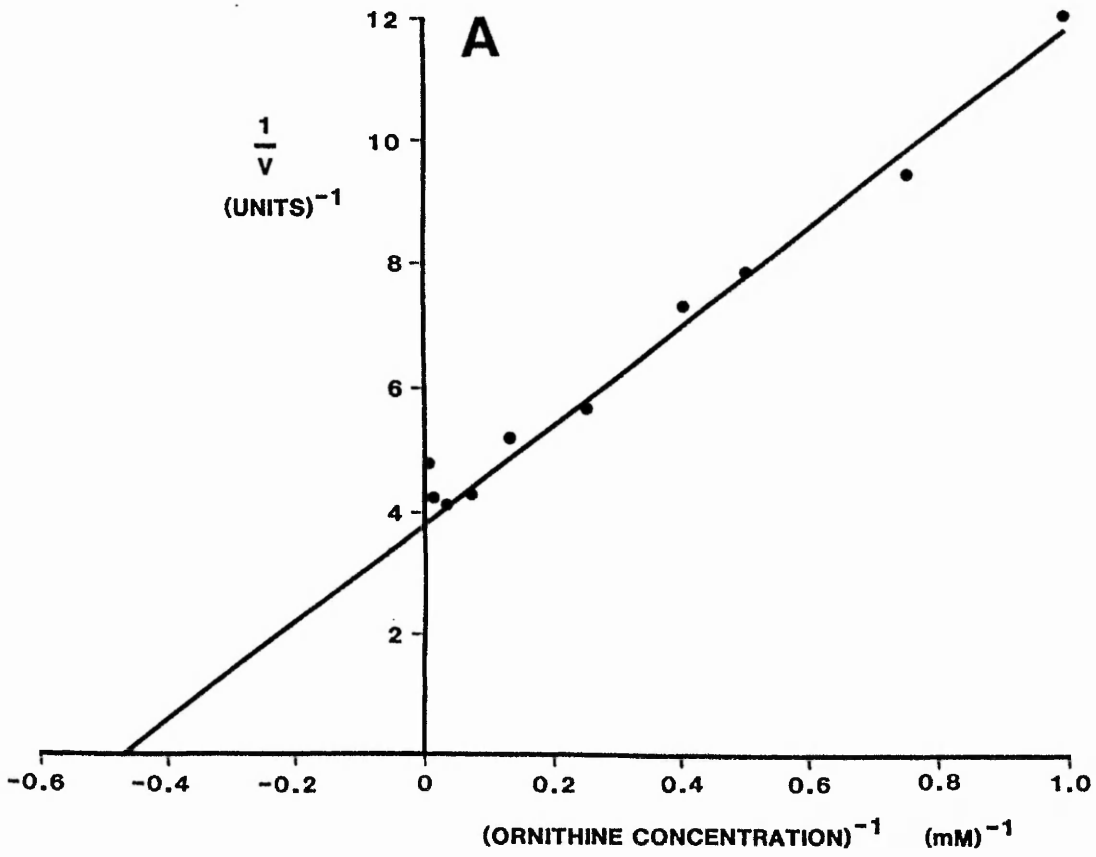
FIGURE 3.5 Determination of apparent Km for substrates using human liver OAT

Lineweaver-Burk plots were constructed to determine the apparent Km for both substrates (varied between 1 and 128 mM) from the intercept of the x-axis (equal to $-\frac{1}{Km}$).

A. Apparent Km (ornithine) = 2.3 mM

B. Apparent Km (2-oxoglutarate) = 2.5 mM

1 unit = μmole Δ^1-pyrroline-5-carboxylate formed per minute at 37°C.


A
12
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
(ORNITHINE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

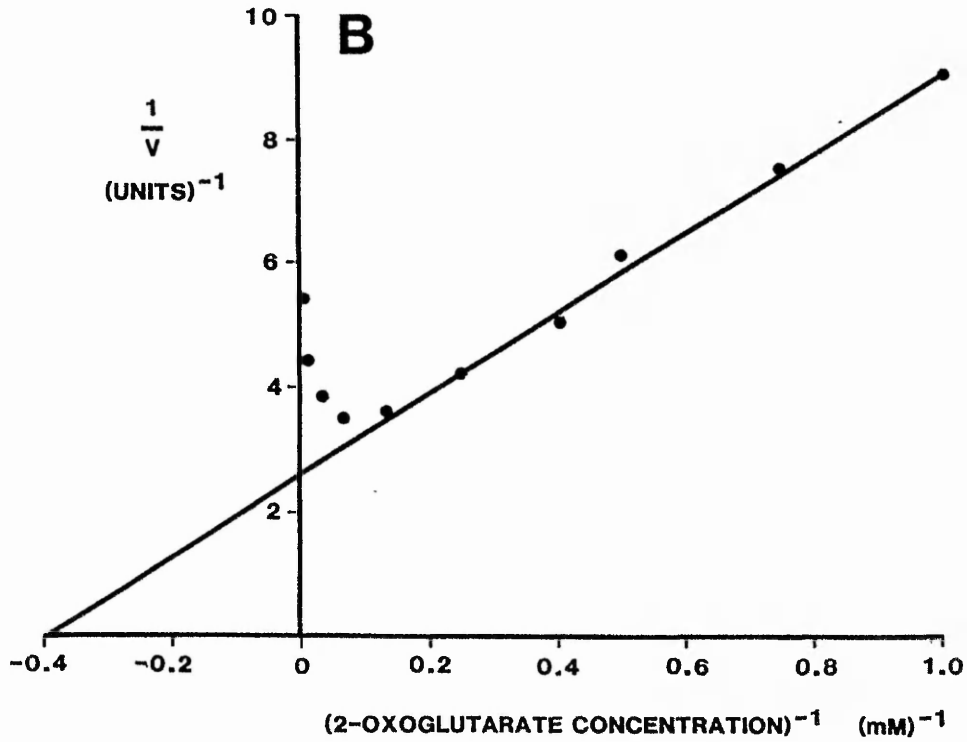

B
10
8
6
4
2
1/V
(UNITS)$^{-1}$
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
(2-OXOGLUTARATE CONCENTRATION)$^{-1}$ (mM)$^{-1}$

FIGURE 3.6 Determination of apparent Km for cofactor using OAT purified from rat and human sources

Lineweaver-Burk plots were constructed to determine the apparent Km for cofactor (varied between 0.25 and 16 μM) from the intercept of the x-axis (equal to $-\frac{1}{Km}$).

▲—▲	Rat kidney	Km (cofactor)	=	4.0 μM
●—●	Rat liver	Km (cofactor)	=	2.0 μM
■—■	Human liver	Km (cofactor)	=	1.0 μM

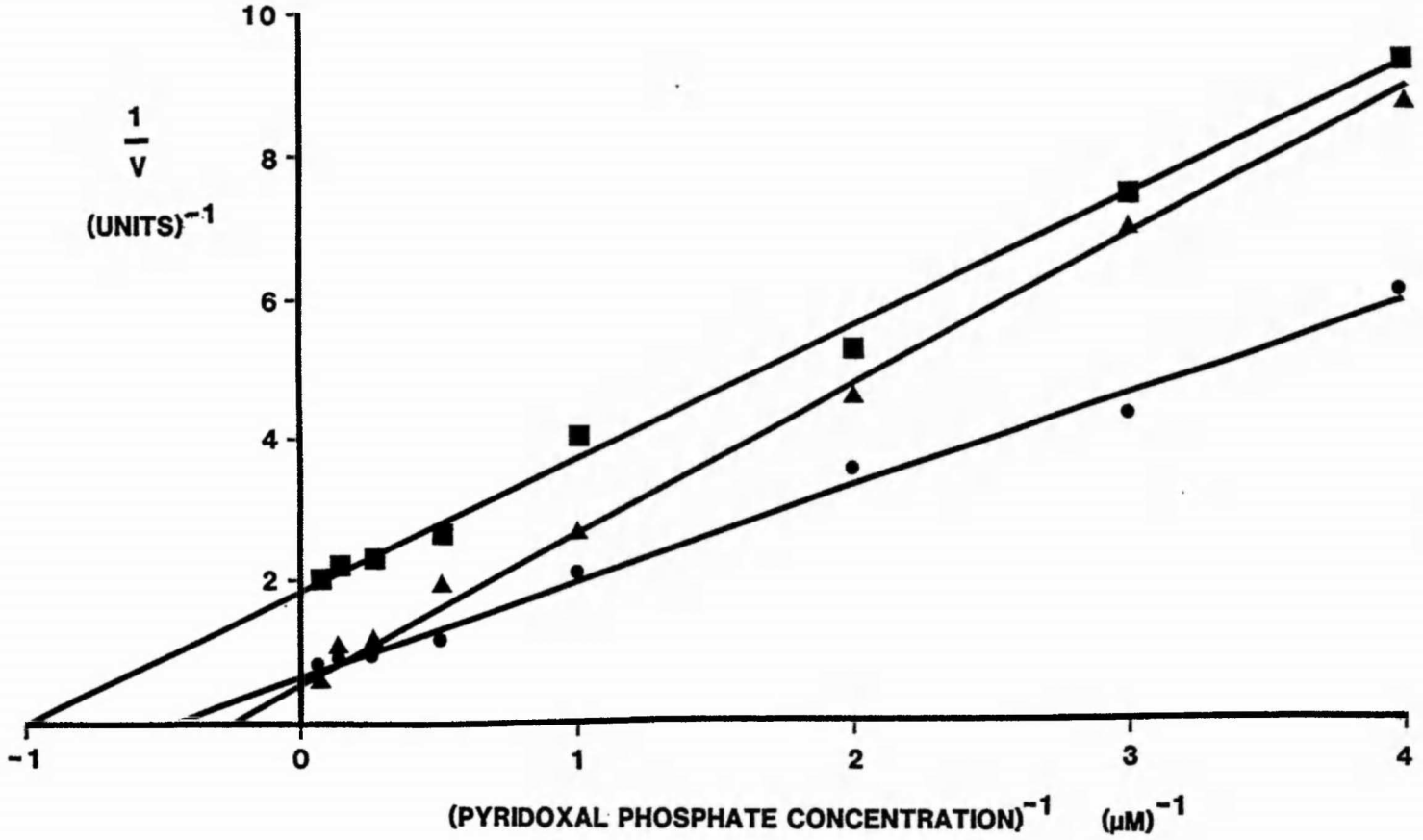

10
8
6
4
2
1/V
(UNITS)$^{-1}$
-1
0
1
2
3
4
(PYRIDOXAL PHOSPHATE CONCENTRATION)$^{-1}$ (μM)$^{-1}$

FIGURE 3.7 Thermal stability of rat kidney OAT with time

Thermal stability of pure rat kidney OAT incubated in 50 mM potassium phosphate buffer containing 2 mM 2-oxoglutarate and 20 μM pyridoxal phosphate. Aliquots (5 ml) of OAT in buffer were heated to a series of temperatures using a water bath as described in the text, for a series of incubation times and then rapidly cooled on ice and assayed. Activity is expressed as a percentage of the activity obtained prior to incubation (the mean of duplicate assays).

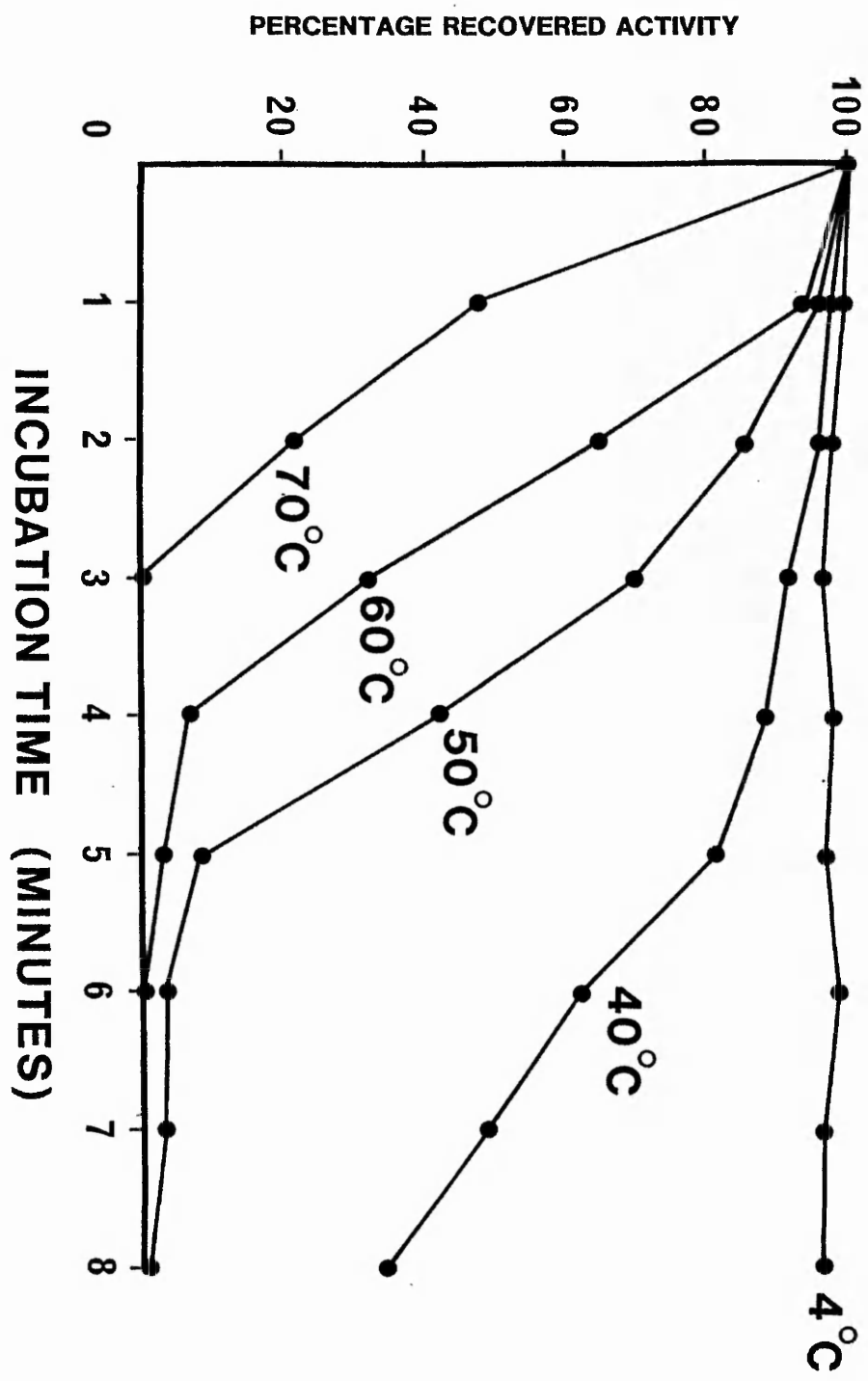

PERCENTAGE RECOVERED ACTIVITY
100
80
60
40
20
0
INCUBATION TIME (MINUTES)
0
1
2
3
4
5
6
7
8
70°C
60°C
50°C
40°C
4°C

FIGURE 3.8 Effect of buffer components upon thermal stability of OAT

Thermal stability of OAT in a series of buffers containing various substrates and cofactor. Aliquots (5 ml) of OAT in buffer were heated to 60°C for 1 minute, then cooled on ice. Samples were dialyzed against buffer (5000 ml for 3 hours) and assayed.

BUFFER: 50 mM potassium phosphate (pH 8.0) containing the following:

	Pyridoxal phosphate (20 μM)	2-oxoglutarate (5 mM)	ornithine 35 (mM)
A.	+	+	-
B.	+	+	-
C.	+	-	-
D.	-	+	-
E.	+	+	+

N.B. Values are expressed as a percentage of the activity obtained for unheated samples (A.). The mean of duplicate assays were used.

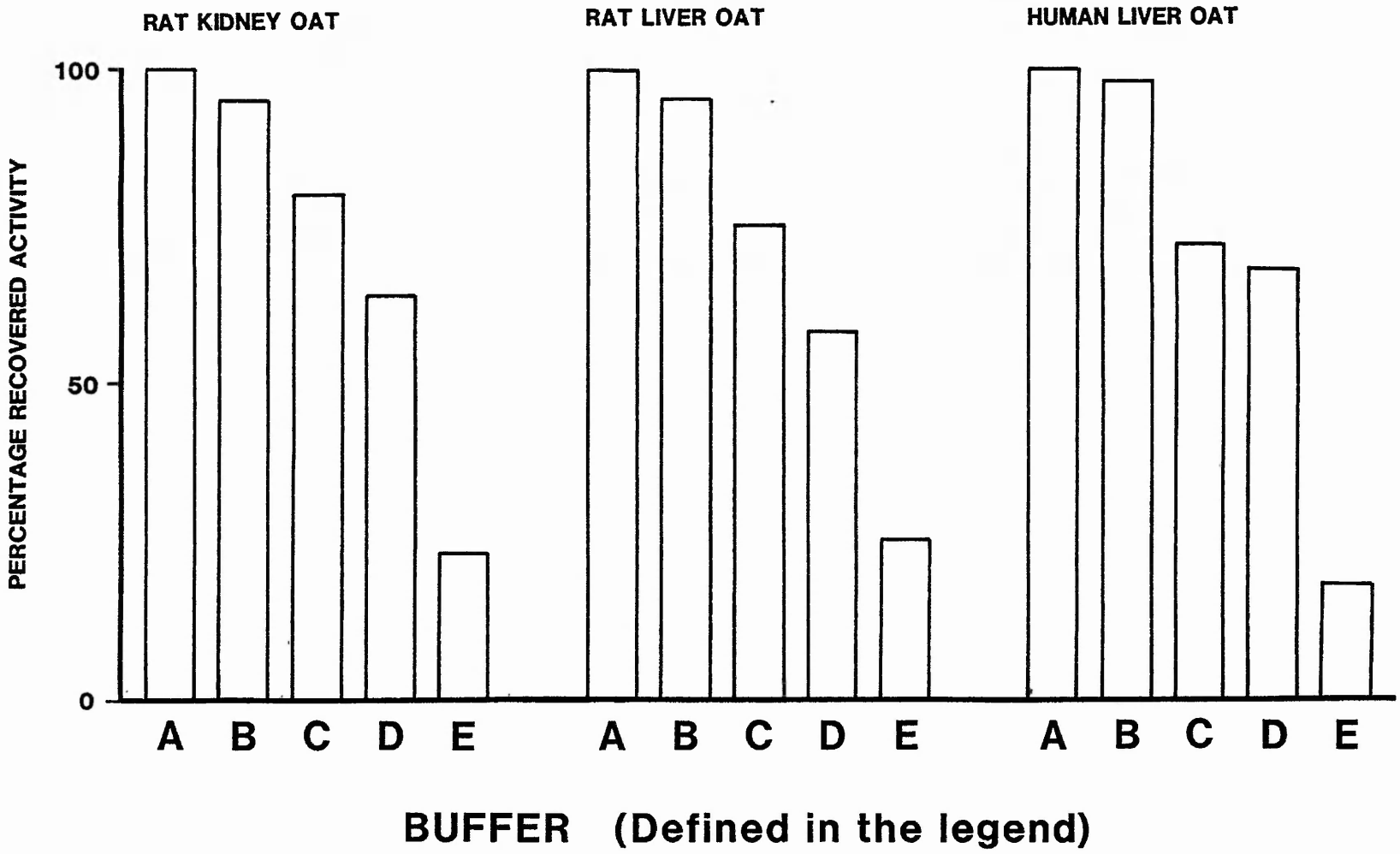

RAT KIDNEY OAT
RAT LIVER OAT
HUMAN LIVER OAT
PERCENTAGE RECOVERED ACTIVITY
100
50
0
A B C D E
A B C D E
A B C D E
BUFFER (Defined in the legend)

FIGURE 3.9 pH-Activity curves for ornithine aminotransferase

Pure enzyme was assayed for activity (section 2.2.1.2) having adjusted the pH of the phosphate buffer component of the incubation mixture accordingly.

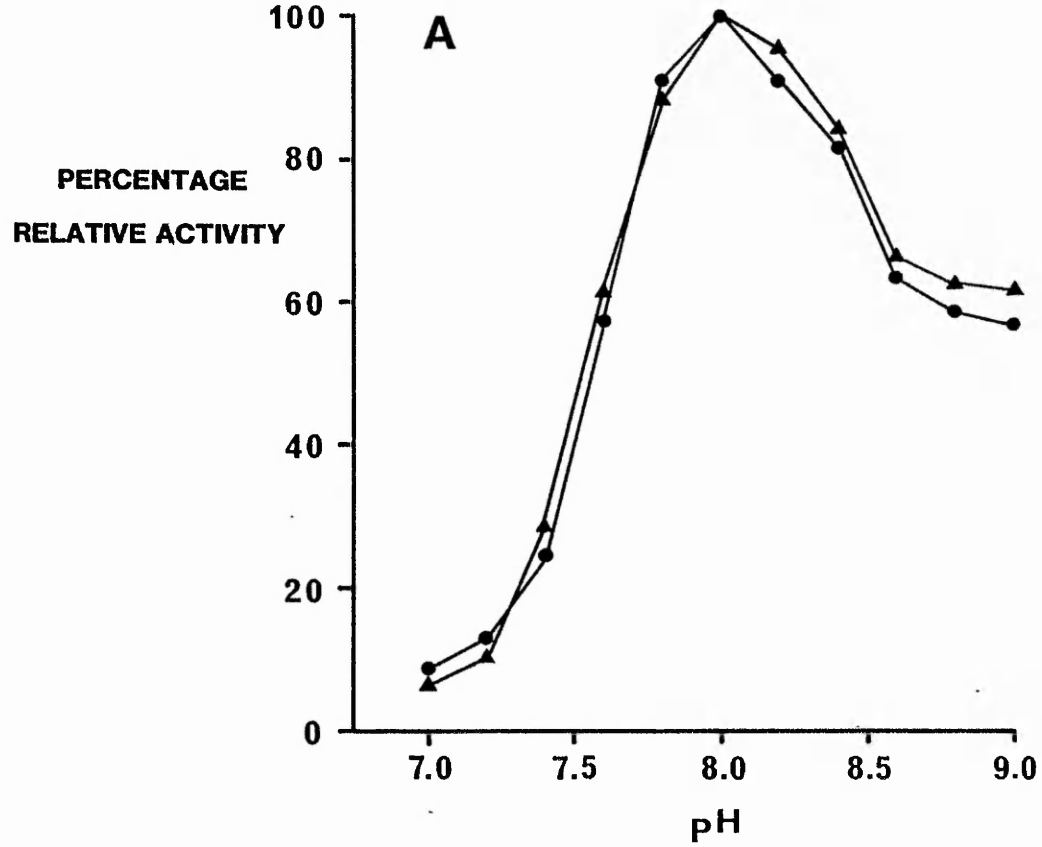
A. pH-Activity curves for OAT purified from:

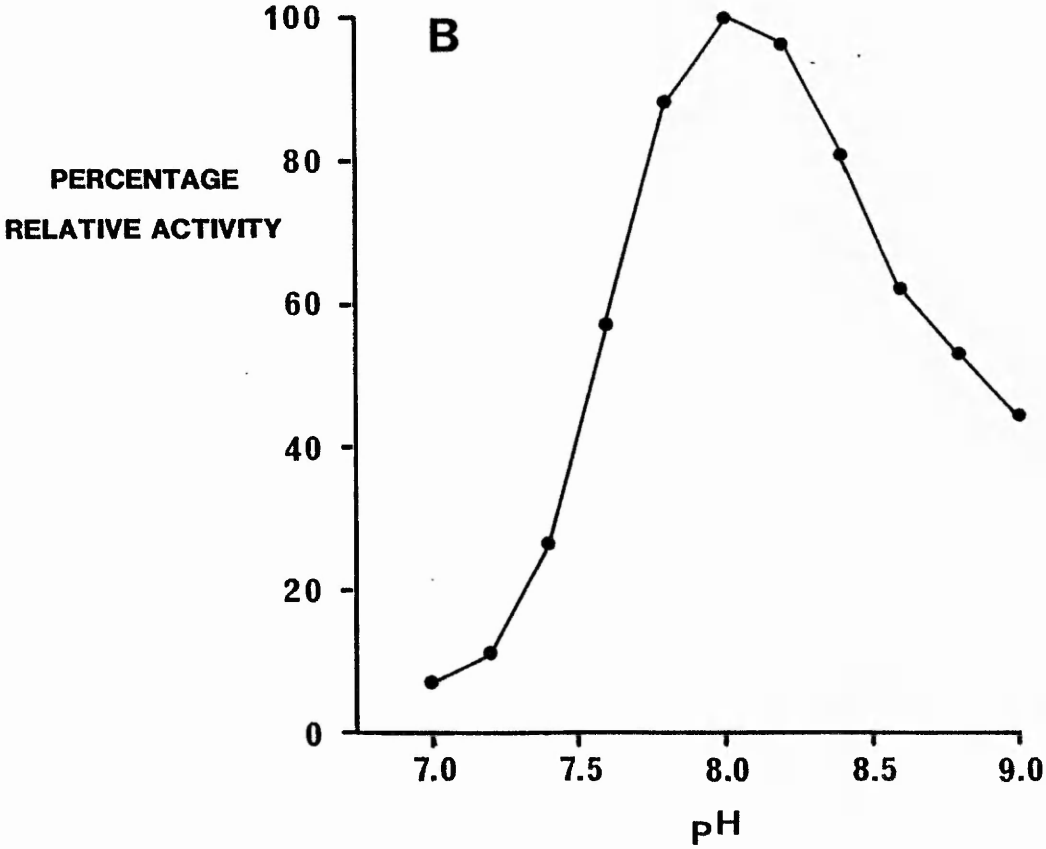
Rat kidney: ▲—▲

Rat liver: ●—●

B. pH-Activity curve for OAT purified from human liver.

Activity was expressed as a percentage of the activity obtained at the pH optimum (8.0).


A
100
80
60
40
20
0
PERCENTAGE
RELATIVE ACTIVITY
7.0
7.5
8.0
8.5
9.0
pH


B
100
80
60
40
20
0
PERCENTAGE
RELATIVE ACTIVITY
7.0
7.5
8.0
8.5
9.0
pH

3.4 Discussion

The molecular weight of monomeric OAT was estimated at 45,000 for human and rat OAT from SDS-PAGE data. This is in close agreement with Ohura et al. (1982) who estimated 44,000. Recent studies in which the elucidation of the primary structure OAT has been reported (Simmaco et al., 1986), have confirmed the molecular weight of rat OAT as 45,749.

Native molecular weight of OAT is dependent upon enzyme concentration since OAT undergoes aggregation with increasing concentration (see section 1.5.4). In view of the observations discussed in section 2 regarding the hydrophobicity of OAT probably being increased as a result of aggregation, the effect of a detergent upon native molecular weight estimations was investigated.

Triton X-100 (a non-ionic surfactant) was found to have no effect upon OAT activity and a detergent concentration of 0.1 %(w/v) was chosen since high concentrations approaching 5 %(w/v) dissociated some of the molecular weight markers used to calibrate the Sephadex G-200 column.

In the absence of detergent, a molecular weight of 282,000 was estimated for rat liver and rat kidney OAT at 10 mg/ml. This suggests that oligomeric OAT is hexameric under these conditions. Recent studies by Markovic-Housley et al., (1987) have also indicated that oligomeric OAT is a hexamer using sedimentation data and X-ray crystallography. They found that hexameric OAT existed over a wide range of OAT concentrations but were unable to show that higher aggregation states exist, in contrast to Boernke et al. (1981) and Peraino et al. (1969). However, the presence of sodium chloride may enhance aggregation possibly by stabilizing repulsing forces between OAT molecules.

In the presence of detergent, the molecular weight of rat liver and kidney OAT was estimated at 188,000 indicating tetrameric OAT at 10

mg/ml. Human liver OAT was estimated at 178,000 in both the presence and absence of detergent, also indicating that OAT is a tetramer at 10 mg/ml. Samples were prepared from lyophilized OAT which may be highly aggregated and therefore not completely dissociated in spite of an overnight incubation period. However, freshly prepared rat kidney OAT samples, concentrated to 10 mg/ml, produced no significantly different estimations in either the presence or absence of detergent.

The presence of detergent appears to prevent aggregation beyond a tetrameric state in the case of rat OAT, although data for human OAT suggests that no further aggregation has occurred at 10 mg/ml without detergent. It may be that human OAT aggregates at higher concentrations than rat OAT. The fact that other workers have reported native OAT as being tetrameric (Peraino et al., 1969; Sanada et al., 1976; Kalita et al., 1976) together with the data obtained in this study, suggests that this state may be the most stable form of oligomer. Sonication of OAT dissociates native OAT into monomers (estimated at 47,000 by gel filtration) although Morris et al. (1974) has shown that monomeric OAT is produced at concentrations of 5 mg/ml or less by overnight incubation.

The rate of dissociation of aggregated OAT has not been investigated and is likely to be dependent upon many factors (pH, ionic strength, temperature). Under the conditions used for this study, single peaks of eluted OAT protein were obtained whereas a broad elution of OAT might be expected if OAT dissociated on the column as the sample was diluted. Presumably, by further experiments where aggregated samples were diluted and left to dissociate over a time course prior to gel filtration, an approximate dissociation rate could be found under specified conditions. No difference was found in specific activity between monomeric OAT (5 mg/ml) and oligomeric OAT (10 mg/ml) with or without detergent. However, Boernke et al., (1981)

has found that the Km for ornithine increases as a result of increasing OAT aggregation and proposed that, *in vivo*, this spares ornithine for use in the urea cycle. The physiological implications of this phenomenon will be discussed later (see general discussion).

Determination of the apparent Km values indicate that the affinity of rat OAT for both substrates is about twice as high as for human OAT. Substrate inhibition of OAT in all cases was observed, indicating that this enzyme is tightly controlled which is not unexpected in view of its metabolic role (see section 1.5.5). Substrate inhibition occurred between 32 and 64 mM ornithine indicating that assay conditions are optimal with 35 mM ornithine approximating to a maximal reaction rate. Inhibition occurred above 16 mM 2-oxoglutarate, assay concentrations of 5 mM being submaximal. No inhibition was observed with cofactor concentrations used for Km determination. This was expected since a low range of concentrations were used (0.25-16 μM). Further investigations using high concentrations of cofactor (50-1000 μM) produced no inhibition and activities approximating to maximal values were obtained. Shih *et al.* (1978) and Kennaway *et al.* (1980) have reported increases in OAT activity of extracts from Gyrate Atrophy patients in the presence of very high cofactor concentrations. Conversely, Peraino *et al.*, (1972) has shown that excess pyridoxal phosphate can partially inactivate OAT possibly by binding to lysine residues in or near the active site.

Investigations into the thermal stability of OAT showed that cofactor is important in protecting OAT activity and that the heat treatment conditions used during purification were optimal for OAT recovery. The high percentage recovery of OAT after a one minute incubation at 60^{o}C using pure OAT is greater than that of crude extracts since denatured contaminating protein tends to trap OAT in the pellet after centrifugation. However, apparently high percentage

recoveries were obtained after this step (section 2., Table 2.3) due to release of OAT sequestered in mitochondria. Addition of ornithine to OAT incubation mixtures appears to destabilize OAT since all activity is lost after 60°C heat treatment and cofactor protection still results in about 75% reduction of recovered activity. Incubation of OAT with 35 mM ornithine at 4°C caused a loss of up to 60% of the original activity within 5 hours. In the absence of ornithine, OAT can be stored on ice for several days with negligible loss of activity.

The pH optimum for OAT was estimated at 8.0 in all cases. This is in close agreement with Peraino (1972) who has determined a value of pH 8.15 for rat liver OAT. Profiles of pH-activity were virtually identical for both rat and human OAT.

In conclusion, rat liver and kidney OAT appear to be the same protein since the physicochemical properties of OAT from these two sources are not significantly different. Human liver OAT also appears to be very similar to rat OAT in this respect although the two can be distinguished. This is supported by Ohura et al. (1982) who have shown that the amino acid composition of human and rat OAT are very similar. OAT from these two sources are compared immunologically in the next section.

4 PRODUCTION OF POLYCLONAL ANTIBODIES

4.1 Introduction

In this study, polyclonal antibodies have been raised to rat and human OAT in rabbits for three purposes: (1) To allow a structural comparison of rat and human OAT by immunodiffusion; (2) To develop an immunoadsorption method of OAT isolation from crude tissue extracts (section 5); (3) To develop a competitive ELISA for OAT capable of detecting low levels of OAT protein in tissue extracts (section 7).

The strength of antibody interaction with a single antigenic determinant is defined as antibody affinity. In the case of polyclonal antibodies, the interaction of antiserum with a multivalent antigen (OAT in this study) is termed functional affinity or avidity. Quantitatively, avidity is a function of the number of binding sites (determinants/epitopes) on the antigen for antibody and the affinities of antigenic determinants for antibody.

Immunodiffusion analysis used in this study relies upon the ability of antigen and antibody to diffuse toward each other in an agar gel having a pore size which traps antigen-antibody complexes. This produces immobilized lines of precipitated complex (precipitin lines) at the point of optimum antigen and antibody concentrations. Because polyclonal antibodies recognize a number of determinants on the OAT molecule, the structural similarity of rat and human OAT can be compared by immunodiffusion since anti-rat OAT antibodies may recognize many determinants on the human OAT molecule indicated by a precipitin line. Additionally, determinants which are unique to either rat or human OAT can be demonstrated by the presence of a precipitin 'spur' which represents antibody reaction with these unique determinants. Immunodiffusion has been used to compare rat OAT purified from several tissues previously (Sanada *et al.*, 1970; Lyons and Pitot, 1976). Ohura *et al.* (1982) have used immunodiffusion analysis to provide evidence

that OAT purified from different human tissues is the same protein and that this is also the case for rat tissues.

The immune response of rabbits to successive doses of OAT antigen has been monitored by a non-competitive ELISA (heterologous enzyme-linked immunosorbent assay). This assay involves immobilizing pure OAT antigen onto plastic wells and then binding rabbit anti-OAT antibody in serum, to the antigen. IgG (immunoglobulin G), the major specific antibody class in immune serum, is then labelled by binding an anti-rabbit IgG-enzyme conjugate to the immobilized complex, bound to the ELISA well. IgG is then indirectly detected by a simple spectrophotometric enzyme assay. The choice of an ELISA system will be discussed in section 6.1.

4.2 Materials and methods

4.2.1 ELISA (non-competitive)

4.2.1.1 Reagents

(1) Phosphate buffered saline (PBS), 0.15 M, pH 7.4

8 g/l NaCl

0.2 g/l KCl

1.15 g/l Na_2HPO_3 (anhydrous)

0.2 g/l KH_2PO_4 adjusted to pH 7.4

(2) 0.05 %(v/v) Tween 80 dissolved in PBS

(3) Blocking agent

1 %(w/v) Bovine serum albumin or 2 %(w/v) 'Marvel' skimmed milk powder dissolved in PBS

(4) Horseradish peroxidase (HRP) conjugated to anti-rabbit IgG (Sigma) diluted in blocking agent (3)

(5) TMB (3,3,5,5 tetramethyl benzidine, Sigma)

The following components were prepared:

(a) 0.1 M sodium acetate buffer adjusted to pH 6.0 with 0.1 M citric acid

(b) 10 mg TMB in 1 ml DMSO (dimethyl sulphoxide), stored in dark at $4^{o}C$

(c) Substrate solution (made up just prior to use):

20 ml buffer (a)

150 µl TMB solution (b)

25 µl 3 %(w/v) H_2O_2

(6) Stopping reagent: 2.5 M H_2SO_4

4.2.1.2 Procedure

Wells of a 96 well polystyrene microtitre plate (Flow Laboratories) were coated with 50 µl aliquots of PBS each containing 1 µg of antigen. The plate was incubated at room temperature for 2 hours or overnight at $4^{o}C$ to allow antigen to bind to the plate. Wells were then aspirated and washed out with blocking agent 2-3 times. Wells were filled with 250 µl of blocking agent and incubated for 2 hours at room temperature to block all binding sites on the wells. Wells were washed 2-3 times in blocking agent and emptied by inversion of the plate onto tissue paper and tapping to remove all fluid. To each well, 50 µl of antibody solution (diluted appropriately in blocking agent) was added and the plate incubated for 2 hours at room temperature or overnight at $4^{o}C$. Wells were aspirated and washed out with PBS containing 0.05 %(w/v) Tween 80, 2-3 times followed by washes with distilled water before emptying by inversion. To each well, 50 µl of HRP-IgG conjugate (diluted 1:1000 in blocking agent) was added, followed by a 2 hour incubation at room temperature. Wells were aspirated and washed out with PBS-Tween 80 solution followed by distilled water. To each well, 100 µl of TMB substrate solution (c) was added and the reaction timed until the chromogen had produced a detectable blue colouration (10-30 minutes). The reaction was stopped by the addition of 50 µl of stopping agent (2.5 M H_2SO_4) producing a

yellow solution. Absorbance of the wells was read at 450 nm using an ELISA reader (Titertek Multiskan, Flow Laboratories).

Control wells without antigen, without antibody and without antigen and antibody were included in each assay.

4.2.2 Antibody production

Antibodies to OAT were produced in adult New Zealand rabbits using the method described by Mayer and Walker (1980).

4.2.2.1 Preparation of antigen

250 µg of lyophilized OAT purified from rat kidney, rat liver and human liver (see section 2) was used for each immunization of respective rabbits. Antigen was dissolved in 0.15 M PBS (pH 7.4) and emulsified in Freund's complete adjuvant (Sigma) for the first immunization, and in Freund's incomplete adjuvant for subsequent immunizations. Emulsification was achieved by adding an equal volume of antigen in 0.15 M PBS (pH 7.4), dropwise to the adjuvant and vortex mixing after each addition. A white creamy emulsion was produced in a total volume of 1 ml per immunization.

4.2.2.2 Control serum

Prior to immunization, rabbits were bled from the marginal ear vein. Approximately 5 ml of blood was obtained from respective rabbits by sectioning of the vein with a sterile scalpel and collecting blood into a centrifuge tube. The blood was allowed to clot overnight at $4^{o}C$ and then detached from the side of the tube with a glass rod. The clot was centrifuged at 2500 x g for 30 minutes at $4^{o}C$ and the supernatant collected, aliquoted and stored at $-20^{o}C$.

4.2.2.3 Immunization procedure

Rabbits were immobilized by being wrapped in a blanket with the head and neck exposed. Antigen was injected subcutaneously at 2-4 sites in the shoulder area using a total

volume of 1 ml of emulsion. Each injection site was wiped with 70% ethanol and aliquots of the emulsion injected using a 2 ml plastic syringe with a 0.8 mm bore needle. A sterile glass syringe was used in cases where the emulsion was viscous.

After the first OAT immunization using Freund's complete adjuvant, a further 3 booster doses using Freund's incomplete adjuvant were made at intervals of 3 weeks.

4.2.2.4 Test bleeding; monitoring immune response

Prior to each successive immunization, approximately 1 ml of blood was taken from the marginal ear vein. The ear was wiped with ethanol and heated gently with warm water to improve blood flow. Blood was taken using a 2 ml syringe with 0.5 mm bore needle and dispensed into microcentrifuge tubes for clotting and serum isolation.

Serum collected from respective rabbits was assayed for IgG directed to OAT using a non-competitive ELISA (section 4.2.1). Serial ten-fold dilutions of the serum were assayed along with control serum diluted similarly. Each dilution was assayed in triplicate.

4.2.2.5 Collection of hyperimmune rabbit serum

At the end of the immunization protocol, when each rabbit had received 4 doses of the respective OAT antigen, animals were bled out by cardiac puncture and section of the neck arteries.

Rabbits were anaesthetized with 120 mg sodium pentobarbitone (Nembutal, 60 mg/ml from Ceva Laboratories) using a dose of approximately 20 mg/Kg body weight. The anaesthetic was injected into the marginal ear vein using a 2 ml syringe with a 0.5 mm bore needle. After 15-30 minutes, blood was removed by cardiac puncture until the heartbeat was no longer detectable and no more blood could be aspirated. The animal was killed by decapitation allowing further

collection of blood from the neck arteries. The amount of blood obtained varied from 60 ml to 120 ml per rabbit. Blood was allowed to clot and serum was collected, aliquoted and stored at -20°C prior to further processing.

4.2.3 Isolation of IgG from rabbit serum

The IgG fraction of 40 ml of the hyperimmune serum obtained from the OAT immunized rabbits was purified as described by Hudson and Hay (1980).

Serum (40 ml) was warmed to 25°C in a water bath and 7.2 g of sodium sulphate was dissolved in it while stirring, to produce an 18 %(w/v) solution. The mixture was incubated at 25°C for 30 minutes and centrifuged at 3000 x g for 30 minutes at 25°C. The pellet was dissolved in 20 ml of distilled water and warmed to 25°C. Sodium sulphate was added to make a 14 %(w/v) solution (about 1.76 g). Once dissolved the solution was incubated for 30 minutes and then centrifuged as before. The pellet was dissolved in 12 ml of distilled water and dialyzed against 3 x 1000 ml of 0.07 M sodium phosphate buffer (pH 6.3). A DEAE-cellulose (Sigma) column (1.6 cm x 30 cm) was packed to give a settled wet volume of 40 ml and equilibrated with 0.07 M sodium phosphate buffer (pH 6.3). The sample was applied to the column and eluted with the column buffer under gravity at room temperature. Fractions were collected in 10 ml volumes and monitored for protein by reading absorbance at 280 nm. The early fractions containing IgG were pooled and concentrated and dialyzed against 3 x 1000 ml of 0.15 M PBS (pH 7.4). The IgG from each rabbit was aliquoted and stored at -20°C.

4.2.4 Double immunodiffusion analysis

The immunodiffusion technique of Ouchterlony was employed using the method of Johnstone and Thorpe (1982).

A 2 %(w/v) solution of agar (Difco Laboratories) was made in 0.15 M PBS (pH 7.4). The agar was melted by autoclaving at 15 p.s.i. (10.3 $N.cm^{-3}$) for 15 minutes and allowed to cool to 60°C in a water bath.

Clean glass plates (10 x 10 cm) were placed on a levelled board and 18 ml of molten agar pipetted onto each plate. When the agar had set, wells were cut into the agar using a cork borer attached to a vacuum aspirator. A template was used to produce a hexagonal well pattern (see figure 4.2). Antiserum was pipetted into the central well and the antigens (OAT) purified from human liver, rat liver and rat kidney were pipetted into surrounding wells. Immunodiffusion gels were incubated at 4°C in a moist sealed container for 2-3 days until white precipitin lines were visible in the gel. To prepare gels for protein staining, the sample wells were carefully washed out and gels immersed in 5000 ml of tap water for 24 hours to allow unbound protein to diffuse out of the gel. Gels were stained with 0.1 %(w/v) Coomassie Brilliant Blue G and destained as described for SDS-PAGE (section 3.2.1.4).

4.3 Results

4.3.1 Assessment of ELISA

Serial 10-fold dilutions of serum produced non-specific binding of protein to the wells of ELISA plates, at serum concentrations above a dilution of 10^{-3} for all sera tested. Absorbance values due to non-specific binding for no-antigen controls using test sera were not significantly different from values obtained for control sera, with antigen present, at all dilutions tested between 10^{-2} and 10^{-7}. Non-specific binding to immobilized antigen (OAT) does not therefore represent a significant cause of increased absorbance. The use of either 1 %(w/v) bovine serum albumin or 2 %(w/v) Marvel produced no significant difference in absorbances of control or test wells. Marvel was therefore chosen as a blocking agent for economic

reasons. Absorbances obtained for control sera were subtracted from test sera at respective dilutions. 'No-antibody' controls were not significantly different from 'No-antigen/antibody' controls, typical absorbances of 0.013 $\pm$ 0.003 (mean $\pm$ S.E., n = 20) were obtained at all dilutions tested. This indicates that the IgG-horseradish peroxidase conjugate does not bind non-specifically to the antigen or blocked ELISA well at the concentration (a dilution of 10^{-3} of stock conjugate) used in the assay (see section 4.2.1.1).

4.3.2 Immune response to OAT

The immune response of rabbits to successive OAT doses was assessed by measuring the dilution of antiserum required to produce 50% saturation of antigen bound to ELISA wells, (example for rat kidney given in figure 4.1A). Antisera obtained after successive antigen doses required successively higher dilutions to produce 50% saturation of bound antigen. Plots of dilution factor against dose of antigen produced similar immune response patterns for OAT from rat and human tissues (figure 4.1B).

4.3.3 Isolation of IgG from serum

Relative antibody activity was estimated as a function of the dilution of each fraction required to produce 50% saturation of antigen bound to an ELISA plate. 'No-antigen' control absorbances were subtracted from test data. These batches of IgG were used for development of a competitive ELISA (section 6). Crude IgG fractions were isolated from antisera raised against rat kidney OAT and human liver OAT, producing purification factors of about 6.5 (Tables 4.1 and 4.2). High percentage recoveries were obtained at the expense of some contamination in order to obtain a representative heterogeneous population of IgG.

4.3.4 Double immunodiffusion data

Precipitin lines were obtained between all 3 antisera reacted with the 3 OAT samples (figure 4.2). No precipitin lines were produced when OAT was incubated with control sera in all cases. Precipitin lines fused between rat kidney and rat liver OAT indicating a reaction of identity, demonstrating that OAT from the 2 rat sources are immunologically identical with respect to anti-rat kidney OAT antiserum (figure 4.2A) and also for anti-human and rat liver OAT antisera. Precipitin lines between human liver OAT and both rat OAT antigens fused with spur formation. This indicates a reaction of partial identity where some antigenic determinants of OAT are identical but additional determinants are recognized as being unique to human OAT (figure 4.2C) and rat OAT (figure 4.2A) with respect to the antisera used.

TABLE 4.1

ISOLATION OF IgG FROM RABBIT SERUM (ANTI-RAT KIDNEY OAT)

FRACTION	VOLUME (ml)	RELATIVE ANTIBODY ACTIVITY (UNITS/ ml)	TOTAL RELATIVE ANTIBODY ACTIVITY (UNITS)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (UNITS/ mg)	% RE- COVERY	PURIF. FACTOR
SERUM AMMONIUM SULPHATE	40	20	800	2816	0.284	100	1
DIALYSATE ION-EXCHANGE	13	58.4	759	853	0.89	95	3.13
DIALYSATE (CONCENT.)	15	46.8	702	378	1.86	88	6.6

TABLE 4.2

ISOLATION OF IgG FROM RABBIT SERUM (ANTI-HUMAN LIVER OAT)

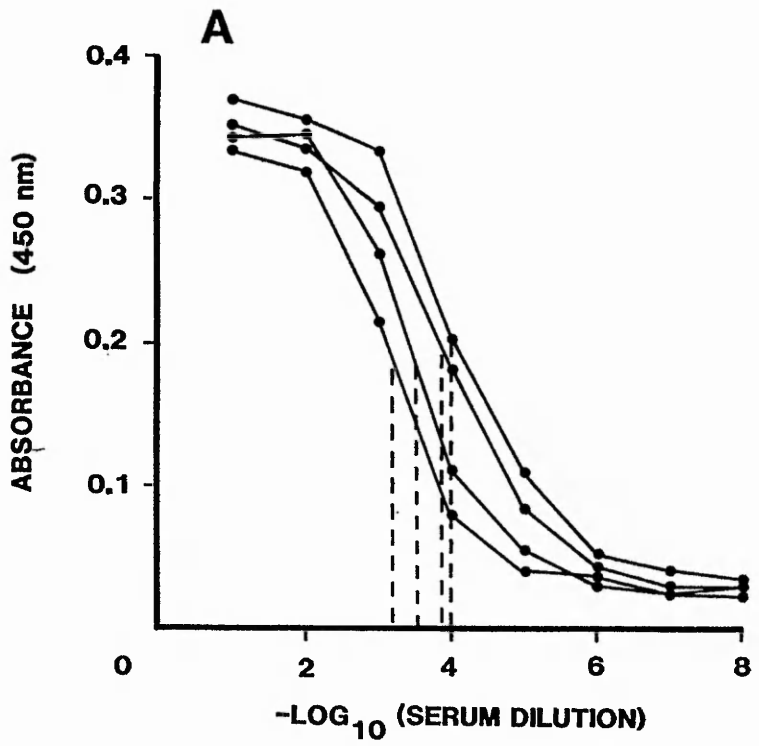
FRACTION	VOLUME (ml)	RELATIVE ANTIBODY ACTIVITY (UNITS/ ml)	TOTAL RELATIVE ANTIBODY ACTIVITY (UNITS)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (UNITS/ mg)	% RE- COVERY	PURIF. FACTOR
SERUM AMMONIUM SULPHATE	40	16	640	2492	0.257	100	1
DIALYSATE ION-EXCHANGE	13	46.3	602	707	0.851	94	3.3
DIALYSATE (CONCENT.)	15	36.4	546	330	1.65	85	6.4

1 unit = antibody dilution factor ($X10^4$) producing 50% saturation of antigen bound to ELISA plates under ELISA conditions (see section 4.2.1).

FIGURE 4.1 Titration of rabbit anti-OAT serum by non-competitive ELISA

A. Titration of test sera obtained from rabbits immunized with rat kidney OAT using the non-competitive ELISA. Serum dilution was estimated at 50% saturation of antigen (indicated by broken lines from left to right corresponding to successive antigen doses) bound to an ELISA plate (50% maximum absorbance).
Mean values (n=3) are plotted.

B. Monitoring of immune response by non-competitive ELISA. Serum dilution factor (reciprocal of serum dilution producing 50% saturation of antigen) was plotted against dose of antigen administered 3 weeks prior to assay.
Immune responses are plotted for rabbits immunized with the following antigens:
   A. Rat kidney OAT
   B. Rat liver OAT
   C. Human liver OAT
Mean values ± S.E. (n=3) are plotted.


A
0.4
0.3
0.2
0.1
0
ABSORBANCE (450 nm)
2
4
6
8
-LOG10 (SERUM DILUTION)

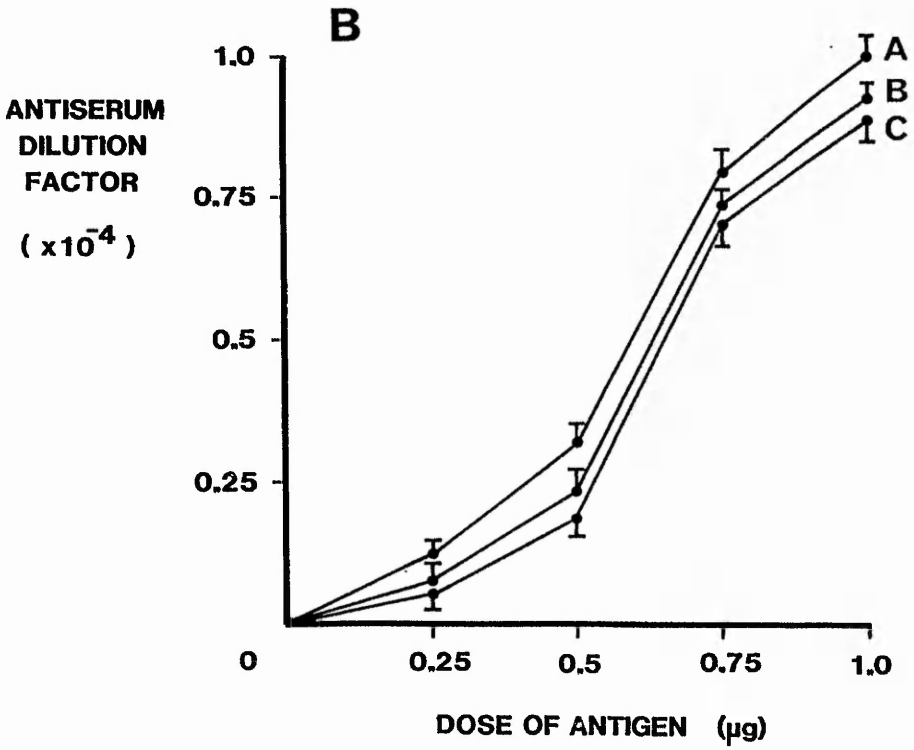

B
1.0
0.75
0.5
0.25
0
ANTISERUM DILUTION FACTOR
(x10-4)
A
B
C
0.25
0.5
0.75
1.0
DOSE OF ANTIGEN (µg)

FIGURE 4.2 Structural comparison of rat and human OAT by immunodiffusion analysis

Double immunodiffusion analysis in 2 %(w/v) agar made up in 0.15 M PBS (pH 7.4). All wells contained 20 µl sample as follows:

HL: Human liver OAT

RL: Rat liver OAT

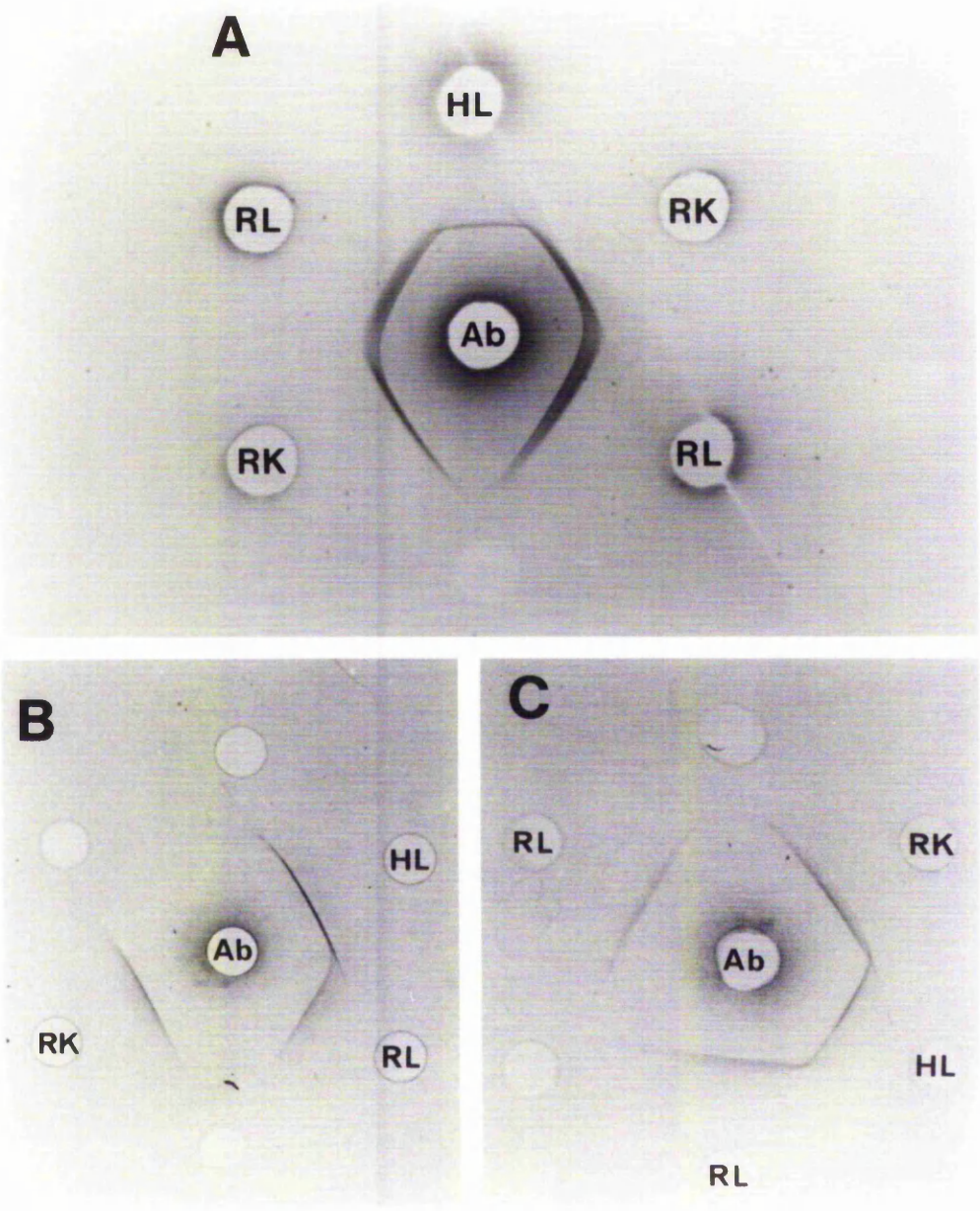
RK: Rat kidney OAT

Ab: Antisera

A. Antiserum raised to rat kidney OAT
   RL: 40 µg
   RK: 40 µg
   HL: 20 µg

B. Antiserum raised to human liver OAT
   RL: 20 µg
   RK: 20 µg
   HL: 20 µg

C. Antiserum raised to rat liver OAT
   RL: 20 µg
   RK: 20 µg
   HL: 20 µg


A
HL
RL
RK
Ab
RK
RL
B
HL
Ab
RK
RL
C
RL
RK
Ab
HL
RL

4.4 Discussion

The non-competitive ELISA used in this study has been successfully employed for assay of IgG in serum. Non-specific binding of serum protein occurs at high concentrations of serum, but this can be accounted for by subtracting identical control assays in the absence of antigen. This enables reliable readings to be made at high protein concentrations of serum. Reproducibility of absorbance values from one assay to another is poor since washing and incubation steps are performed manually and enzyme activity of the horseradish peroxidase conjugate is likely to vary. However, since titres of serum are being determined at the point of 50% saturation of antigen, this estimation should be reproducible since the defined titre is independent of absolute absorbance values.

Titres of IgG used as a function of immune response are not strictly comparable in spite of being very similar for human and rat OAT (see figure 4.1B). This is due to the difference between human and rat OAT antigen and the difference in IgG populations in each antisera, since the ELISA measures IgG as a function of the net avidities of the IgG population and not just the amount of IgG protein. Immune response patterns appear to be reaching a plateau after the fourth dose of antigen, indicating a near maximal hyperimmune serum. It will be shown later that the final titre of IgG is equivalent to a high yield of IgG as measured by quantitative precipitin data (see section 5).

Isolated crude IgG from serum should contain a representative population of IgG molecules of both high and low affinity. These will be used for development of a competitive ELISA (section 7). Removal of contaminating proteins should eliminate non-specific binding at high concentrations of antibody and purified IgG is necessary for efficient coating of ELISA wells during development and application of a competitive ELISA.

Immunodiffusion data provide further evidence that rat OAT purified from rat kidney, liver and other tissues is the same protein and that human and rat OAT are structurally similar. Sanada et al. (1970) have shown that OAT purified from rat kidney, liver and small intestine was immunologically identical, an observation also supported by Lyons and Pitot (1976). Immunodiffusion data reported by Ohura et al. (1982) demonstrated a reaction of partial identity between human and rat OAT and this is in agreement with data obtained in this study. The similarity of human and rat OAT is exploited in the work described in Section 5, where anti-rat kidney OAT antiserum has been used to purify human OAT.

5 DEVELOPMENT OF AN IMMUNOADSORPTION METHOD OF OAT PURIFICATION FROM HUMAN TISSUE

5.1 Introduction

The similarity of rat and human OAT has been discussed in previous sections. Furthermore, OAT has been relatively easily purified from rat kidney whereas human OAT has been purified by complex procedures from liver by the only method reported for human tissue (Ohura et al., 1982).

The use of immunoadsorption for affinity purification of specific antibodies is an established procedure (Hudson and Hay, 1980) but has limited application to antigen purification since deforming agents often required for desorption frequently denature and inactivate labile proteins. Low affinity antibodies require less severe desorption conditions, however, and electrophoretic desorption (Dean et al., 1977; Mayer and Walker, 1980) avoids the use of chemical desorption altogether.

In this study, the close structural identity of human and rat OAT has been exploited to produce an OAT-specific, species cross-reacting immunoadsorbent. Relatively high recovery purification of human liver OAT was achieved by employing electrophoretic desorption of the immunoadsorbent.

The purpose of developing such a procedure is to provide a method of isolating pure OAT from crude tissue extracts. This method could be used to isolate small amounts of OAT which may be present in tissues from patients with Gyrate Atrophy. If this can be achieved then the enzyme could be characterized to assess the possibility of an abnormal OAT protein being produced in these patients. Immunoadsorption of OAT is further employed in section 7 from blood platelets which may provide a suitable human tissue for study.

5.2 Materials and methods

5.2.1 Quantitative precipitin test

A quantitative precipitin test was performed as described by Hudson and Hay (1980) on rat kidney OAT antiserum. PBS containing pure rat kidney OAT ranging from 0-450 ug protein in 0.45 ml was aliquoted into microcentrifuge tubes. To each tube, 0.1 ml of rat kidney OAT antiserum was added. Tubes were incubated at 4^{o}C for 7 days to allow immunoprecipitation to occur. Tubes were centrifuged at 3000 x g for 5 minutes in an angle-head microcentrifuge. The white precipitate was washed twice by centrifugation with 1 ml PBS. The final pellets were dissolved in 1 ml of 0.1 M sodium hydroxide. Absorbances were read at 280 nm and a plot of absorbance of dissolved precipitate against amount of antigen added was constructed. A preliminary test was performed in order to choose a close range of OAT concentrations around the point of equivalence. The concentration of OAT and IgG required to produce an absorbance of 1.0 at 280 nm was determined. Results were used to determine the IgG content and antigenic valency of rat kidney OAT antiserum.

5.2.2 Affinity purification of IgG

IgG was affinity-purified from rabbit serum using rat kidney OAT immobilized to cyanogen bromide activated Sepharose 4B (Sigma Chemical Co.) using the method of Dalchau and Fabre (1986). 20 mg of lyophilised rat kidney OAT was dissolved in 4 ml of 'coupling' buffer (pH 8.3), containing 0.1 M sodium carbonate dissolved in 0.5 M hydrochloric acid. Pre-swollen cyanogen bromide activated Sepharose 4B equilibrated with coupling buffer was mixed with OAT (5 mg protein per ml of gel) overnight at 4^{o}C by end-over-end rotation. This mixture was transferred to a scintered glass funnel and sucked dry under vacuum. The fluid was recovered and assayed for protein to assess coupling efficiency. The gel was washed thoroughly with coupling buffer and

mixed as before, for 2 hours with 1 M ethanolamine (pH 8.0). The gel was sucked dry and washed with coupling buffer. Protein non-covalently adsorbed to the gel was removed by washing the gel with alternating buffers of low pH (0.1 M sodium acetate, 1 M sodium chloride, pH 4.0) and high pH (0.025 M Tris, 1 M sodium chloride, pH 8.2). The OAT-linked Sepharose was washed with 0.15 M PBS (pH 7.4) which contained 0.1 M sodium azide during storage at 4°C.

Quantitative precipitin test results were used to choose a suitable serum volume of known IgG content for affinity purification. 8 ml of antiserum was mixed with the OAT-linked Sepharose 4B prepared as described, by end-over-end rotation for 1 hour at 4°C. The gel was transferred to a mini-column and washed with 100 ml of 0.15 M PBS (pH 7.4) until collected fractions gave negligible readings at 280 nm. A batch of IgG was eluted with 20 ml of 0.1 M glycine-hydrochloric acid buffer (pH 2.5). A further batch of higher affinity IgG was eluted with 0.1 M glycine-hydrochloric acid (pH 2.5) containing 10 %(w/v) dioxane (BDH Ltd., Dorset, UK.). All fractions (5 ml aliquots) were immediately adjusted to pH 8.0 with solid Tris. The two populations of IgG fractions were pooled separately, and concentrated by collodion bag filtration (Sartorius Instruments Ltd., Surrey, UK) prior to dialysis against 5000 ml of 0.15 M PBS (pH 7.4). 4 ml of the lower affinity IgG was dialyzed against the coupling buffer (pH 8.3), adjusted to 5 mg/ml protein and mixed with 4 ml of swollen cyanogen bromide activated Sepharose 4B. The IgG was coupled by the method described for OAT coupling.

5.2.3 Immunoadsorption of OAT from human liver

Frozen human liver tissue was thawed, minced and homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 5 mM 2-oxoglutarate and 20 uM pyridoxal phosphate using a Waring blender at top speed for 30 seconds. All steps were performed at 4°C. This produced a 20 %(w/v)

homogenate which was centrifuged at 18,000 x g for 20 minutes. The supernatant was heated to 60^{o}C for one minute and rapidly cooled in ice. Precipitated protein was removed by centrifugation at 18,000 x g for 10 minutes. The supernatant was adjusted to pH 7.4 and mixed with Sepharose 4B at 1 ml per 10 ml of supernatant. Mixing by end-over-end rotation for 1 hour removed any proteins likely to adsorb non-specifically to the immunoadsorbent. The supernatant fluid was isolated by vacuum filtration through scintered glass and mixed with 1.5 ml of the prepared immunoadsorbent pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.4) by end-over-end rotation at 4^{o}C overnight. The mixture was transferred to a vacuum filter to remove unbound protein and washed with 150 ml of 10 mM potassium phosphate buffer (pH 7.4). The immunoadsorbent was transferred to the eluting column of a preparative gel electrophoresis system (Bethesda Research Laboratories Ltd., UK) which was set up as shown (figure 5.1). Both reservoirs, eluting column and collecting chamber contained 10 mM potassium phosphate buffer (pH 7.4). Dialysis membrane covered the base of the collecting chamber and the top of the eluting column. Once the immunoadsorbent was packed, a current of 10 mA with variable voltage was applied across the two reservoirs for 40 minutes at 4^{o}C. After desorption the current was switched off, the top of the reservoir removed and buffer decanted above the immunoadsorbent. The desorbed protein was removed through a valve in the collecting chamber by syringe. The protein was dialyzed against 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and 20 µM pyridoxal phosphate.

5.3 Results

5.3.1 Quantitative precipitin data

IgG produces an absorbance at 280 nm of 1.0 at a concentration of 0.695 mg/ml in 0.1 M sodium hydroxide. Similarly, rat kidney OAT at a concentration of 1.15 mg/ml produced an absorbance of 1.0.

From the precipitin curve (figure 5.2), at the point of equivalence, the absorbance at 280 nm of the dissolved precipitate equals 0.84 where 120 µg of antigen is assumed to be bound to antibody. These data were used to make the following calculations:

(i) 120 µg of OAT is equivalent to an absorbance (280 nm) of 0.104.

(ii) Absorbance (280 nm) due to bound antibody = 0.84 - 0.104 = 0.736.

(iii) 0.1 ml of antiserum contained (0.736 x 0.695)mg IgG.

(iv) IgG content of rat kidney OAT antiserum = 5.1 mg/ml.

5.3.2 Estimation of antigenic valency

At extreme antibody excess, every antigenic determinant is likely to be covered by a separate antibody molecule. If the amount of antibody in the precipitate is calculated at this point, the ratio of antibody to antigen gives the antigenic valency of the antibody. Ratios of antibody:antigen molecules were determined using quantitative precipitin data at antibody excess. The molecular weight of IgG was assumed to be 150,000 and that of rat kidney OAT, 45,000. By plotting these ratios against total antigen used in the quantitative precipitin test (figure 5.3) and extrapolating the line, the theoretical ratio at infinite antibody excess was determined. An antigenic valency of 4 was estimated for rat kidney OAT antiserum.

5.3.3 Coupling of OAT to cyanogen bromide activated Sepharose 4B

Of the 20 mg of pure rat kidney OAT protein incubated with 4 ml of activated Sepharose 4B, 18.6 mg of protein bound to the gel producing a binding efficiency of 93% and OAT-linked Sepharose 4B at a capacity of 4.65 mg of OAT per ml of gel.

5.3.4 Affinity purification of anti-rat kidney OAT antibody

A volume of 8 ml of rat kidney OAT antiserum containing an estimated 40.8 mg of specific IgG from quantitative precipitin data, was incubated with the OAT-Sepharose 4B. Out of a total serum protein content of 422.4 mg, an estimated 45 mg bound to the gel, since 377.4 mg protein was recovered. Unbound protein contained no significant IgG activity by non-competitive ELISA. Assuming a binding efficiency of 100%, the binding capacity of OAT-Sepharose 4B was estimated at 11.25 mg of IgG bound per ml of gel.

The recovery of IgG desorbed from the OAT-Sepharose 4B by 0.1 M glycine-hydrochloric acid buffer (pH 2.5) was 73% (batch 1) and a further 15% (batch 2) was recovered by elution with the same buffer containing 10 %(w/v) dioxane. After concentration and dialysis of the lower affinity IgG (batch 1), recovery was reduced to 61%.

5.3.5 Coupling of IgG to cyanogen bromide activated Sepharose 4B

Of the 20 mg of IgG (batch 1) incubated with 4 ml of activated Sepharose 4B, 16.8 mg bound to the gel producing a binding efficiency of 84% and IgG-linked Sepharose 4B at a capacity of 4.2 mg IgG per ml of gel.

5.3.6 Assessment of antibody affinity

In order to assess antibody affinity, a non-competitive ELISA was performed on serial dilutions of respective antibody batches. IgG concentration was determined at 50% saturation of the antigen and this value used as an indicator of apparent antibody affinity in each case (figure 5.4). The lower the IgG concentration needed for 50%

saturation, the higher the affinity of the antibody for the antigen. Results for antibody titrated against rat OAT antigen were as follows: anti-rat OAT antiserum, 0.2 nM; IgG (batch 1), 0.36 nM; IgG (batch 2), 0.02 nM. This indicates that IgG (batch 1) has a twenty-fold lower affinity than IgG (batch 2) for rat OAT. IgG (batch 1) titrated against human OAT produced a value of 0.63 nM suggesting a slightly lower affinity for human antigen than for rat antigen.

5.3.7 Immunoadsorption of OAT from human liver

Purification of human liver OAT by immunoadsorption (Table 5.1) produced a specific activity of 28.13 units/mg for the desorbed enzyme and a yield of 56%. Assessment of OAT purity (figure 5.5, lane D) by SDS-PAGE showed a single band with very minor contaminants desorbed from the immunoadsorbent. The extent of purification can be seen by a comparison with crude extracts (figure 5.5, lanes B and C).

5.3.8 Assessment of binding capacity and chemical desorption

The maximum binding capacity of the immunoadsorbent was determined by mixing 250 µl aliquots of gel with 1 ml aliquots of rat kidney OAT at 1,5 and 10 mg/ml respectively, overnight at $4^{o}C$. The immunoadsorbent contained 1 mg of immobilized antibody per 250 µl and the maximum binding of OAT was 0.42 mg at 10 mg/ml. The effect of chemical desorption was briefly assessed by incubating unbound rat kidney OAT with 1 M propionic acid (pH 2.6) and 50 mM diethylamine (pH 11.5) respectively, both containing 0.5 g/100 ml Triton X-100. A 20 minute incubation followed by dialysis against 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and 20 uM pyridoxal phosphate resulted in total loss of activity under acidic conditions and 40% loss under alkaline conditions.

5.3.9 Immunoadsorption of OAT from rat kidney

A similar immunoadsorbent of anti-rat kidney OAT antibody, IgG (batch 1) linked to Sepharose 4B as described in this section, was used to

purify OAT from a crude rat kidney extract. The binding capacity of the immunoadsorbent was 4.4 mg IgG per ml of gel. The purification method was identical to that used for human liver (section 5.3.2). The purified enzyme had a specific activity of 34.7 units/mg (Table 5.2) and a yield of 50%. Minor contaminants just visible after SDS-PAGE (figure 5.5) have similar relative mobilities to those present in the purified human enzyme.

TABLE 5.1

PURIFICATION OF HUMAN LIVER OAT BY IMMUNOADSORPTION

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	% YIELD	SPECIFIC ACTIVITY (units/mg)	PURIFICATION FACTOR
HOMOGENATE	100	3080	100	0.0104	1
60°C SUPERNATANT	81	1758	103[a]	0.0188	1.81
DESORBED PROTEIN	4	0.64	56	28.13	2705

[a] Yield increases due to mitochondrial release of sequestered OAT.

1 unit = 1 μmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

TABLE 5.2

PURIFICATION OF RAT KIDNEY OAT BY IMMUNOADSORPTION

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	% YIELD	SPECIFIC ACTIVITY (units/mg)	PURIFICATION FACTOR
HOMOGENATE	100	4000	100	0.033	1
60°C SUPERNATANT	82	738	77	0.14	4.24
DESORBED PROTEIN	4.3	1.9	50	34.7	1051

1 unit = 1 μmole of Δ'-pyrroline-5-carboxylate formed per minute at 37°C.

FIGURE 5.1 Diagrammatic representation of the electrophoretic desorption apparatus

Diagrammatic representation of the electrophoretic desorption system described in the text. Dialysis membrane (D) was held in position by rubber 'O' rings on the top of the eluting column and on the base of the collecting chamber. Desorbed protein was removed via valve A after draining the immunosorbent via valve B.

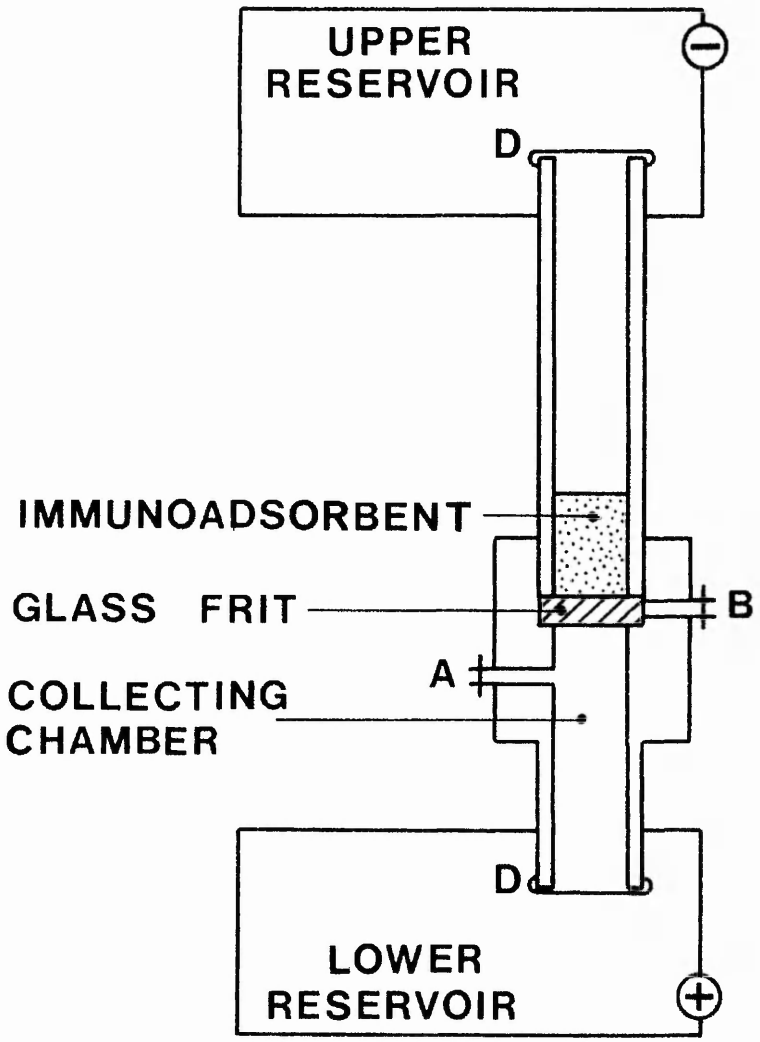

UPPER
RESERVOIR
D
IMMUNOADSORBENT
GLASS FRIT
B
A
COLLECTING
CHAMBER
D
LOWER
RESERVOIR

FIGURE 5.2 Quantitative precipitin curve of rat kidney OAT with rat kidney OAT antiserum

Increasing amounts of antigen were added to fixed concentrations of antiserum and the absorbance of the precipitate determined. The equivalence point, when all the antibody and antigen is complexed in the precipitate, occurs just before the point of maximum precipitation.

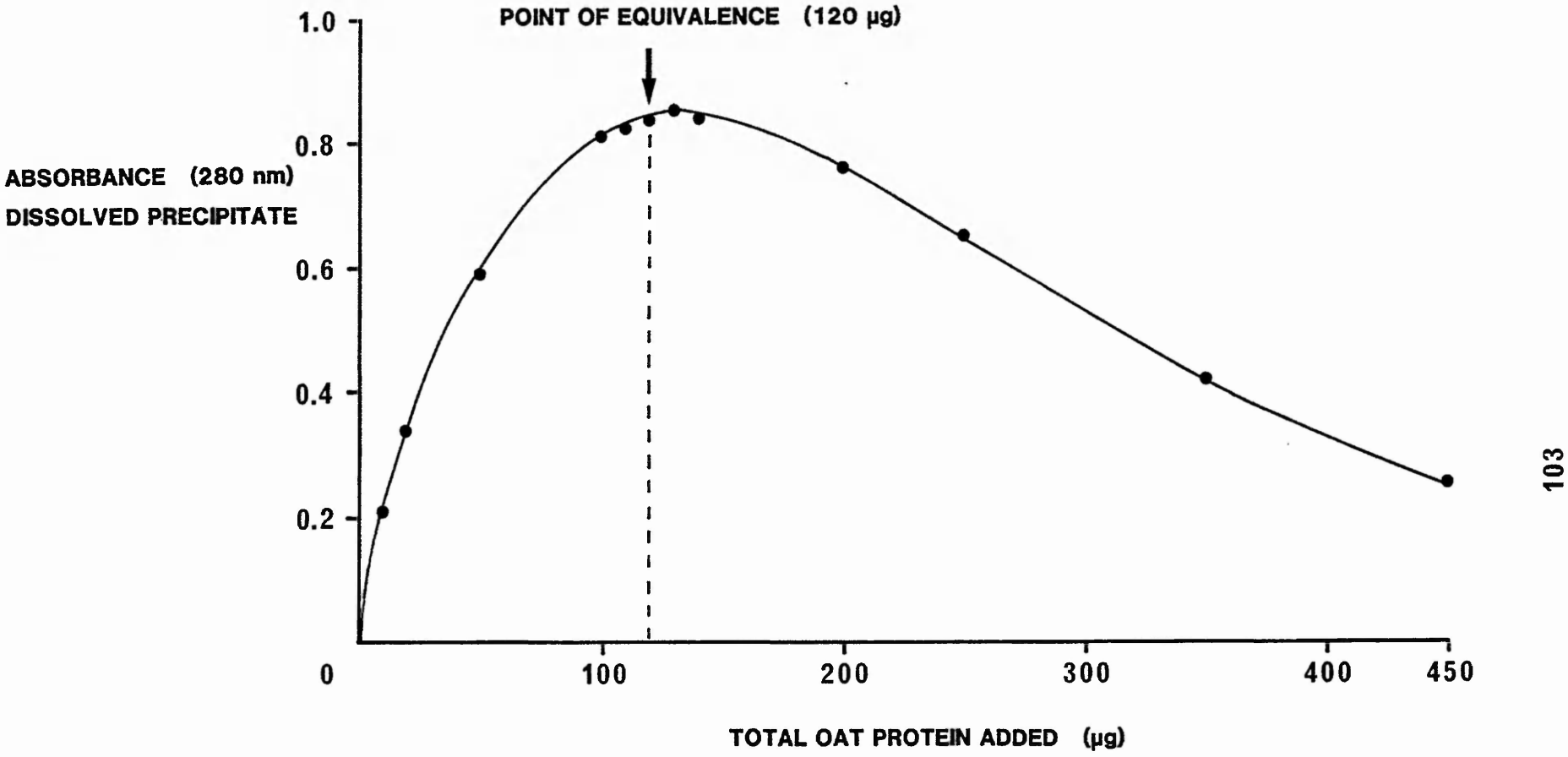

POINT OF EQUIVALENCE (120 µg)
ABSORBANCE (280 nm)
DISSOLVED PRECIPITATE
1.0
0.8
0.6
0.4
0.2
0
100
200
300
400
450
TOTAL OAT PROTEIN ADDED (µg)

FIGURE 5.3 Estimation of the antigenic valency of rat kidney OAT antiserum

A regression line, fitted by the method of least squares, was extrapolated back to determine the ratio of antigen to antibody molecules at infinite antibody excess. Antigenic valency was estimated at 4 IgG molecules per molecule of OAT. Data was obtained from a quantitative precipitin curve (Figure 5.2).

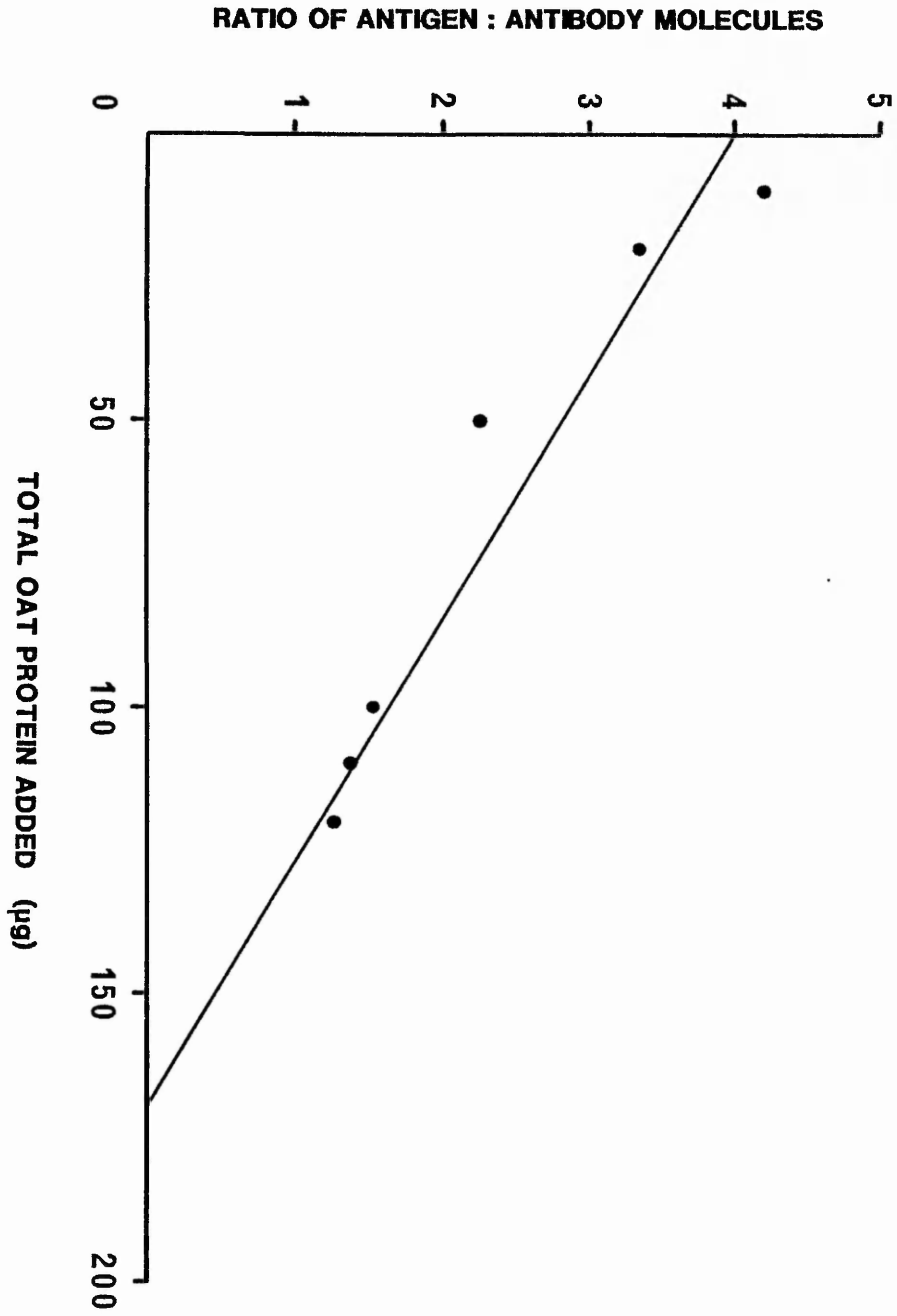
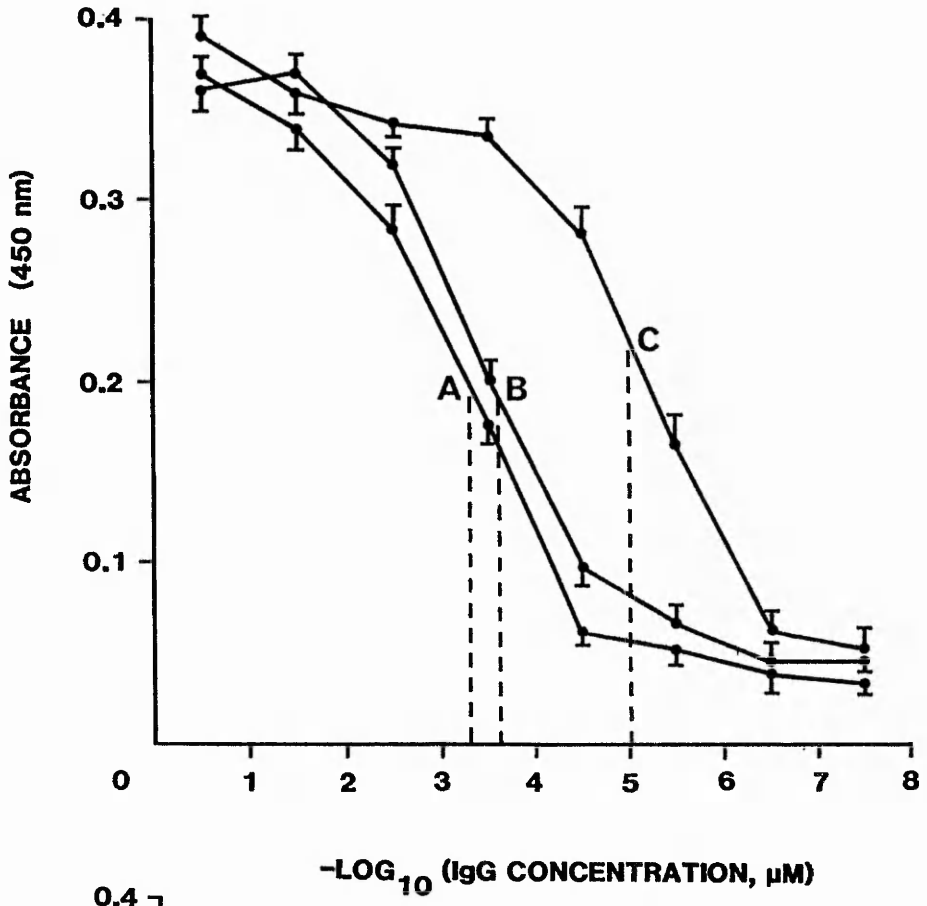

RATIO OF ANTIGEN : ANTIBODY MOLECULES
0
1
2
3
4
5
50
100
150
200
TOTAL OAT PROTEIN ADDED (µg)

FIGURE 5.4 Assessment of antibody affinity

IgG concentration was determined at 50% saturation of antigen by non-competitive ELISA and this value used as an indicator of apparent antibody affinity.

A. IgG (batch 1) titrated against human OAT
B. IgG (batch 1) titrated against rat OAT
C. IgG (batch 2) titrated against rat OAT
D. Anti-rat OAT antiserum titrated against human OAT
E. Anti-rat OAT antiserum titrated against rat OAT.


0.4
0.3
0.2
0.1
0
ABSORBANCE (450 nm)
A
B
C
1
2
3
4
5
6
7
8
-LOG10 (IgG CONCENTRATION, μM)

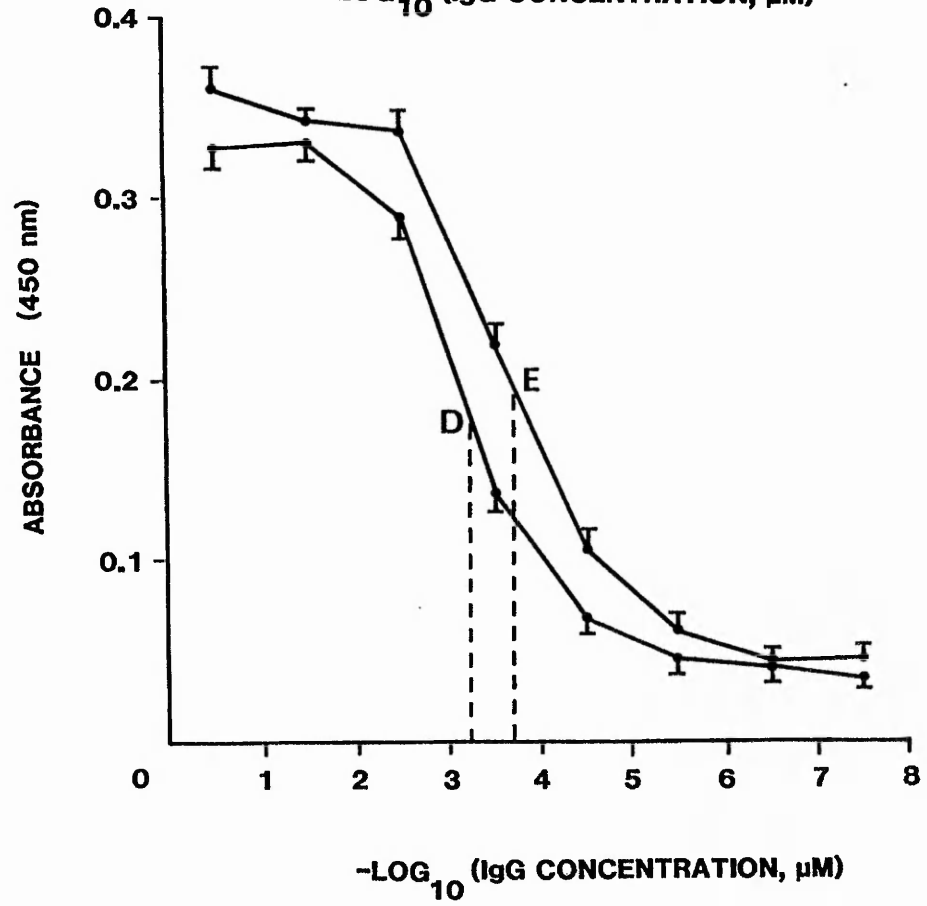
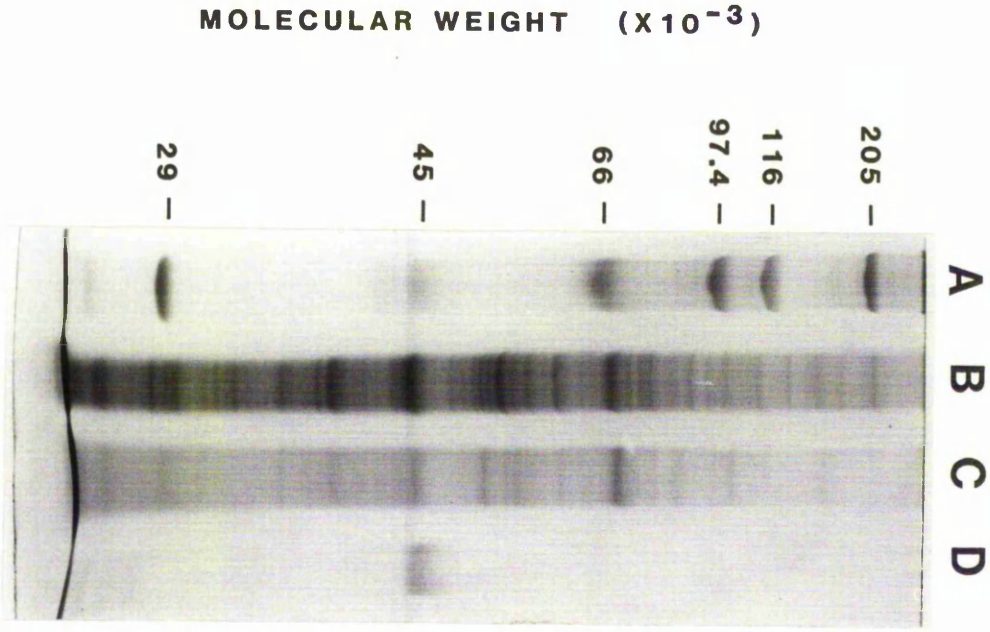
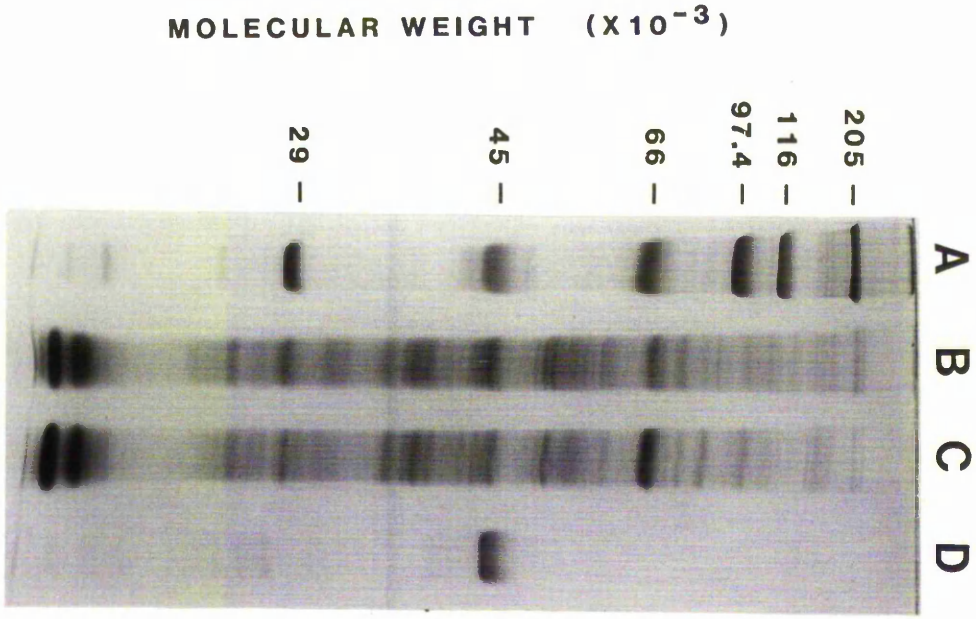

0.4
0.3
0.2
0.1
0
ABSORBANCE (450 nm)
D
E
1
2
3
4
5
6
7
8
-LOG10 (IgG CONCENTRATION, μM)

FIGURE 5.5 SDS-PAGE of human liver and rat kidney fractions before and after immunoadsorption of OAT

Both rat and human homogenates (lane B) were subjected to heat treatment as described in the text to produce a particle free extract of soluble proteins containing OAT (lane C). OAT protein was immunoadsorbed and desorbed from the soluble extract as described in section 5.2.3.
Samples of human extract were as follows:
lane B, homogenate (60 µg)
lane C, 60°C supernatant (60 µg)
lane D, desorbed protein (6 µg)
Samples of rat extract were as follows:
lane B, homogenate (60 µg)
lane C, 60°C supernatant (30 µg)
lane D, desorbed protein (3 µg)
Protein markers (lane A) were as follows:
carbonic anhydrase (28,000)
egg albumin (45,000)
bovine serum albumin (66,000)
phosphorylase B (97,400)
β -galactosidase (116,000)
myosin (205,000)


MOLECULAR WEIGHT (X10⁻³)
205
116
97.4
66
45
29
A
B
C
D
RAT KIDNEY


MOLECULAR WEIGHT (X10⁻³)
205
116
97.4
66
45
29
A
B
C
D
HUMAN LIVER

5.4 Discussion

The conventional methods needed to purify human liver OAT require the use of a series of different processes which can be costly and time consuming. Each additional step in purification usually results in loss of yield and often necessitates concentration and dialysis before work can continue (see figure 2.3). As a result, low yields of pure protein are obtained and large volumes of crude extract often have to be processed in order to obtain sufficient end-product. By using an immunoadsorbent, however, most of these problems can be overcome.

The specific adsorption of human liver OAT onto the immunoadsorbent used in this study has relied on the close structural similarity between human and rat OAT, the latter being easily purified from rat kidney. It was therefore possible to purify human OAT using anti-rat OAT antibodies for immunoadsorption. Initial purification of human OAT by more complex methods can thus be avoided and would not be required for antibody production nor affinity purification of specific IgG. The advantage of being able to use IgG raised to rat kidney OAT is that the affinity for the human source is likely to be lower and therefore more easily desorbed from an immunoadsorbent. Purification of rat kidney OAT by immunoadsorption produced a recovery of 50% compared to 56% for human OAT. This may be due to a higher affinity of rat kidney IgG for the rat kidney enzyme.

Assessment of apparent antibody affinity by ELISA suggests that the IgG used for immunoadsorption (batch 1) was of lower affinity than the original antiserum and that it was separated from a small population of much higher affinity IgG (batch 2) prior to immobilization. It is important to note that the term 'affinity' applied to polyclonal antibodies refers to the functional affinity (avidity) of antibody molecules and, therefore, assessment of apparent antibody affinity by ELISA can only provide an indication of the

average affinity of the total number of binding sites in the antibody population. The test is more valid for monoclonal antibodies (Heyningen, 1986). A low affinity immunoadsorbent should prolong its useful shelf-life by preventing irreversible binding of OAT onto high affinity IgG after successive OAT purifications.

The maximum theoretical binding capacity of the immunoadsorbent occurs when both binding sites of every immobilized antibody molecule bind a molecule of OAT. Assuming the molecular weights of OAT and IgG are 45,000 and 160,000 respectively, 1 mg of immobilized IgG could bind 0.563 mg of OAT. The capacity of the immunoadsorbent used in this study (4.2 mg IgG bound per ml of Sepharose 4B) was estimated at 0.42 mg of OAT bound per mg of immobilized IgG (75% of the theoretical maximum). This estimation enables the immunoadsorbent to be used at a submaximal binding capacity with tissue extracts. However, OAT-linked Sepharose 4B has a higher binding capacity for IgG since a number of IgG molecules can bind to the OAT molecule depending upon antigenic valency. The valency of OAT for IgG raised to rat kidney OAT was estimated at four, calculated from quantitative precipitin data (section 5.3.1).

The binding of OAT from large volumes of crude tissue extract onto small volumes of immunoadsorbent appears to be very efficient and effectively concentrates the enzyme. Dean et al. (1977) were able to purify ferritin by a factor of 50,000 from 1000 ml of plasma using 1.5 ml of immunoadsorbent. Non-specific adsorption of human liver proteins was prevented by pre-incubation of the extract with Sepharose 4B which effectively removed these proteins prior to immunodasorption.

The use of the electrophoretic desorption apparatus was modified to produce conditions similar to those described previously (Dean et al., 1977). The desorbed protein could be concentrated in the collecting chamber (figure 5.1) by reducing the chamber volume,

although Mayer and Walker (1980) have noted the use of a sample concentrator (ISCO Model 1750, USA) which can elute and concentrate the antigen simultaneously. Desorption of proteins from immunoaffinity (or other types of affinity) resins by electrophoresis has not as yet been properly exploited and remains mostly unused. This is perhaps surprising since such a desorption method would appear to be a good solution to the problem of harsh chemical desorption which is the only major drawback of immunoadsorption or immunoaffinity chromatography applied to enzyme purification. Where a specific immunoadsorbent cannot be produced it may be possible to combine the technique of preparative gel electrophoresis with desorption by overlaying an acrylamide gel with immunoadsorbent, for example. Desorbed proteins could then be separated through the gel and collected as fractions. By avoiding the use of chemical desorbing agents, the useful life of the immunoadsorbent and the recovery of desorbed native protein should be greatly improved. Electrophoretic desorption of higher affinity adsorbents may, however, not be as effective and could result in build up of tightly bound antigen. This may necessitate chemical desorption in order to restore binding capacity after several uses.

The development of an OAT immunoadsorbent provides a useful tool for the purification of small amounts of OAT from crude tissue extracts. This provides the potential for purification of any OAT protein in tissue extracts from patients with Gyrate Atrophy. This would enable characterization of OAT from such a patient and may determine whether an abnormal enzyme is being produced or whether low levels of normal OAT are present. The feasibility of the methodology developed in this section for OAT purification from human tissue extracts will be investigated later.

6 DEVELOPMENT OF A COMPETITIVE ELISA FOR OAT

6.1 Introduction

Immunoassays involve the specific binding of antigen with antibody to allow determination of the concentration of antigen or antibody in unknown samples. Radioimmunoassays which employ a radiolabelled antigen or antibody are the most common immunoassay techniques but more recently alternative labels such as enzymes (ELISA) or fluorochromes (fluorescence immunoassay) have started to replace radioisotopes.

In this study, development of an ELISA for OAT was chosen because of the simplicity, safety and economy of the reagents and techniques used. The disadvantages of a radioimmunoassay is that the radioisotopes needed for assay are relatively expensive and produce an additional hazard not encountered with enzyme-antibody conjugates. The ELISA technique described earlier (section 4.2.1) was a non-competitive assay since antibody was binding onto antigen immobilized onto ELISA plate wells prior to labelling with enzyme-antibody conjugate. This assay was used to measure antibody (IgG) whereas the competitive ELISA developed in this section measures OAT antigen. The assay is competitive since competition for binding sites on free antigen and bound antigen is involved in the presence of a fixed limiting amount of specific anti-OAT antibody (IgG).

The purpose of developing a competitive ELISA for OAT is to produce a sensitive assay capable of detecting deficient levels which may be found in the tissues of Gyrate Atrophy patients and which can detect both active and inactive OAT. This ELISA is applied to OAT measurement in rat and human tissues in section 7.

6.2 Materials and methods

6.2.1 Determination of optimal bound antigen concentration

Human and rat OAT were bound to wells of an ELISA plate at concentrations of 1 µg, 0.2 µg and 0.02 µg in an incubation volume of 50 µl containing 2 %(w/v) 'Marvel' in PBS. Isolated IgG (prepared as described in section 4.2.1) raised to human and rat OAT were titrated against respective bound antigens using the non-competitive ELISA method described previously (section 4.2.3) using an incubation time of 20 minutes for assay of the horseradish peroxidase (HRP) conjugate.

6.2.2 Determination of subsaturating antibody concentration

Isolated IgG raised against human and rat OAT was titrated against respective antigen at the optimal concentration for binding, using a non-competitive ELISA. The IgG concentration at which 50% of the bound antigen was saturated was determined and a suitable subsaturating concentration of IgG selected for competitive ELISA development.

6.2.3 Competitive ELISA protocol

OAT antigen was bound to ELISA plate wells at 1 µg per well in a volume of 50 µl and wells blocked with 2 %(w/v) 'Marvel' as described earlier (section 4.2.3). A second blocked ELISA plate (containing no bound antigen) was used for incubation of free antigen (serial dilutions of OAT standards) with fixed subsaturating IgG concentration for 2 hours at room temperature. Equal volumes of IgG (25 µl) and antigen (25 µl) were used for incubation. After 2 hours, the 50 µl of reacted material was transferred to the aspirated wells of the blocked ELISA plate containing bound OAT. Any remaining free IgG was then left to bind specifically to the bound antigen by incubation overnight at $4^{o}C$. Wells were then aspirated and washed out 3 times with PBS containing 0.05 %(w/v) Tween 80. To each well, 50 µl of HRP - IgG conjugate (diluted 1:1000 in PBS containing 2 %(w/v) 'Marvel') was added and ELISA plates incubated for 2 hours at room temperature. Wells were

aspirated and washed with PBS containing 0.05 %(w/v) Tween 80 prior to HRP assay (as described in section 4.2.3), with an incubation time of exactly 20 minutes.

A broad range of free antigen ccncentrations from 1000 ng to 0.001 ng were used initially to determine the linear range of the ELISA. A further range of free antigen concentrations were used to assess the limits of the assay, using subsaturating IgG at 50% saturation and also at 30% saturation of bound antigen with an increase in HRP assay incubation time to 30 minutes.

6.3 Results

6.3.1 Optimal bound antigen determination

A maximum absorbance was obtained by binding OAT antigen to ELISA wells at a concentration of 1 µg per well (figure 6.1) and produced well-defined maximum and minimum plateaus on the titration curve as determined by a non-competitive ELISA. At 0.2 µg of OAT bound per ELISA well, the titration curve was less steep and the maximum absorbance reduced by about 25% in both rat and human OAT assays. At 0.02 ug of OAT bound per ELISA well, poor titration curves were produced, not suitable for assay.

6.3.2 Optimal subsaturating IgG concentration

An anti-rat IgG concentration of 1×10^{-4} µM produced 50% saturation of OAT antigen bound at 1 µg per ELISA well (figure 6.1). The concentration of anti-human IgG required for 50% saturation was slightly higher (1.58×10^{-4} µM). This suggests that the anti-rat IgG may have a higher functional affinity for rat OAT than the anti-human IgG for the human enzyme, as discussed in section 5.3.6.

6.3.3 Competitive ELISA development

Preliminary competitive ELISA titrations using IgG concentrations produced 50% saturation of antigen (bound at 1 µg per well) indicated that the curve was approximately linear over a wide range of free OAT

levels, from 100 ng to 0.01 ng (figure 6.2) for both human and rat OAT assays. Lower subsaturating concentrations of IgG produced lower absorbance ranges and narrower linear titration ranges for free OAT. IgG concentrations producing 40% OAT saturation reduced the upper limit of assay sensitivity from 100 ng to about 1 ng of free OAT in both human and rat assays while the lower limit of sensitivity remained largely unchanged. Maximum absorbance was reduced by about 25% and the slope of the linear titration range was not significantly different from ELISA using IgG producing 50% OAT saturation. At IgG concentrations producing 30% OAT saturation, maximum absorbances were reduced to below 0.2 and titration curves were shallow and poorly defined.

At closer ranges of free OAT using IgG concentrations producing 50% saturation of bound OAT, the linearity of both human and rat ELISA titration curves departed from linearity above 10 ng free OAT and below 0.005 ng free OAT (figure 6.3). At fixed IgG concentrations producing 40% saturation of OAT where the incubation time for HRP assay was increased from 20 minutes to 30 minutes to increase absorbance values, a good linear titration curve was obtained. The upper limit of detection was reduced from 10 ng to 1 ng and the lower limit was virtually unchanged. However, the gradient of the linear portion of the titration curve was slightly steeper at the lower IgG concentration with both human and rat OAT assays. This indicates an increased sensitivity within the detection ranges, since larger absorbance changes were produced over the same range of free OAT levels compared to those at IgG concentrations producing 50% saturation and an HRP incubation time of 20 minutes.

The competitive ELISA procedure outlined in figure 6.4 was therefore selected as the optimal assay method for OAT in human and rat tissues.

FIGURE 6.1 Non-competitive ELISA of IgG to determine optimal bound OAT and subsaturating IgG

Determination of a subsaturating concentration of IgG titrated against an optimal amount of OAT antigen bound to wells of an ELISA plate for competitive ELISA development. A non-competitive ELISA was used to titrate isolated IgG (see section 4.2.3) against OAT antigen bound at 1 µg, 0.2 µg and 0.02 µg per well (as indicated).

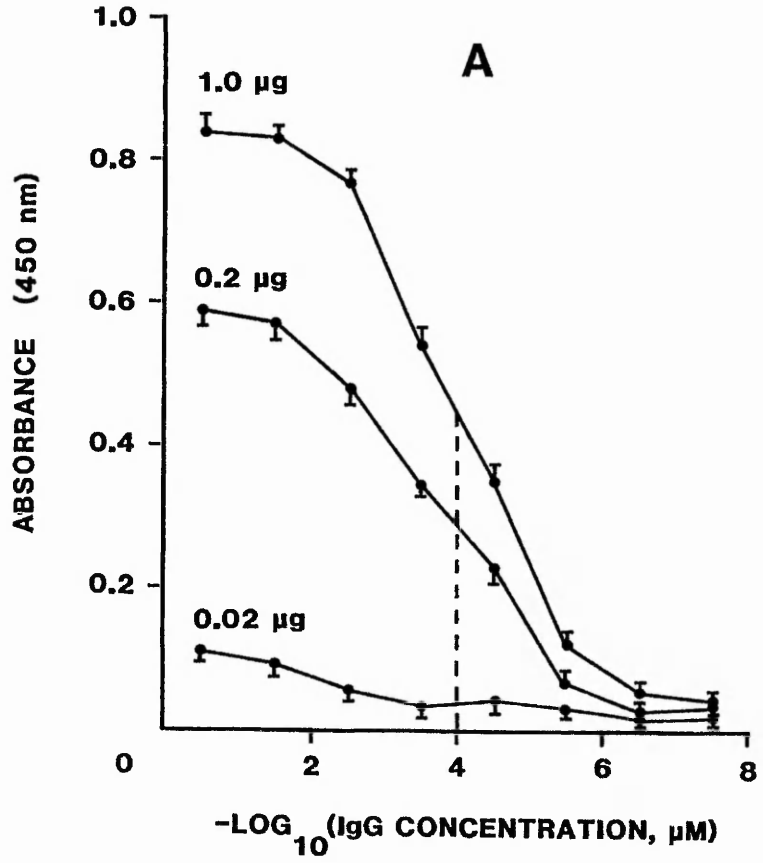
A. IgG raised to rat kidney OAT titrated against rat kidney OAT.

B. IgG raised to human liver OAT titrated against human liver OAT.

Incubation time for HRP assay = 20 minutes.

50% saturation of bound antigen is indicated by dashed lines.

Results are expressed as mean ± S.E. (n=4).


A
1.0 µg
0.2 µg
0.02 µg
ABSORBANCE (450 nm)
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
-LOG10(IgG CONCENTRATION, µM)

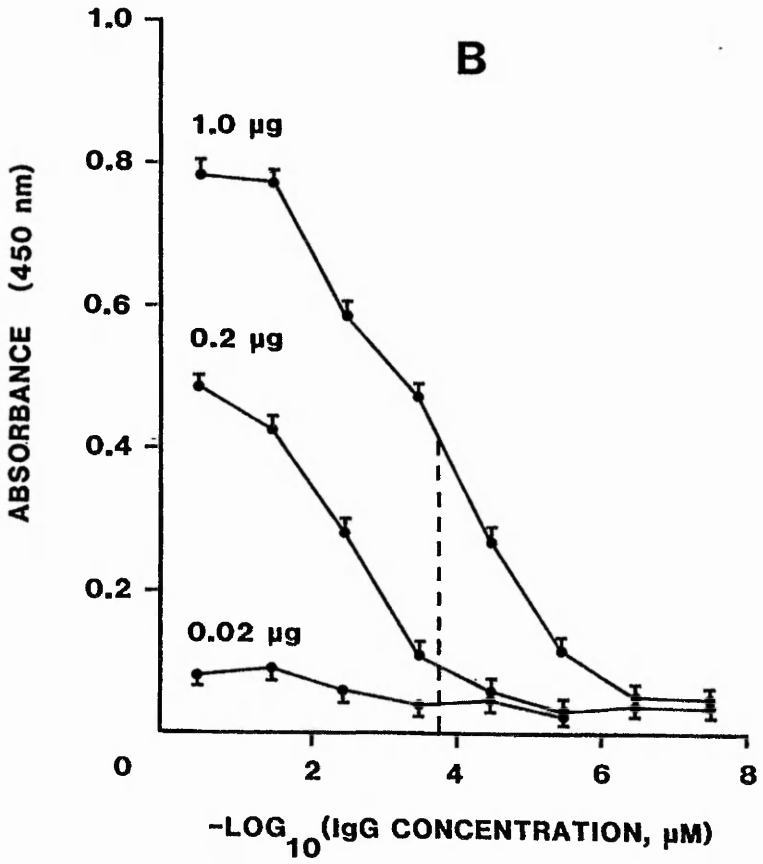

B
1.0 µg
0.2 µg
0.02 µg
ABSORBANCE (450 nm)
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
-LOG10(IgG CONCENTRATION, µM)

FIGURE 6.2 Competitive ELISA of OAT at optimal and sub-optimal IgG

Fixed subsaturating IgG (50%, 40% and 30% saturation of OAT antigen bound at 1 µg per ELISA well) was incubated with a broad range of 10-fold serial dilutions of free OAT antigen. Absorbances of control wells containing no bound antigen were subtracted from test wells.

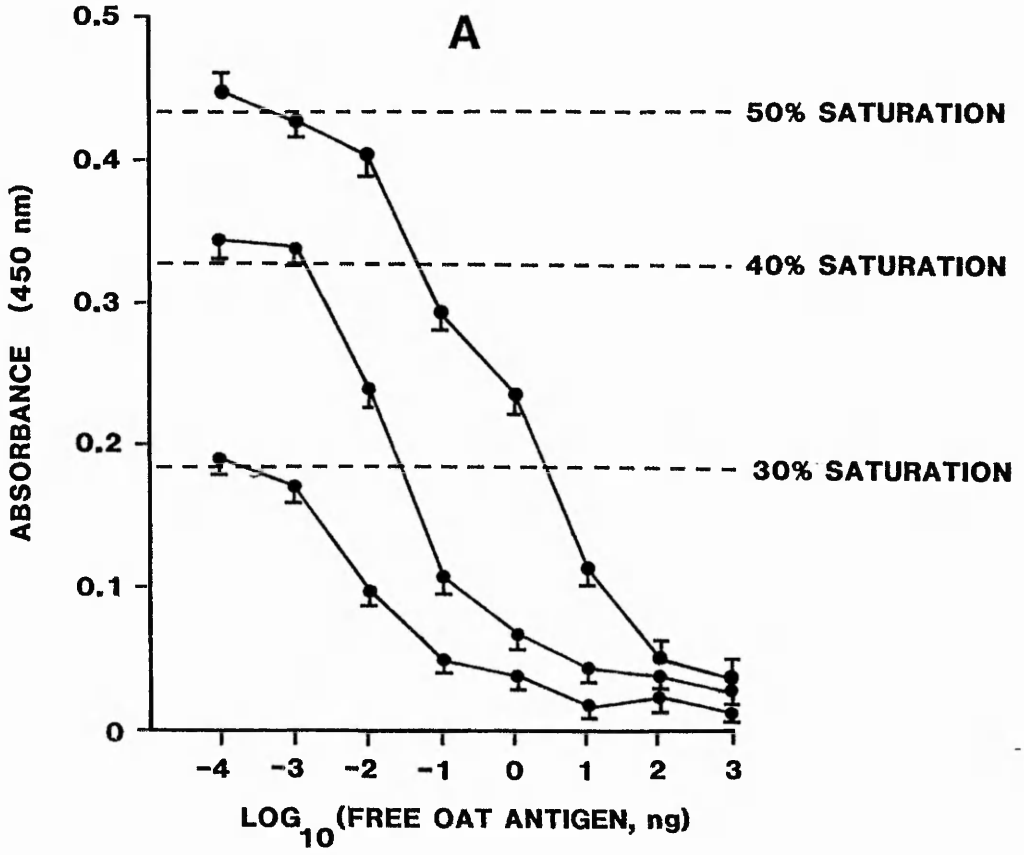
A. Competitive ELISA of rat OAT using IgG raised to rat OAT.

B. Competitive ELISA of human OAT using IgG raised to human OAT.

Incubation time for HRP assay = 20 minutes.

Dashed lines indicate saturation of 50%, 40% and 30% of bound antigen by IgG where no free antigen was present.

Results are expressed as mean $\pm$ S.E. (n=4).


A
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (450 nm)
50% SATURATION
40% SATURATION
30% SATURATION
-4
-3
-2
-1
0
1
2
3
LOG10 (FREE OAT ANTIGEN, ng)

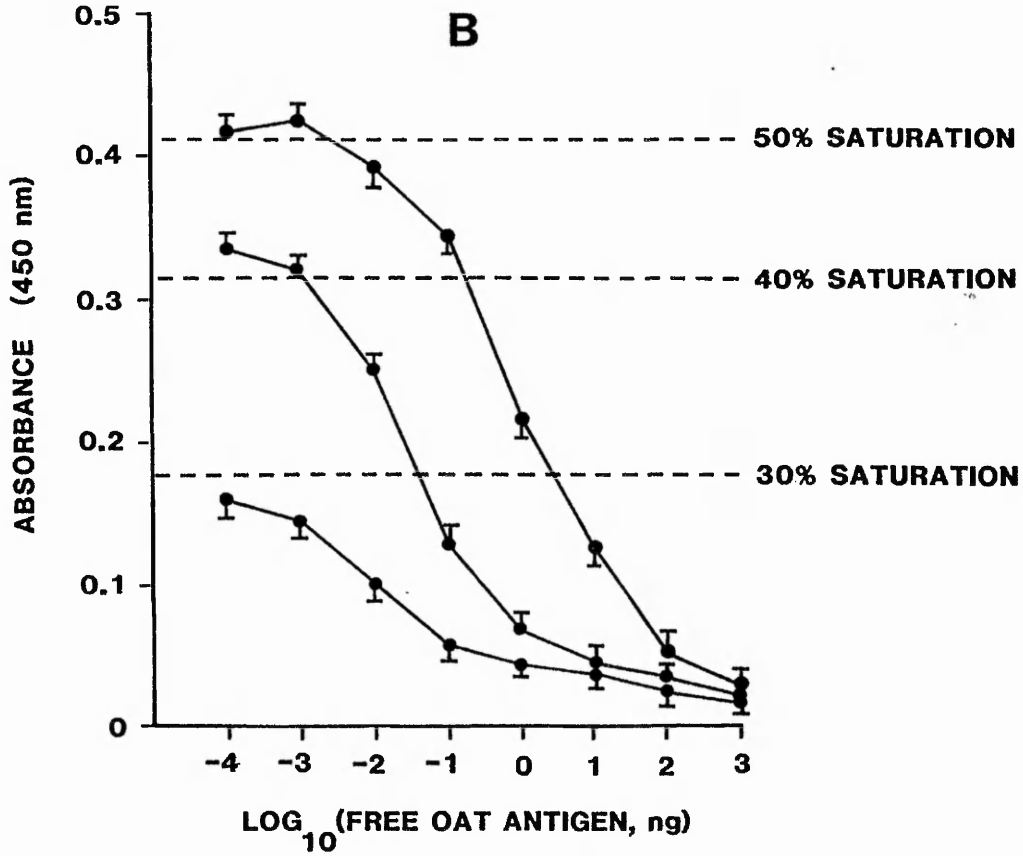

B
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (450 nm)
50% SATURATION
40% SATURATION
30% SATURATION
-4
-3
-2
-1
0
1
2
3
LOG10 (FREE OAT ANTIGEN, ng)

FIGURE 6.3 Competitive ELISA of OAT under optimal conditions

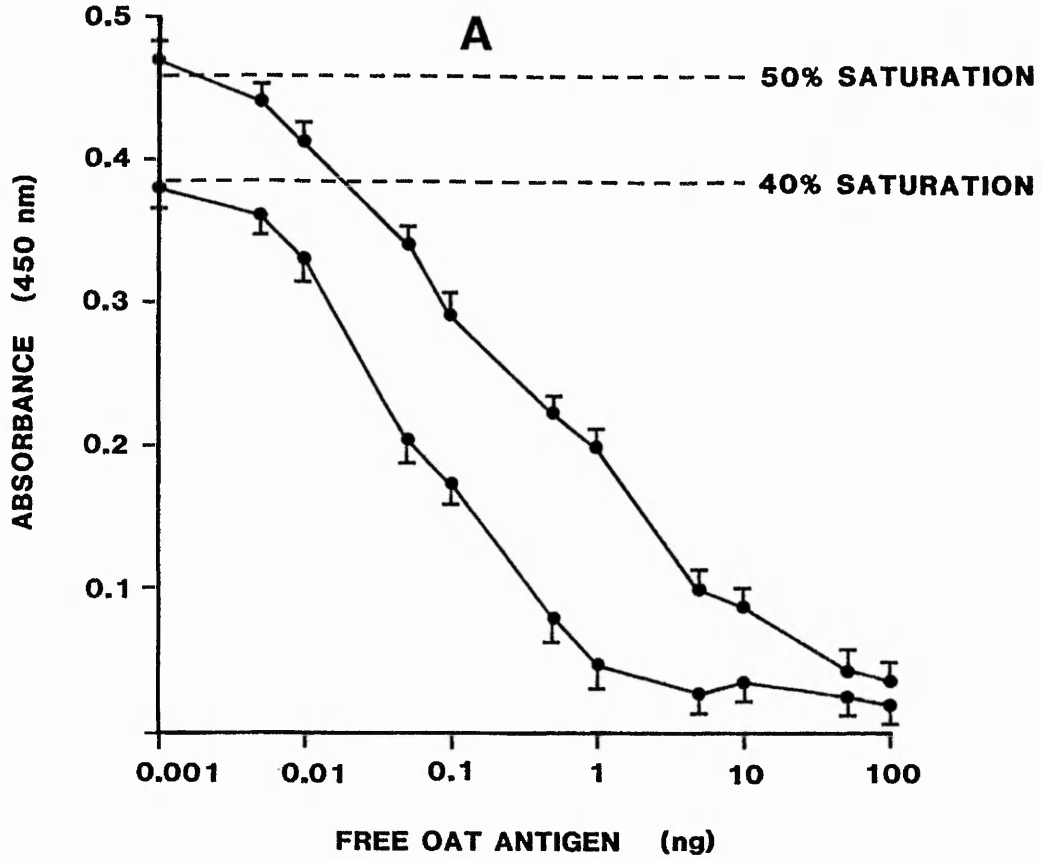
Fixed subsaturating IgG was incubated with varying free OAT antigen concentrations. Absorbances of control wells containing no bound antigen were subtracted from test wells. A competitive ELISA was performed at IgG concentrations producing 50% saturation of bound antigen using an incubation time for HRP assay of 20 minutes. A second assay was performed at IgG concentrations producing 40% saturation of bound antigen using an incubation time for HRP assay of 30 minutes.

A. Competitive ELISA of rat OAT using IgG raised to rat OAT.

B. Competitive ELISA of human OAT using IgG raised to human OAT.

Dashed lines indicate saturation of 50% and 40% of bound antigen where no free antigen was present.

Results are expressed as mean $\pm$ S.E (n=4).


A
50% SATURATION
40% SATURATION
ABSORBANCE (450 nm)
0.5
0.4
0.3
0.2
0.1
0.001
0.01
0.1
1
10
100
FREE OAT ANTIGEN (ng)

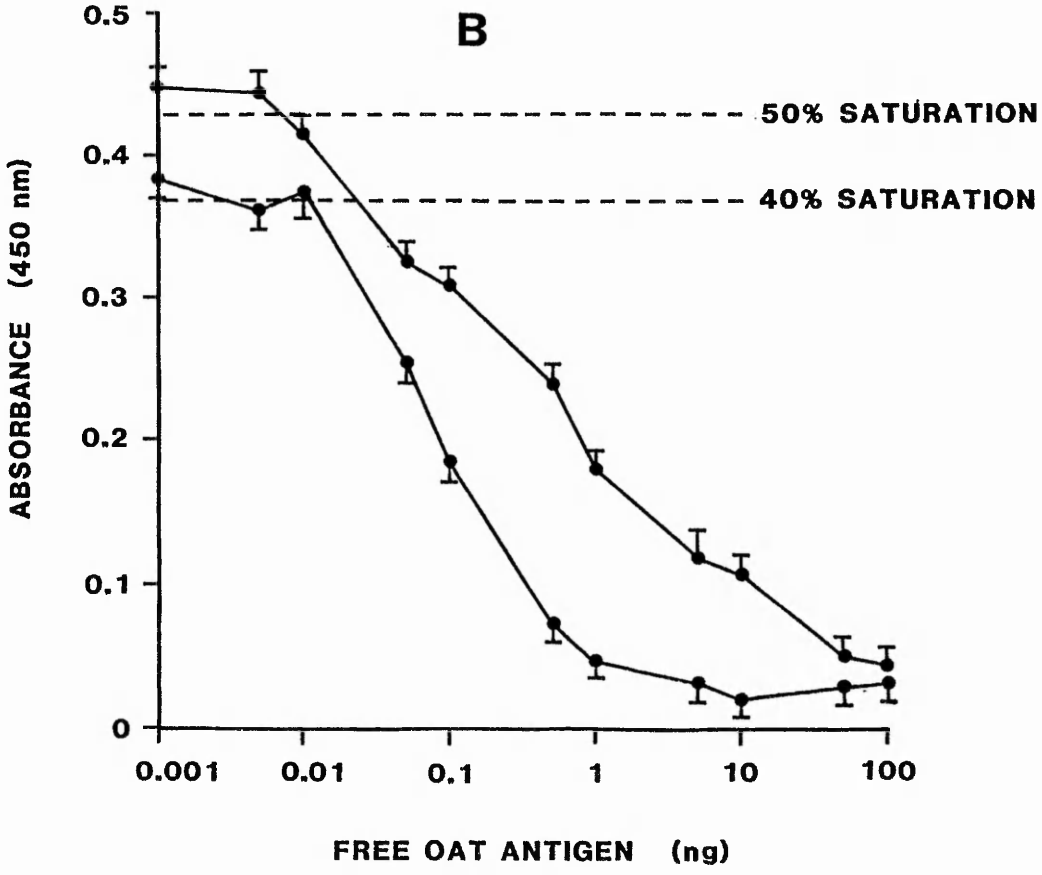

B
50% SATURATION
40% SATURATION
ABSORBANCE (450 nm)
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
100
FREE OAT ANTIGEN (ng)

FIGURE 6.4 Competitive ELISA procedure under optimized conditions

Rat or human OAT was bound to wells of ELISA plates at 1 µg per well (incubation volume: 50 µl) which was then blocked with 2 %(w/v) Marvel. A second ELISA plate was used to pre-incubate free OAT with free OAT specific IgG at the optimal subsaturating concentration of bound antigen determined from figure 6.3a. Any free IgG remaining was then transferred in the incubation mixture (50 µl) to the ELISA plate containing bound OAT. Free material was removed by washing, prior to incubation of the immobilized antigen:antibody complex with anti-rabbit IgG-HRP conjugate. After washing, the appropriate substrates were added (see section 4.2.1.2) and enzyme assay allowed to occur for 30 minutes.

COMPETITIVE ELISA PROCEDURE

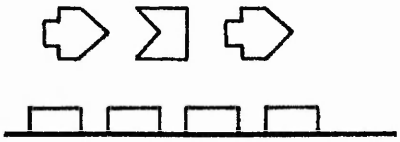
1. OAT ANTIGEN BOUND TO ELISA WELL AT 1 µg/50 µl

OAT MOLECULE

2. EXCESS BINDING SITES ON WELL BLOCKED

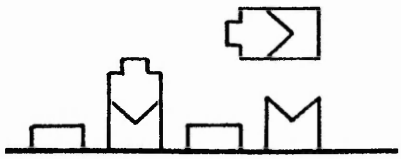
BLOCKING AGENT

3. PRE-INCUBATE STANDARD AMOUNTS OF FREE OAT ANTIGEN OR UNKNOWN SAMPLES WITH FIXED SUBSATURATING IgG CONCENTRATION (40% SATURATION OF BOUND ANTIGEN) IN BLOCKED ELISA WELLS WITH NO BOUND ANTIGEN

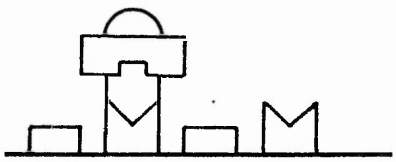


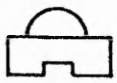
OAT SPECIFIC RABBIT IgG

4. TRANSFER REACTED COMPLEXES AND FREE ANTIBODY TO ELISA WELLS CONTAINING BOUND ANTIGEN TO ALLOW ANY FREE ANTIBODY TO BIND TO ELISA WELL



5. WASH OFF UNBOUND MATERIAL AND INCUBATE WELL WITH ANTI - RABBIT IgG - HRP CONJUGATE PRIOR TO WASHING AND ENZYME ASSAY





ANTI - RABBIT IgG - HRP CONJUGATE

6.4 Discussion

The optimal conditions for competitive ELISA allow detection of OAT protein down to 0.005 ng (0.1 femtomoles). This level of detection is comparable with the detection level achieved by Kato et al. (1977) who used IgG adsorbed onto silicone pieces to develop an immunoassay which permitted OAT detection down to 0.03 femtomoles of protein, although these workers may not have used the monomeric molecular weight of OAT in their calculations.

The effect of reducing the concentration of IgG from 50% to 40% saturation of bound is to decrease the maximum absorbance readings. This is expected since less IgG is available for maximum binding to bound OAT. However, by increasing the incubation time for HRP assay from 20 to 30 minutes, absorbance is increased with no loss of reproducibility or linearity of the titration curve. The upper limit of OAT assay is reduced since less free OAT is required to bind all of the free IgG. The lower limit of OAT detection should also be reduced since less free antigen is required for competition with bound antigen. This is not clearly demonstrated in figure 6.3 where the lower detection limit of free OAT at both 50% and 40% saturation of bound OAT remains virtually constant.

Because of the number of incubation steps involved in the competitive ELISA procedure, absorbances vary from one assay to another, especially since all procedures are performed manually. However, variations are consistent within assays and standard curves are reproducible.

The major initial problem encountered during ELISA development was the poor results obtained when free OAT and bound OAT were incubated simultaneously with IgG in the same ELISA plate. Virtually no decrease in absorbance was observed with increasing free OAT under these conditions, suggesting that IgG binds to bound OAT preferentially,

thereby preventing competitive binding. However, recent work reported by Chiancone and Gattoni (1987) may provide another explanation for this phenomenon. These workers have reported the use of immobilized enzyme subunits of α-chymotrypsin as potentially useful for the purification of this enzyme which undergoes self-assocation. Similarly, OAT bound to an ELISA plate may be binding free OAT by self-association and therefore depleting free OAT for reaction with IgG in a competitive assay. The introduction of a pre-incubation step (see figure 6.4) into the assay procedure produces the results shown in figure 6.3. Pre-incubation avoids direct competition since free OAT is first allowed to bind to IgG before any interaction with bound OAT occurs. The consequence of self-associating enzymes being used in competitive immunoassays may have important implications for other proteins when developing the assay procedure.

Reliable measurements of OAT by competitive ELISA rely on the IgG being monospecific for the enzyme protein and the non-interference of contaminating proteins present in crude extracts containing OAT. In section 7, the IgG used for assay (which has not been affinity-purified) will be assessed for cross-reactivity in crude extracts by Western blotting and subsequent measurement of OAT protein in tissue extracts will be reported.

7 OAT STUDIES ON HUMAN AND RAT TISSUES

7.1 Introduction

In this section, the competitive ELISA and immunoadsorption methods developed have been applied to tissue extracts. Rat tissue extracts were assayed for OAT activity and OAT protein. Assay of human and rat hair roots, human skin fibroblasts and rat blood platelets were performed in order to determine whether these tissues would provide a suitable source of OAT for both OAT measurement in patients with Gyrate Atrophy and also as a source of OAT protein for purification. If pilot studies prove successful, they will provide the methodology for isolating and characterizing a possibly abnormal OAT protein from Gyrate Atrophy patients.

Since the competitive ELISA can measure both active and inactive OAT, measurements of abnormal OAT protein or active protein in crude extracts where a possible inhibitor of OAT is present, can still be made. Comparison with OAT activity assay measurements (section 2.2.1.2) makes it possible to detect both inactive and active OAT protein quantitatively.

7.2 Materials And Methods

7.2.1 Assay of OAT activity in rat tissues

Tissues from five male and five female Sprague-Dawley rats were used for OAT assay of tissue homogenates. Rats weighed between 200-250 g and were fed *ad libitum* on a diet containing 20% protein. Freshly dissected rat tissues were homogenized in PBS to produce a 25 %(w/v) homogenate using a glass Potter Elvehjem homogenizer. Aliquots of tissue homogenate were diluted where appropriate in PBS and assayed as described earlier (section 2.2.1.2) except that the incubation time was increased from 20 minutes to 1 hour to improve the detection limit of the assay. All steps were performed at $4^{o}C$.

7.2.2 Preparation of hair roots for assay

Human and rat hair root homogenates were prepared as described by Janssen et al. (1981) with modifications. Fifty hairs removed from the human scalp or rat body were cut off above the sheath and root, and homogenized in 200 µl of a buffer containing 0.25 M Tris-HCl, 25 mM magnesium chloride and 0.2 %(v/v) Triton X-100 at pH 8.0. Hair roots were homogenized in a glass 250 µl Potter Elvehjem microhomogenizer. The homogenate was centrifuged in a microcentrifuge at 7,500 x g for 3 minutes. The supernatant was kept on ice while the pellet was resuspended in 50 µl of homogenization buffer and centrifuged as before. The supernatants were combined and immediately used for assay of OAT activity and competitive ELISA.

Assay of protein in all homogenates was performed by the method of Lowry et al (1951).

7.2.3 Tissue culture of human skin fibroblasts

7.2.3.1 Materials

All vessels were supplied sterile or were autoclaved at 15 p.s.i. (10.3 $N.cm^{-3}$) for 15 minutes. Aseptic procedures were performed in a flow cabinet (Gelaire BSB4). Human skin fibroblasts from an established cell line were obtained from Flow Laboratories. Cells were grown in monolayer culture supplied in a 75 ml flask at a passage number of 24 (P-24) containing Earle's minimal essential medium with 10 %(v/v) foetal calf serum (FCS). Dulbecco's modified Eagle's medium (DMEM) was obtained from Imperial Laboratories without sodium bicarbonate and made up to 13.65 g/l in distilled pyrogen-free water as instructed by the suppliers. This solution was filter-sterilized aseptically through a double filter system (0.45 µm and 0.2 µm pore size) into sterile media bottles in a flow cabinet. A stock solution containing penicillin and streptomycin (pen./strep.) at 5000 units/ml and 5 mg/ml respectively in 0.9 %(w/v) sodium chloride was obtained from Sigma. FCS, 0.4 %(w/v)

trypan blue in 0.9 %(w/v) sodium chloride and 0.5 %(w/v) trypsin with 0.2 %(w/v) ethylene-diamine tetra-acetic acid (EDTA) in 0.9 %(w/v) sodium chloride were also obtained from Sigma.

Growth media was prepared as follows: 83 ml of DMEM solution was added to 15 ml of FCS and 2 ml of stock pen./strep. solution in a sterile measuring cylinder in a flow cabinet. This solution was stored at $4^{o}C$.

Petri-dishes were obtained from Sterilin and tissue culture flasks obtained from Nunclon (Denmark).

7.2.3.2 Trypsinization and subcultivation of cells

Upon arrival, fibroblasts were incubated overnight at $37^{o}C$ in an atmosphere of 10% carbon dioxide in an incubator, the lid of the culture flask being loosened to allow equilibration. Confluent cells were then subcultivated as follows: the Earle's minimal essential medium was aseptically aspirated from the 75 ml flask and 7 ml of calcium- and magnesium-free PBS was added to the flask to wash the cells (the flask was gently rocked). The PBS was then aspirated and 1 ml of 0.05 %(w/v) Trypsin-EDTA solution (diluted from stock solution in calcium and magnesium free PBS) added dropwise from a Pasteur pipette. The flask was gently agitated to spread the trypsin solution over the cells. The flask was then sealed and transferred to an inverted microscope for cell examination. After about 2 minutes, cells had become rounded and detached from the flask. A fine white suspension of cells was observed with the naked eye. Trypsin activity was immediately quenched by addition of 12 ml of growth media (DMEM + 2 %(w/v) stock pen./strep. solution + 15 %(v/v) FCS). Repeated pipetting was used to wash off all trypsinized cells from the flask. The cell suspension was aliquoted into three universal bottles (4 ml in each) and centrifuged at 2000 x g for 5 minutes. The supernatant media was aspirated and cells resuspended in the following media in petri-dishes:

5 ml of Earle's minimal essential medium + 10 %(v/v) FCS, 2.5 ml of Earle's minimal essential medium + 10 %(v/v) FCS + 2.5 ml of DMEM/FCS, and 5 ml of DMEM/FCS. Petri-dishes were incubated at 37°C in a 10% carbon dioxide atmosphere and examined periodically for cell attachment and growth. Subsequent passages of cells grown to confluency were grown in DMEM + 10% FCS + 2 %(v/v) pen./strep. stock solution.

7.2.3.3 Viable and total cell counts

At each subcultivation of cells, total cell count and viable cell count were estimated by haemocytometry. Small aliquots of suspended cells were aseptically removed and counted on a haemocytometer using an inverted microscope. Cells were also diluted by a factor of two, in 0.4 %(w/v) trypan blue, and examined for dye exclusion prior to viable cell count.

7.2.3.4 Cell growth

Cells were found to grow poorly in petri-dishes and were subsequently subcultivated into 25 ml tissue culture flasks. A split ratio of 1:2 was used for each passage of cells which typically took 4-6 days to reach confluency. Media were changed every 48 hours by aspiration, the volume of medium being kept constant at 5 ml per flask. Cells were examined microscopically each day to check for growth and contamination.

7.2.3.5 Preparation of cells for assay

Cells were harvested having grown to confluency after four subcultivations producing cells with a total passage number of 28 (P-28). These cells were trypsinized as described previously and then washed three times in PBS by centrifugation at 2000 x g for 5 minutes. Cells were finally sonicated at 10 kilocycles per minute for 5 minutes prior to assay.

7.2.4 Competitive ELISA

Tissue extracts from rat organs were diluted in serial ten-fold dilutions in PBS containing 2 %(w/v) Marvel. A 25 µl aliquot of each dilution was incubated with subsaturating IgG as described in section 6.2.3. Human and rat hair root homogenates, fibroblasts, and platelet preparations were prepared similarly. All extracts were assayed for protein (Lowry et al, 1951).

7.2.5 Preparation of platelets from female rat blood

Platelets were isolated from whole blood by the method of Corash (1980). Fresh blood (10 ml) was mixed with 0.1 ml of 10 %(w/v) disodium EDTA in a plastic centrifuge tube and stored at room temperature. The 10 ml of blood was then mixed with 5.7 ml of washout buffer containing 360 mM glucose and 473 mM sodium chloride, made up as follows: 8.1 g sodium chloride, 1.22 g sodium hydrogen phosphate, 0.218 g sodium dihydrogen phosphate dihydrate, and 2 g glucose were dissolved and made up to 1000 ml in distilled water and adjusted to pH 7.2.

The blood/buffer mixture was centrifuged at 600 x g for 4 minutes and the supernatant pipetted off, avoiding white cell contamination at the red cell interface. The supernatant was stored on ice and the pellet resuspended in 20 ml of washout buffer and centrifuged as before. This procedure was repeated five times and the supernatants were pooled. The supernatant solution which contained the platelets was centrifuged at 100,000 x g for 30 minutes and the pelleted platelets resuspended in 300 µl of PBS. This extract was finally sonicated at 10 kilocycles per minute for 5 minutes prior to assay.

7.2.6 Electroblotting procedures

7.2.6.1 Electroblotting after SDS-PAGE

Electroblotting of proteins from polyacrylamide gels was performed as described by Towbin et al. (1979). Electroblotting buffer was made up to produce 25 mM Tris/192 mM glycine as follows: 18.18 g Tris base and

86.4 g glycine were dissolved in 1000 ml of distilled water and adjusted to pH 8.0. This stock solution was diluted to 6000 ml with distilled water and cooled to 4^{o}C prior to use.

Tissue homogenates were subjected to SDS-PAGE as described previously (section 3.2.1). After electrophoresis, the edge of the gel was cut to mark the position of the sample lanes. The gel was then immersed in 100 ml of electroblotting buffer to allow partial removal of SDS. Six sheets of filter paper (Whatman No.1) were cut to slightly larger than the size of the gel. A Scotch-Brite pad was placed onto the cathode electrode plate of the electroblotting apparatus (EC-Apparatus corporation). Three sheets of filter paper were soaked in electroblotting buffer and placed onto the pad. Air bubbles were removed between successive layers by gently rolling a 10 ml pipette over the sheets. The equilibrated gel was carefully placed onto the filter paper. A sheet of nitrocellulose (pore size: 0.45 µm, Gelman Sciences Inc.) was cut to the size of the cut filter papers (N.B. gloves were worn) and was soaked in buffer. The nitrocellulose was placed onto the gel, air bubbles excluded and the position of the gel was marked by piercing the nitrocellulose at the corners of the gel. A further 3 filter papers were soaked in buffer and placed over the nitrocellulose and finally the cathode electrode plate positioned on top. The two electrodes were held firmly together with rubber bands and waterproof tape and transferred to the electroblotting tank and immersed in the 6000 ml of buffer. A current of 200-250 mA was applied across the electrodes for 17 hours. After transfer, the gel was stained with Coomassie blue (section 3.2.1.4) to assess transfer, and the nitrocellulose blot was cut into separate sample lanes. Blots were either stained for protein with 0.1 %(w/v) Amido black (dissolved in 45 %(v/v) methanol plus 10 %(v/v) acetic acid) for 5 minutes followed by

destaining as for SDS-PAGE gels (section 3.2.1.4) or OAT bands detected immunochemically (Western blotting).

7.2.6.2 Immunoblotting and OAT detection

Western blotting for OAT protein was performed as follows. The remaining binding sites on the nitrocellulose were blocked by incubating the blot in buffer A (100 mM Tris-HCl; 150 mM sodium chloride; 0.05 %(v/v) Tween 80; 2 %(w/v) Marvel, pH 9.0) for 1 hour at room temperature. The blot was rinsed with buffer B (50 mM Tris-HCl; 200 mM sodium chloride; 0.1 %(v/v) Tween 80, pH 7.4). Rabbit IgG (antisera, isolated IgG or affinity purified IgG) raised to OAT from either rat or human tissue was diluted in buffer A to produce 5 µg/ml IgG protein. In the case of antisera, the IgG content was assumed to be 5 mg/ml (see section 5.3.1). The diluted IgG was incubated with the blocked blot overnight at 4^{o}C. The blot was rinsed thoroughly with buffer B and transferred to a new incubation tray. The blot was incubated with anti-rabbit IgG-biotin conjugate (Sigma) diluted 1:1000 in buffer B containing 2 %(w/v) Marvel, for 2 hours at room temperature. The blot was washed with buffer B and transferred to a new tray. HRP-avidin conjugate (Sigma) was diluted 1:500 in buffer B and incubated with the blot for 30 minutes at room temperature. The blot was rinsed with distilled water and equilibrated with PBS for 10 minutes. The blot was finally incubated with chloronaphthol reagent at room temperature until mauve bands appeared (10-15 minutes). The reaction was stopped by rinsing the blot with distilled water.

Chloronaphthol reagent was made up just prior to use as follows: 20 mg 4-chloro-1-naphthol (Sigma) was dissolved in 2.5 ml DMSO; this solution was added dropwise to 47.5 ml PBS with constant stirring; to this solution, 15 µl of 30 %(w/v) hydrogen peroxide was added prior to immediate incubation with the blot.

7.3 Results

7.3.1 OAT levels in rat tissues

Specific activities of OAT in rat tissue homogenates were determined in 5 male and 5 female rats (Table 7.1) and compared to the abundance of OAT protein per mg total protein, determined by competitive ELISA. Where no significant difference was found between male and female tissues, the combined averages of both were expressed. Levels of OAT estimated from activity measurements approximated well with levels determined by ELISA and whilst no significant difference between these assay measurements was observed, there was a trend towards higher levels of OAT estimated by ELISA. This may be due to the inherent assay error or may represent partial inactivation of OAT protein during tissue processing.

Female rat kidney tissue was found to have the highest abundance of OAT, the male rat kidney having about 50% of female OAT levels. This sex difference was also present in adrenal tissue. The major sites of OAT in rat tissues were kidney, liver and small intestine. Levels in testis and eye homogenates may also be of significance as will be discussed later. Leg muscle and ovary tissue had the lowest specific activities of OAT.

7.3.2 Assessment of competitive ELISA

The standard error of assay determinations was typically $\pm$ 10% of the mean (n=3). Assay of OAT in crude extracts was reproducible at the dilutions used. Samples were diluted sufficiently to detect OAT below 1 ng protein. However, attempts to assay whole rat blood as part of the studies on platelets, produced inconsistent results at all dilutions tested. This may be due to interference by contaminating proteins or by non-specific binding. Data shown in Table 7.1 suggest that OAT protein is being reliably measured since values are consistent with activity measurements. This therefore shows that only OAT protein

is being detected and that no structurally similar OAT protein (such as precursor OAT) is being detected. This was investigated further by Western blotting.

7.3.3 Western blotting of tissue extracts

Non-specific binding occurred in all tissue extracts incubated with antisera diluted 1:1000, an observation confirmed by incubating blotted extracts with control serum diluted similarly. Isolated IgG raised to rat or human OAT, or affinity purified anti-rat kidney OAT antibody, produced a single band corresponding to OAT by SDS-PAGE. This was achieved in all cases when used at a concentration of 5 µg/ml of IgG protein. Higher concentrations of IgG produced non-specific binding. Examples of Western blots are shown in figures 7.1 and 7.2. Single OAT bands could be detected in all tissue extracts tested with the exception of whole rat blood. An OAT band could be detected using isolated IgG diluted to 1:100, but non-specific binding also occurred. IgG raised to human OAT was used to detect human hair root OAT, the OAT band being just visible.

7.3.4 OAT detection in hair roots

Hair roots prepared from rat and human sources produced 400 - 700 µg protein per homogenate of 50 hairs. This was not sufficient to detect OAT activity. Competitive ELISA of rat hair root OAT produced a value of 6.6 $\pm$ 1.26 ng OAT per mg of protein (mean $\pm$ S.E., n=6 rats). Three male and three female rats were used for assay of hair roots, no sex difference of OAT levels was observed. Competitive ELISA of human hair roots produced varying levels of OAT from the same individual on three separate occasions. Values of OAT abundance in hair roots were as follows: 7.1; 5.8; 2.7 ng OAT per mg protein (values are means of 3 determinations).

7.3.5 OAT detection of cultured skin fibroblasts

Fibroblasts became re-established in monolayer culture after 2 passages of the original supplied culture. However, trypsinization of cells produced a loss of up to 25% of viable cells as determined by dye exclusion. Dead cells rapidly became detached and were removed by renewing the media. Cells were subcultivated at a split ratio of two and a cell count of $1 - 5 \times 10^5$ cells per ml medium. Confluent cells produced a total cell count of $0.5 - 1.0 \times 10^7$ cells per 25 ml flask. A major problem encountered with growth of this cell line was the slow growth and high mortality after trypsinization. This may have been due to the age of the cells, the media or the 10% carbon dioxide atmosphere used for growth (such cells are more usually grown in a 5% carbon dioxide atmosphere).

No activity was detected in a protein extract from 2×10^6 cells (740 ug protein); however OAT protein was detected by competitive ELISA of cell extracts. OAT levels were not significantly affected by passage number (P), the following results being obtained:

2.7 ± 0.35 (P-26); 2.26 ± 0.17 (P-27); 2.33 ± 0.23 (P-28).

Values are means $\pm$ S.E. (n=3), expressed as ng OAT per mg protein.

7.3.6 OAT detection in blood platelets

Attempts to measure OAT in whole rat blood produced inconsistent results by competitive ELISA and activity could not be determined. Total platelet protein isolated from 10 ml of rat blood was 4.2 mg. This protein was found to contain 302.4 ng of OAT protein by competitive ELISA. The specific activity of the platelet extract was estimated at 72 ± 6.5 ng OAT per mg protein (mean $\pm$ S.E., n=3). Assay of 3 mg of the platelet protein produced no activity.

7.3.7 Immunoadsorption of OAT from blood platelets

The immunoadsorption method developed in section 5 was applied to the purification of OAT from platelets isolated from the whole blood of 11

female rats. A platelet extract containing 28.56 mg of protein was obtained from 68 ml of blood. Extracts from each rat were stored at -20^{o}C as a pellet and finally thawed and pooled. The combined extracts were resuspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM 2-oxoglutarate and 20 µM pyridoxal phosphate prior to sonication at 10 kilocycles per minute for 5 minutes. The sonicated extract was centrifuged at 100,000 x g for 30 minutes and the supernatant used for immunoadsorption as described in section 5.2.3.

A specific activity of 83 $\pm$ 6.8 ng OAT per mg protein (mean $\pm$ S.E., n=3) was determined for the platelet supernatant extract by competitive ELISA. Since the 28.56 mg of platelet protein has an estimated OAT content of 2.37 µg OAT, the protein recovered after desorption was freeze-dried and dissolved in sample buffer prior to SDS-PAGE. Since the amount of OAT protein extracted was too low for protein assay, the specific activity could not be determined. Platelet extracts and desorbed platelet OAT protein after SDS-PAGE are shown in figure 7.3.

TABLE 7.1

MEASUREMENT OF OAT IN RAT TISSUES[a]

TISSUE	SPECIFIC ACTIVITY (units/mg) x 10^3	ESTIMATED OAT LEVELS[b] (ng/mg)	MEASURED OAT LEVELS[c] (ng/mg)
Kidney[d] (female)	43 ± 6.5	1228 ± 186	1384 ± 193
Kidney[d] (male)	20 ± 3.0	577 ± 86	631 ± 81
Liver	23.7 ± 3.4	676 ± 97	693 ± 87
Small intestine	15 ± 2.6	431 ± 74	462 ± 82
Testis	5.9 ± 1.8	169 ± 51	218 ± 45
Eye[e]	2.7 ± 1.0	77 ± 29	105 ± 23
Eye lens	1.7 ± 0.6	49 ± 17	65 ± 21
Adrenal[d] (female)	2.9 ± 1.2	82 ± 34	95 ± 23
Adrenal[d] (male)	1.4 ± 0.4	41 ± 11	57 ± 16
Spleen	1.8 ± 0.7	52 ± 21	60 ± 20
Brain (anterior)	1.6 ± 0.6	44 ± 17	57 ± 23
Brain (posterior)	1.1 ± 0.3	30 ± 9	45 ± 9
Lung	1.8 ± 0.6	51 ± 18	59 ± 21
Heart	1.6 ± 0.5	44 ± 16	43 ± 18
Ovary	0.97 ± 0.3	28 ± 9	38 ± 12
Muscle	0.7 ± 0.2	20 ± 6	24 ± 11

[a] OAT levels were measured in tissues from 10 rats. Values are expressed as mean ± S.E.

[b] Values estimated from activity assuming pure OAT has a specific activity of 35 units/mg protein.

[c] Measurements by competitive ELISA.

[d] n=5 rats

[e] lens and aqueous humour removed.

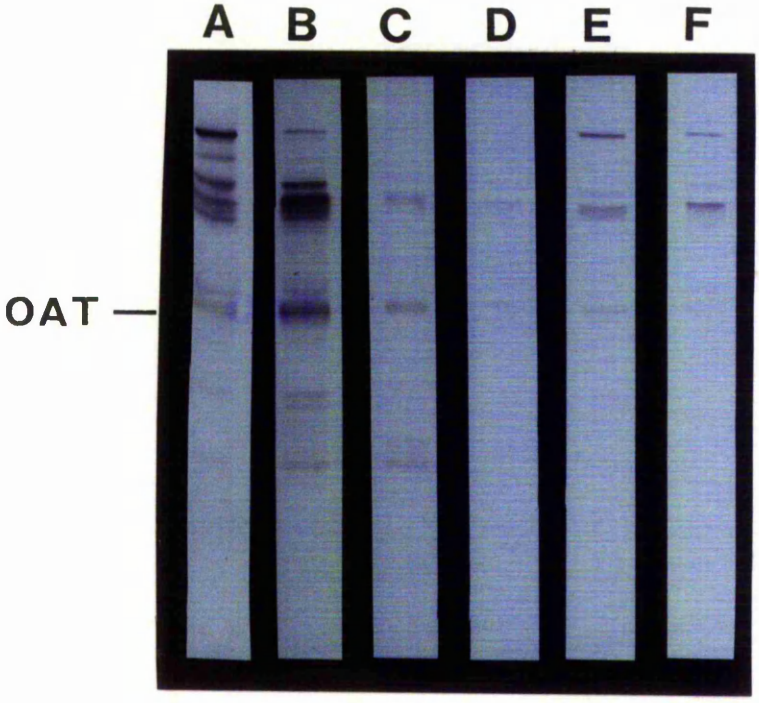
FIGURE 7.1 Western blots of rat tissue extracts

Examples of Western blots produced as described in the text, after protein separation by SDS-PAGE.

7.1a Non-specific binding of antibody to rat tissue homogenates produced at high anti-rat kidney OAT antibody concentration. Tissue homogenates are defined as follows:
A, small intestine; B, kidney; C, liver; D, muscle;
E, eye; F, eye (lens removed).

7.1b Demonstration of the monospecificity of anti-rat kidney OAT (isolated crude IgG) to rat OAT upon elimination of non-specific binding by reduction of antibody concentration. Tissue homogenates are defined as follows:
A, kidney; B, liver; C, small intestine; D, testis;
E, blood platelets; F, hair root extract.

7.1a



7.1b

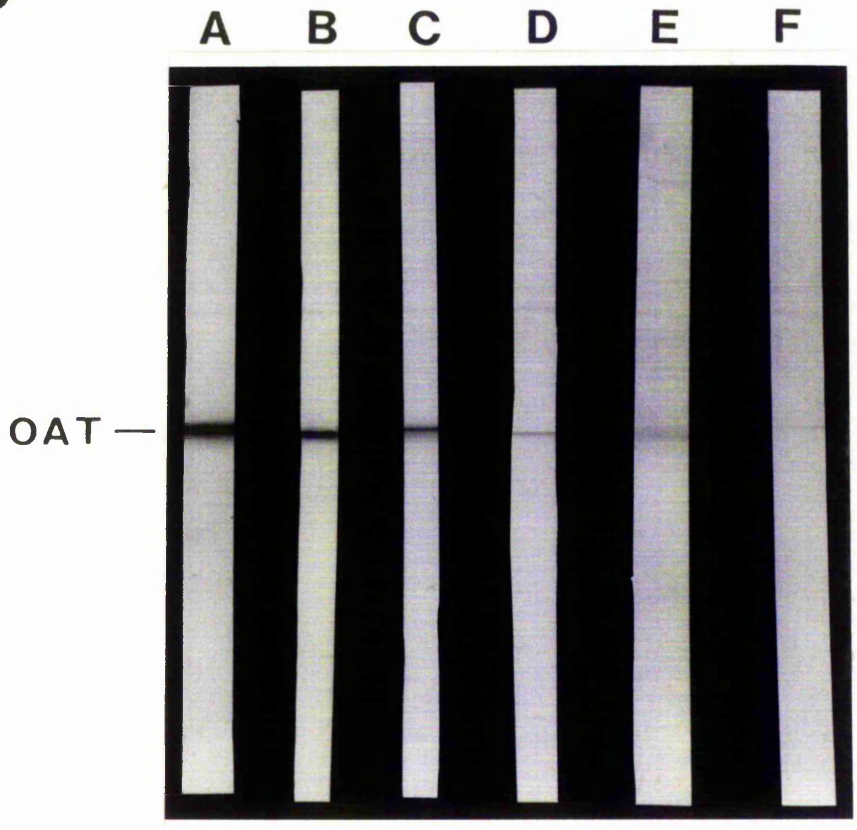


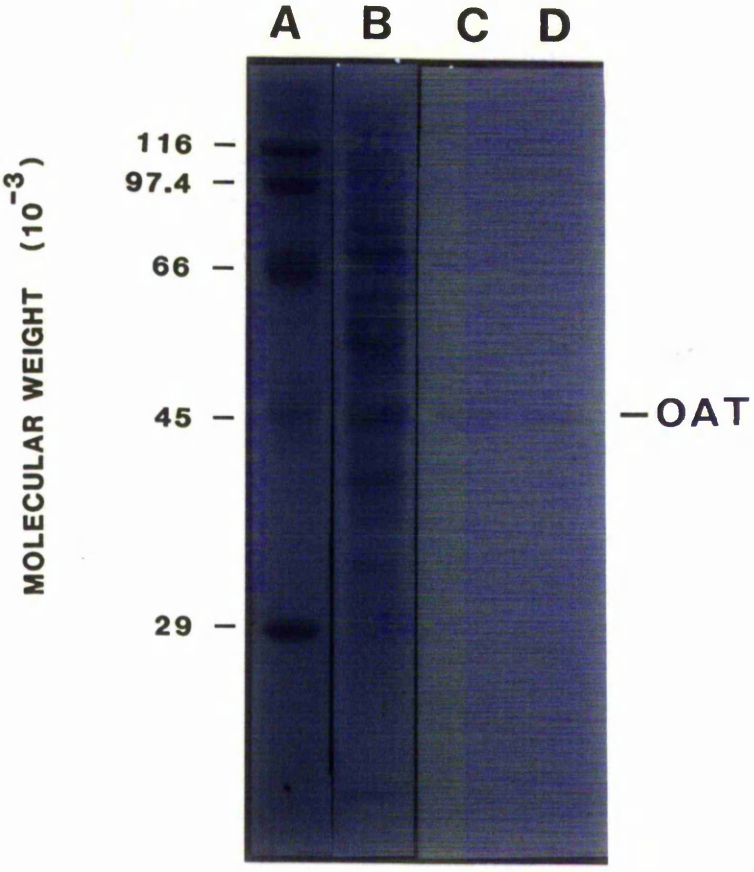
FIGURE 7.2 Western blots of human tissue extracts

Examples of Western blots produced as described in the text, after protein separation by SDS-PAGE.

7.2a Detection of OAT in human skin fibroblast extracts. Lane A, electroblotted molecular weight markers (defined in the text) stained for protein. Lane B, electroblotted fibroblast homogenate stained for protein. Lanes C and D, Western blots of fibroblast homogenates.

7.2b Demonstration of OAT detection and anti-human OAT antibody monospecificity in human tissues. Lane A, liver homogenate. Lanes B and C, hair root extracts.

7.2a



7.2b

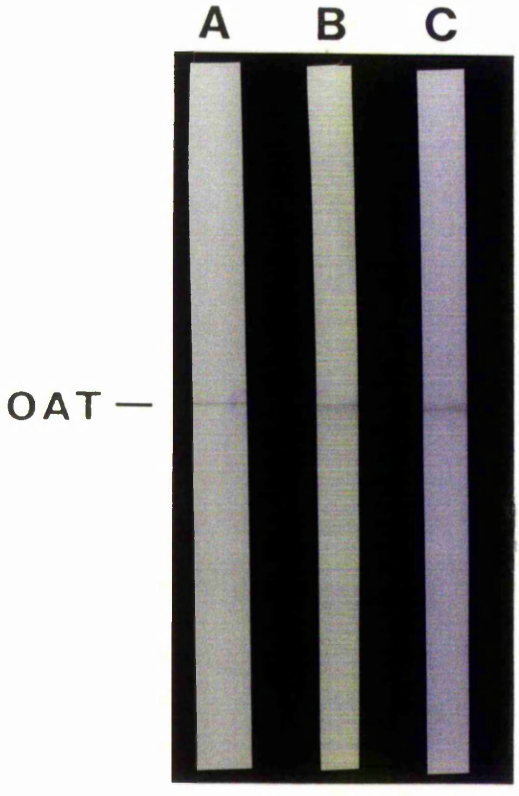
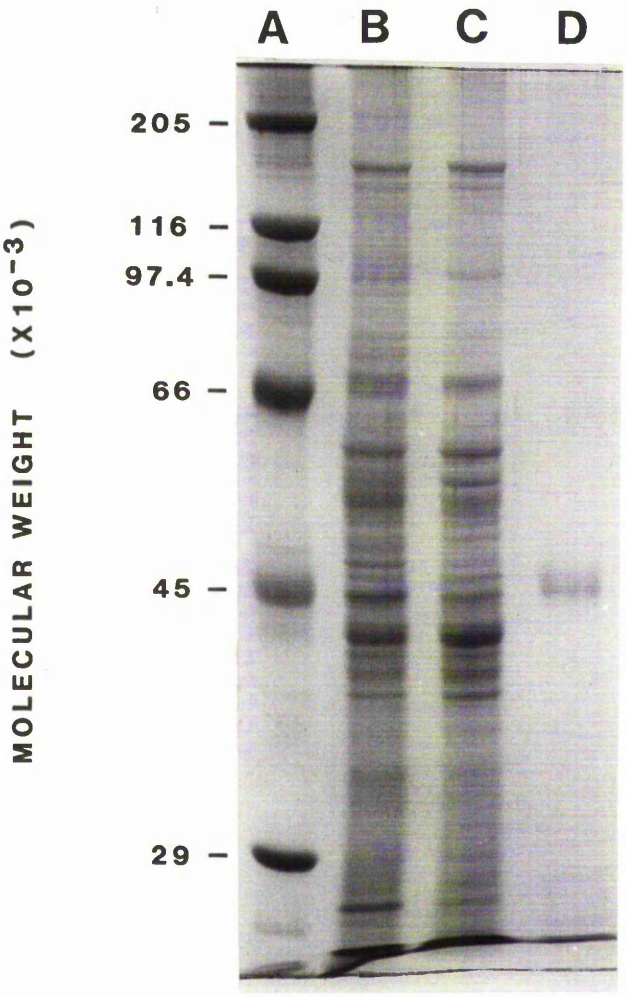


FIGURE 7.3 Immunoadsorption of OAT from rat blood platelets

Rat OAT was isolated from blood platelet extract by the immunoadsorption procedure developed in section 5. Samples were subject to SDS-PAGE. Lane A, molecular weight markers (defined in the text). Lane B, whole platelet extract. Lane C, soluble platelet extract. Lane D, desorbed OAT protein.


A
B
C
D
205
116
97.4
66
45
29
MOLECULAR WEIGHT (X10^{-3})

7.4 Discussion

7.4.1 Significance of OAT levels in rat tissues

The difference in OAT abundance in rat kidney and adrenal gland, between male and female tissues, is due to hormonal factors as reviewed previously (section 1.5.3.3). The physiological significance of high OAT levels in rat kidney and small intestine remain unclear. It is known that arginase activity is very low in kidney tissue and consequently negligible amounts of ornithine would be produced from arginine. It may be possible that the OAT reaction is reversed in these tissues, producing ornithine from Δ^1-pyrroline-5-carboxylate to drive the urea cycle reactions to produce arginine. The reversibility of the OAT reaction has been demonstrated in developing rat small intestine (Herzfeld and Raper, 1976) which also suggests that the reverse reaction may occur in adult rats, necessitating a high abundance of OAT.

The high specific activity of OAT in rat testis can probably be explained by the abundance of mitochondria in this tissue needed for spermatozoa production and motility. The specific activity of OAT in crude eye and eye lens homogenates were not notably high although whole eye extract had about twice the specific activity of rat lens tissue. Further dissection of tissues of bovine eye (Hayasaka et al., 1980) has shown that the retinal pigment epithelium has the highest specific activity of OAT (about ten-fold higher than liver OAT levels). However, bovine eye lens has no reported OAT activity, although human lens tissue has been used for clinical studies (Rao et al., 1981). The importance of eye tissues for studying OAT has been discussed previously in association with Gyrate Atrophy (section 1.6.2.7).

OAT abundance in rat brain was not significantly different in cerebral and hind-brain tissue. Sadasivudu and Indira (1974) have shown that OAT and arginase activity are highest in rat cerebellum.

This suggests that urea production is not significant in brain tissue and that arginine is formed in order to produce glutamic acid via arginase and OAT. Wong et al. (1982) have shown that OAT activity in the frontal cortex of the brain is reduced in Huntington's disease and this suggests that OAT may be involved in neurotransmitter synthesis in the brain.

The low levels of OAT in rat muscle homogenates suggest that proline synthesis from ornithine is not a significant pathway in this tissue. However, structural abnormalities have been reported in muscle tissue from Gyrate Atrophy patients, suggesting a possible impairment of metabolism of proline, the major component of collagen.

7.4.2 Synthesis and degradation of OAT

Western blotting studies on rat tissue homogenates have shown that only mature OAT protein is present in significant quantities and that no precursor OAT can be detected. Precursor OAT produced in the cytosol has a molecular weight of 49,000 and would be detected as a separate band above mature OAT after SDS-PAGE. It is very unlikely that the polyclonal IgG raised to OAT would fail to recognize precursor OAT and so it can be concluded that the precursor is either unstable or is produced in very small quantities and has a rapid turnover.

It has been reported previously that OAT levels are controlled by OAT synthesis (section 1.5.3.3) and that degradation is probably rapid since no inactive pool of OAT has been detected (Kominami and Katunuma, 1976). Data obtained in this study confirm this report, since all OAT protein in tissue homogenates can be accounted for by activity measurements (Table 7.1).

7.4.3 Tissue suitable for clinical OAT studies

Janssen et al. (1981) have described the use of hair roots for assay of OAT activity in order to determine homozygotes and heterozygotes for Gyrate Atrophy. The spectrophotometric assay used in this study was

not sufficiently sensitive to detect OAT activity, but assay of OAT by competitive ELISA has allowed sensitive detection of OAT protein. However, the specific activity of OAT was variable due to differing amounts of total protein present in the hair root extract. The heterogeneity of Gyrate Atrophy and the wide range of OAT activities reported in patients has been reviewed previously (section 1.6.2.2) and suggests that assay of hair roots may only provide a qualitative measurement of OAT. Therefore, assay of hair roots could provide a presumptive diagnosis of Gyrate Atrophy in high risk individuals and is non-invasive, but is of limited use for clinical studies.

Human skin fibroblasts grown in tissue culture have provided a good source of tissue for study of most inherited metabolic disorders. Probably due to the age of the cell-line used, OAT levels were very low and are comparable to specific activities found in Gyrate Atrophy patients (Ohura et al., 1984). However, this demonstrates that the competitive ELISA developed here would be capable of measuring OAT levels present in Gyrate Atrophy patients. Ohura et al. (1984) have shown that repeated subculture of fibroblasts can affect OAT levels, and that fibroblasts from Gyrate Atrophy patients lost 80-85% of OAT activity present in the first subculture by the third subculture. This suggests that cultured skin fibroblasts are of limited use in this instance and would not provide an opportunity for extended OAT studies or represent a good source of OAT protein for purification. Additional problems include the biopsy which is invasive to the patient and also the cost and work of maintaining cells in culture.

Preliminary studies on rat platelets appear to represent a good source of OAT and a convenient means of obtaining tissue from a human patient. The small number of mitochondria present in platelets has provided sufficient protein to assay several enzymes associated with mitochondria (Corash, 1980). A pilot study applying the

immunoadsorption methods developed in section 5 to rat blood platelets has been successful although the amount of blood used was insufficient to permit biochemical characterization of OAT. However, platelets may provide an ideal source of OAT for purification from Gyrate Atrophy patients, although a large volume of blood (500-600 ml) would be needed to allow characterization of any abnormal OAT protein. Problems were encountered with competitive ELISA of whole rat blood and this may also occur in the human assay system. However, provided that the recovery of platelets is calculated by performing counts before and after platelet isolation, it should be possible to measure OAT levels in whole blood without difficulty.

8 DEVELOPMENT OF SYSTEMS FOR AMINO ACID ANALYSIS: APPLICATION TO QUANTITATIVE MEASUREMENT OF SERUM AND URINE

8.1 Introduction

This section describes the development and application of two analytical systems for the purpose of sensitive detection and quantitative measurement of amino acids in physiological fluids. The more rapid analytical system (reversed phase HPLC) will be applied to investigation of intracellular ornithine and other amino acid pools in section 9.

The original methods developed for amino acid analysis by classical ion-exchange chromatography (Spackman et al., 1958; Hamilton, 1963) employed post-column detection with ninhydrin and provided a detection limit of 250 picomoles per amino acid. Improvements in sensitivity by fluorimetric detection methods such as o-phthalaldehyde/2-mercaptoethanol (OPA/2-ME) derivatization (Benson and Hare, 1975) have allowed amino acid detection at the 100 picomole level, by ion-exchange chromatography.

More recently, advances in the development of micro-particulate resins has enabled rapid and more sensitive methods of amino acid analysis using reversed phase HPLC, (Turnell and Cooper, 1982; Griffin et al., 1982; Price et al., 1984). At present, the main types of derivative for reversed phase amino acid analysis are prederivatization with phenylisothiocyanate to form the phenylthiocarbamyl derivative (Picotag system, Water Associates), prederivatization with 9-fluorenylmethyl chloroformate (Aminotag system, Varian) or prederivatization with OPA/2-ME reagent.

Each system has its advantages and disadvantages but in general the OPA system is probably the most versatile when considering ease of derivatization, method of detection and complexity of columns and gradients required for efficient separation of amino acids. It does,

however, suffer from the disadvantage of being unable to detect the amino acids proline and hydroxyproline and, in common with the other reversed phase systems available, is limited in the number of components which can be separated when dealing with complex mixtures. For the analysis of complex mixtures such as physiological fluids, a dedicated ion-exchange system is generally required. This system is very costly, has little versatility and in most laboratories can only be justified if a large number of samples are to be analysed on a continuous basis. The relative merits of ion-exchange and reversed phase HPLC have been reviewed previously (Palmer, 1985; Dong and DiCesare, 1982).

In this section, an ion-exchange amino acid analyzer (constructed from existing HPLC equipment) is compared with a reversed phase HPLC system in order to determine which system is best suited to amino acid analysis of physiological fluids and specific amino acid abnormalities.

8.2 Materials And Methods

8.2.1 Ion-exchange system

8.2.1.1 Reagents

Amino acid standards, o-phthalaldehyde (OPA), 2-mercaptoethanol (2-ME) and Brij 35 were purchased from Sigma Chemical Co (London, UK). Physiological standards, DL-norleucine, lithium citrate diluent buffer (pH 2.2), lithium citrate Picobuffer system IV, were purchased from Pierce Chemical Co, (Chester, UK). Ion-exchange resin DC-4A (sulphonated polystyrene 8% cross-linked with divinylbenzene) supplied in the sodium form from Dionex (Hampshire, UK) was converted to the lithium form as instructed by Benson (1976). 'Analar' grade methanol, boric acid, potassium hydroxide were purchased from BDH Chemicals (Poole, UK).

O-Phthalaldehyde/2-mercaptoethanol (OPA/2-ME) reagent used for derivatization of amino acids was made up as follows: 50 g of boric

acid, 44 g of potassium hydroxide and 3.5 ml of Brij 35 were dissolved in double distilled water, adjusted to pH 10.4 and made up to 1000 ml. 600 mg of OPA were dissolved in 7.5 ml of methanol and 5.0 ml of 2-ME and the solution added to the boric acid buffer. The reagent was vacuum-filtered through 0.22 µm Durapore filters (Waters Associates), degassed and stored in a dark bottle under nitrogen during use. Picobuffers were degassed and filtered similarly and stored under nitrogen during use.

8.2.1.2 Ion-exchange apparatus

An M-45 pump (Waters Associates) was employed to pump OPA/2-ME reagent to a post-column low volume mixing manifold (Waters Associates) at a flow rate of 0.2 ml/min from which a two minute reaction time was permitted prior to fluorescence detection. A Perkin-Elmer 1000 'm' fluorimeter fitted with a 25 µl flow cell, 339 nm interference filter and an emission wavelength of 455 nm, was used for detection. Peak areas were determined using a Spectra Physics SP 4270 integrator. The buffer pump used at a flow rate of 0.2 ml/min was a Waters Type 501, fitted with a Rheodyne 7125 injector and 100 µl sample loop. A stainless steel column with an internal diameter of 0.3 cm and length 35 cm was used to support the ion-exchange resin. A purpose-built glass jacket surrounded the column to which water was supplied by a Churchill circulator (Chemlab Instruments Ltd, UK).

Elution buffers were stored in stoppered air tight glass containers kept under a nitrogen pressure of 0.35 bar. Buffer reservoirs were connected to a Rheodyne pressure regulated selection valve using teflon (Anachem) tubing connected by flanged connectors (Pierce).

A BBC model B microcomputer was used fitted with disk drive (Acorn) and interface as described in the technical information for the ion-exchange system (Appendix A).

8.2.1.3 Preparation of physiological samples for amino acid analysis

Preparation methods were those described by Benson and Hare (1976). Deproteinization was performed by vortex mixing 0.8 ml of urine with 0.2 ml of 10 %(w/v) sulphosalicylic acid followed by centrifugation at 7,500 x g for 5 min to remove precipitated protein. The supernatant was adjusted to pH 2.0-2.2 with 0.3 M sodium hydroxide (approximately 0.2 ml) producing a dilution factor of 1.5. The sample was diluted a further ten-fold with 0.067 M lithium citrate diluent buffer (pH 2.2) before analysis.

Deproteinization of serum or plasma samples was performed by first drying 0.1 ml of 12.5 %(w/v) sulphosalicylic acid in 95% ethanol in a microfuge tube. The dried residue was mixed with 0.1 ml serum sample until dissolved and then centrifuged as described for urine to remove the resulting protein precipitate. The serum supernatant was removed and diluted five-fold in 0.067 M lithium citrate diluent buffer (pH 2.2) and applied to the analyzer.

8.2.2 Reversed phase HPLC system

The HPLC system has been described previously (Griffin et al., 1982). A Zorbax C8 column (25 x 0.46 cm, DuPont Instruments) was employed for separation of amino acid derivatives with a guard column packed with Co:Pell ODS (Whatman). The chromatography system (system 8821, DuPont Instruments) consisted of a series 8800 gradient controller, and 870 pump module and a DuPont column compartment fitted with a Rheodyne injector with a 50 ul injection loop. Fluorescence detection and peak area determination were performed as described for the ion-exchange system. Gradients were formed from two solvents: potassium acetate buffer (20 mM, pH 5.5) and methanol. Both solvents contained 1 %(w/v) tetrahydrofuran. A flow rate of 1.8 ml/minute was used to run a gradient from 20% to 90% methanol with inclusion of two isocratic stages. Most amino acids were eluted within 25 minutes.

The derivatization procedure was that described by Price et al. (1984), which gave improved sensitivity over the method previously described (Griffin et al., 1982). Immediately before use, 250 µl of 0.4 M boric acid buffer (pH 9.4) was added to 25 µl of 2-ME, the components being rapidly mixed. A 250 µl aliquot of this mixture was vortex mixed with 25 µl of prepared sample for exactly 2 minutes prior to injecting 50 µl onto the column. Urine and serum samples were prepared by deproteinizing a maximum of 80 µl sample with 40 µl methanol and 20 µl of 0.4 mM 2,4-diaminobutyric acid (internal standard).

8.2.3 Estimation of urinary creatinine

Urine samples were diluted 1:100 with distilled water. To 3 ml of diluted sample, 1 ml of saturated picric acid solution and 1 ml of 0.75 M sodium hydroxide were added and mixed. Tubes were incubated at room temperature for 15 minutes prior to absorbance measurement at 500 nm. Standard creatinine samples were treated identically to produce a standard curve constructed between 6 µg/ml and 60 µg/ml.

8.3 Results

8.3.1 Assessment of ion-exchange system

Assessment of reproducibilities of column retention times and peak areas (Table 8.1) was performed by applying a series of physiological standards to the column. The percentage coefficients of variation (% CV) for retention times ranged from 0.3 to 4.0%, the majority of components having a % CV of about 2.0%. Peak areas were more variable, the majority producing a % CV of between 1 and 5%. Components giving a notably high % CV were α-amino adipic acid and ammonia. Amino acid standards were used to assess the correlation between peak area ratio and concentration. For chosen concentration range of between 1 and 12.5 nmoles, each amino acid per 100 µl injection produced a linear relationship to peak area ratio, for all amino acids tested. The

quantitative detection limit of the system was 10 pmoles, below which baseline noise interfered with poorly fluorescent derivatives.

Analysis of a mixture of physiological standards (figure 8.1) showed good separation of nearly all amino acids enabling quantitative analysis of over 30 components. Elution conditions used for separation of components in complex mixtures such as physiological fluids were found to be adequate for continuous analyses every five hours, which included column equilibration time.

8.3.2 Assessment of reversed HPLC system

The % CV for retention times ranged between 0.25 and 1.4% and for peak area, % CV ranged between 2.0 and 8.7% (Table 8.2), determined after applying standard amino acid mixtures to the column. High % CV values were obtained for aspartic acid, citrulline and α-amino adipic acid, the latter compound possibly being subject to degradation during processing. α-amino adipic acid was found to elute before asparagine, not after as reported previously (Griffin et al., 1982). The relationship between peak area and concentration for most amino acids, was linear down to 1 pmole which was the quantitative detection limit. Below this value, baseline noise affected quantitative measurement. The reversed phase HPLC system was capable of separating over 20 amino acids within 25 minutes, in a standard mixture (figure 8.4).

8.3.3 Application of analytical systems to plasma and urine analysis

Plasma and urine samples from normal individuals and a patient with Gyrate Atrophy, analyzed by the ion-exchange system (figures 8.2 and 8.3) and by the reversed phase HPLC system (figures 8.5 and 8.6) were used for comparison of the two systems.

As a brief check of measurement reliability, a normal plasma sample was analyzed by the ion-exchange system and compared to literature values (Table 8.3). Only glutamine was found to be present

at levels below the normal range and this may be explained by the inherent instability of this amino acid in stored plasma (Palmer et al., 1973). Ammonia determination using the ion-exchange system can only be assessed on a semi-quantitative basis due to buffer contamination which is evident in figure 8.2 where a broad raised plateau surrounds the sharper sample peak (peak 24).

Comparison of amino acid measurements by the two systems of normal urine and urine from a Gyrate Atrophy patient (Table 8.4) demonstrated a good correlation between most values and showed how several estimations by the reversed phase HPLC system were incorrectly estimated due to coelution of amino acids. In this example, glutamine, threonine and arginine were all higher by reversed phase HPLC analysis compared to ion-exchange. However, urinary ornithine measurement of the Gyrate Atrophy sample by both systems was comparable, (362 and 346 µmoles/mmole creatinine by reversed phase HPLC and ion-exchange respectively). Plasma ornithine values were 1012 and 987 µM by reversed phase HPLC and ion-exchange analysis respectively, for the Gyrate Atrophy sample, (normal range of plasma ornithine, 30-130 µM). A plasma sample from a second Gyrate Atrophy patient had an ornithine concentration of 412 µM compared to a normal value estimated at 27 µM by reversed phase HPLC. However, the reversed phase HPLC system could not be used to determine citrulline which has metabolic and transport links with ornithine, whereas the ion-exchange system revealed a normal plasma citrulline concentration of 10 µM in addition to raised plasma ornithine (987 µM) in the Gyrate Atrophy sample. Attempts to detect the lactam of ornithine by ion-exchange analysis proved unsuccessful with pure lactam samples (the synthesis of which is described in section 9), suggesting that the compound might have broken down during sample processing or storage, or may have remained on the column during the normal elution period.

TABLE 8.1

Reproducibility of retention times and peak areas for separation of amino acids by the ion-exchange system. The data represent the mean values from 5 separate experiments. Elution conditions were those described in Appendix A. Norleucine was used as an internal standard for calculation of peak areas.

AMINO ACID COMPONENT	RETENTION TIME: MEAN (min)	% CV	PEAK AREA: % CV
PHOSPHOSERINE	6.8	3.9	0.7
TAURINE	11.3	2.0	1.4
PHOSPHOETHANOLAMINE	13.6	2.1	1.8
ASPARTIC ACID	39.0	2.2	6.0
THREONINE	51.5	1.9	5.5
SERINE	54.7	1.7	5.2
GLUTAMIC ACID	59.8	0.6	4.9
α-AMINO ADIPIC ACID	88.4	3.7	7.3
GLYCINE	100.0	1.3	6.5
ALANINE	103.6	2.6	4.8
CITRULLINE	106.3	2.3	2.8
VALINE	116.5	2.8	4.8
CYSTINE	120.9	2.3	3.7
METHIONINE	129.7	2.4	2.3
ISOLEUCINE	142.5	2.7	2.0
LEUCINE	148.7	2.4	3.3
NORLEUCINE	153.9	1.7	--
TYROSINE	158.4	1.7	3.8
β-ALANINE	168.1	1.6	2.3
PHENYLALANINE	170.1	1.9	3.3
AMMONIA	192.9	1.0	6.7
HYDROXYLYSINE	198.4	1.6	4.8
ORNITHINE	208.9	1.7	4.5
LYSINE	222.5	1.8	3.2
1-METHYL HISTIDINE	227.5	1.8	5.6
HISTIDINE	232.7	1.9	3.2
3-METHYL HISTIDINE	244.6	1.8	4.1
TRYPTOPHAN	253.9	0.29	4.9
CARNOSINE	255.2	0.47	1.3
ARGININE	258.6	0.46	3.8

TABLE 8.2

Reproducibility of retention times and peak areas for separation of amino acids by the reversed phase HPLC system. The data represent the mean values from 5 separate experiments.

2,4-diaminobutyrate was used as an internal standard for calculation of peak areas.

AMINO ACID	RETENTION TIME: MEAN (minutes)	% CV	PEAK AREA: % CV
ASPARTIC ACID	2.31	1.1	8.7
GLUTAMIC ACID	2.42	1.25	5.6
α-AMINO ADIPIC ACID	3.68	1.25	6.1
ASPARAGINE	5.88	1.22	4.7
SERINE	7.75	1.20	4.3
GLUTAMINE	8.49	1.4	3.6
HISTIDINE	8.94	1.0	5.5
GLYCINE	10.36	0.7	2.4
THREONINE	10.69	1.2	7.1
ARGININE	11.77	0.52	3.2
TAURINE	12.77	0.61	4.0
TYROSINE	13.32	0.61	4.8
METHIONINE	18.15	0.50	4.6
TRYPTOPHAN	18.78	0.42	5.5
PHENYLALANINE	19.29	0.3	4.5
AMMONIA	19.81	1.3	5.4
ISOLEUCINE	20.70	0.3	3.1
LEUCINE	21.04	0.3	2.9
2,4-DIAMINOBUTYRATE	21.92	0.34	--
ORNITHINE	22.36	0.3	2.0
LYSINE	22.99	0.25	2.0

TABLE 8.3

Assessment of measurement reliability of the ion-exchange system. As an example, amino acid measurements obtained for a normal human serum sample were compared with literature values for adult serum (Ibbott, 1974).

AMINO ACID COMPONENT	CALCULATED SERUM VALUES (μM)	NORMAL SERUM RANGES (μM)
TAURINE	51	35 - 140
ASPARTIC ACID	42	11 - 54
THREONINE	78	75 - 250
SERINE	90	61 - 190
GLUTAMIC ACID	108	0 - 120
GLYCINE	240	130 - 490
ALANINE	246	170 - 500
CITRULLINE	15	12 - 55
γ -AMINO-N-BUTYRIC ACID	8	8 - 35
VALINE	123	120 - 330
METHIONINE	36	13 - 39
ISOLEUCINE	36	35 - 100
LEUCINE	72	69 - 160
TYROSINE	60	32 - 87
PHENYLALANINE	63	34 - 120
LYSINE	150	90 - 260
HISTIDINE	60	56 - 120
ARGININE	49	46 - 150
GLUTAMINE	310	420 - 760

TABLE 8.4

Comparison of urinary amino acid determinations made by the two analytical systems. Values in brackets indicate anomalous HPLC estimations due to co-elution of components. Concentrations expressed as μmoles per mmole creatinine. N.D. = Not Detected

AMINO ACID	NORMAL URINE:		HYPERORNITHINURIA:	
	REVERSED PHASE HPLC	ION-EXCHANGE	REVERSED PHASE HPLC	ION-EXCHANGE
ASPARTIC ACID	3	4	26	24
GLUTAMIC ACID	5	5	(177)	60
SERINE	26	27	59	71
GLUTAMINE	28	26	1	2
HISTIDINE	62	56	51	43
GLYCINE	87	84	196	221
THREONONE	17	16	(40)	15
ARGININE	5	5	(26)	13
TAURINE	70	67	228	216
TYROSINE	(9)	3	25	22
METHIONINE	5	3	13	14
ISOLEUCINE	0.2	N.D.	2	2
LEUCINE	0.3	0.5	5	6
ORNITHINE	N.D.	N.D.	362	346
LYSINE	4	5	73	75

FIGURE 8.1 Ion-exchange separation of amino acid standards

Separation of a standard physiological amino acid mixture (10 nanomoles per component) by the ion-exchange system described in the text. Elution conditions are given in Appendix B.
Numbered peaks are defined as follows:
Peak 1, taurine; 2, aspartic acid; 3, threonine; 4, serine; 5, glutamic acid; 6, α-amino adipic acid; 7, glycine; 8, alanine; 9, citrulline; 10, α-amino-n-butyric acid; 11, valine; 12, cystine; 13, methionine; 14, L-cystathionine; 15, isoleucine; 16, leucine; 17, norleucine (internal standard); 18, tyrosine; 19, (not determined); 20, phenylalanine; 21, β-alanine; 22, DL-β-amino-isobutyric acid; 23, γ-amino-n-butyric acid; 24, ammonia; 25, ornithine; 26, lysine; 27, 1-methyl histidine; 28, histidine; 29, 3-methyl histidine; 30, tryptophan; 31, arginine; 32, phosphoserine; 33, glutamine.

AMINO ACID STANDARDS

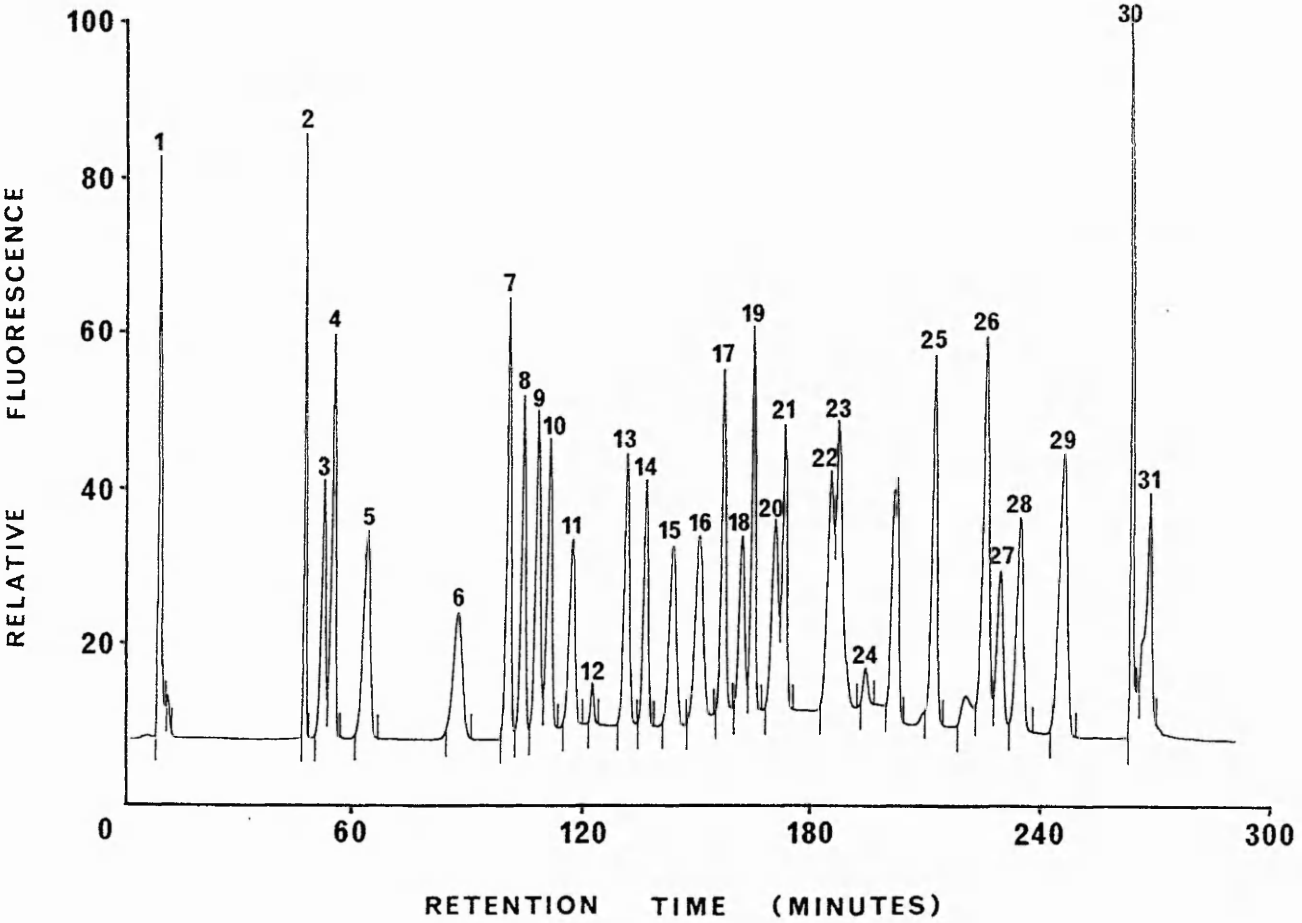
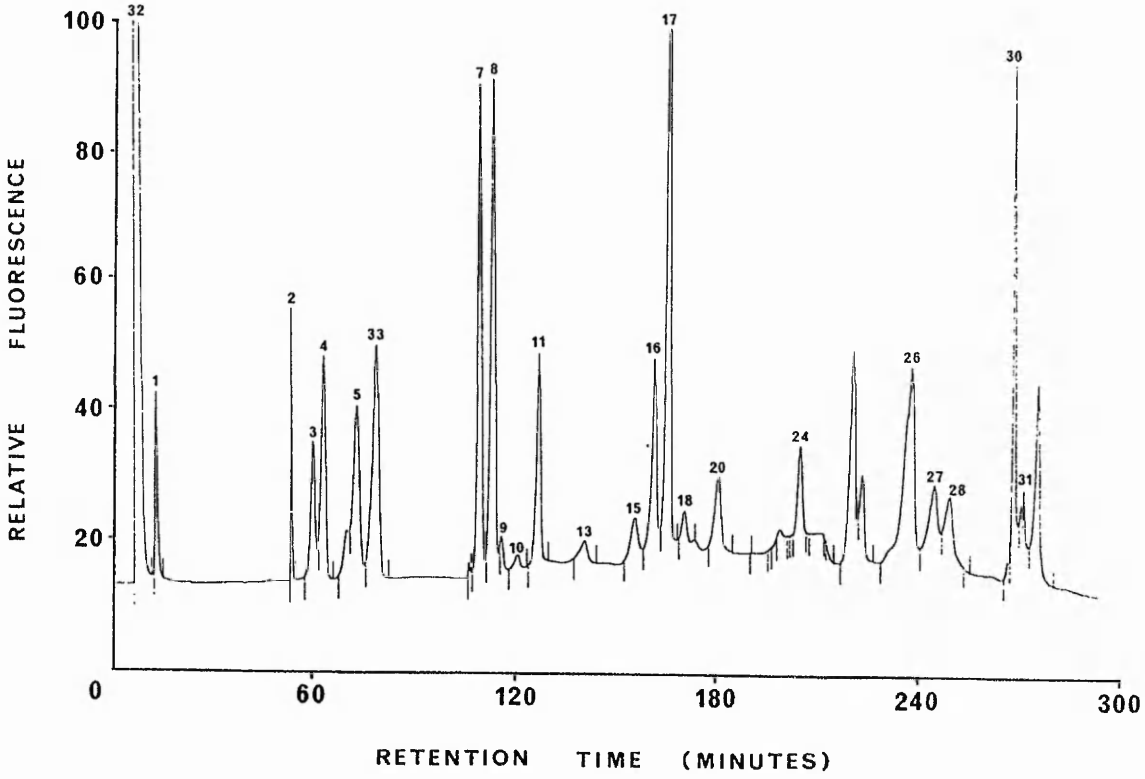


FIGURE 8.2 Ion-exchange amino acid analysis of plasma samples

Separation of amino acids in normal plasma and plasma from a patient with Gyrate Atrophy, by ion-exchange chromatography. Hyperornithinaemia is clearly demonstrable (peak 25).
Numbered peaks are defined as follows:
Peak 1, taurine; 2, aspartic acid; 3, threonine; 4, serine; 5, glutamic acid; 6, α-amino adipic acid; 7, glycine; 8, alanine; 9, citrulline; 10, α-amino-n-butyric acid; 11, valine; 12, cystine; 13, methionine; 14, L-cystathionine; 15, isoleucine; 16, leucine; 17, norleucine (internal standard); 18, tyrosine; 19, (not determined); 20, phenylalanine; 21, β-alanine; 22, DL-β-amino-isobutyric acid; 23, γ-amino-n-butyric acid; 24, ammonia; 25, ornithine; 26, lysine; 27, 1-methyl histidine; 28, histidine; 29, 3-methyl histidine; 30, tryptophan; 31, arginine; 32, phosphoserine; 33, glutamine.

NORMAL PLASMA



HYPERORNITHINEMIA

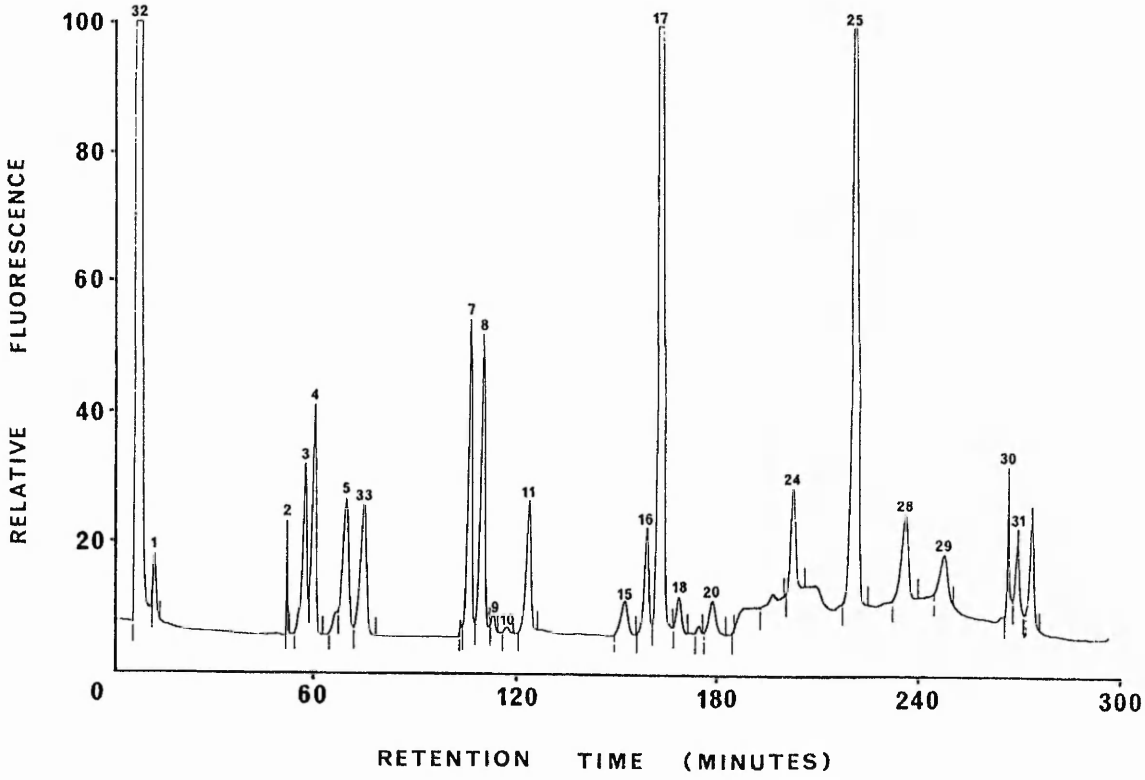


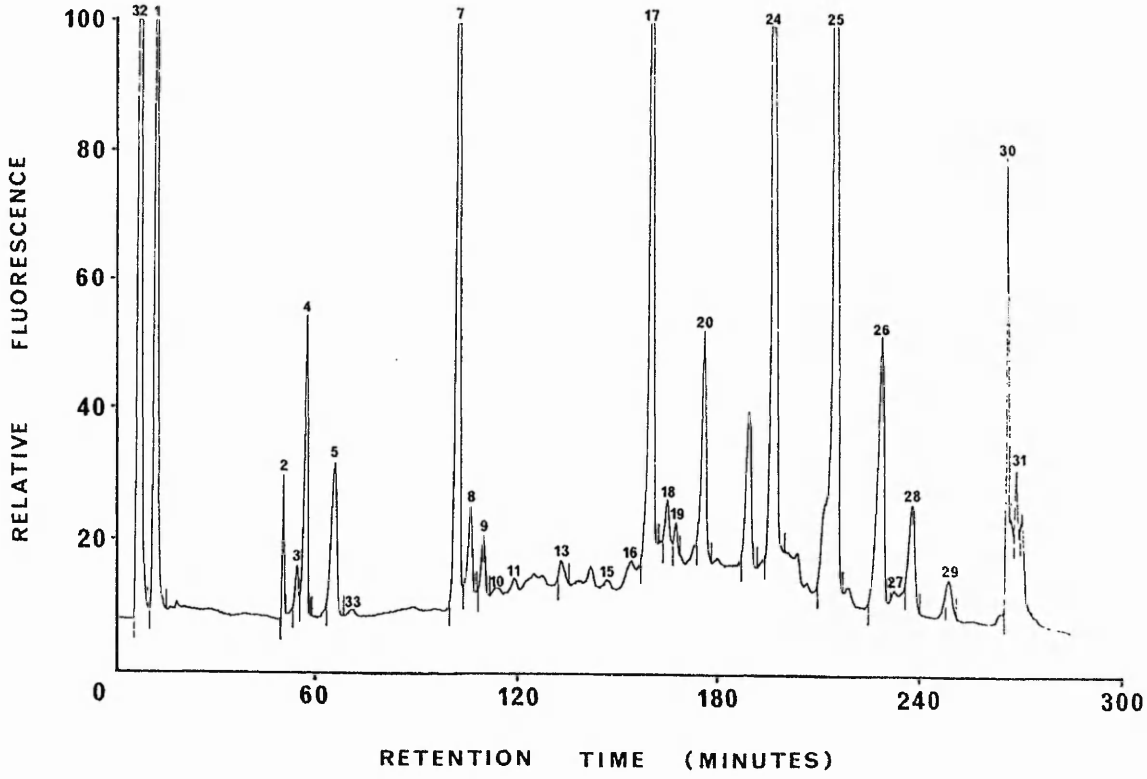
FIGURE 8.3 Ion-exchange amino acid analysis of urine samples

Separation of amino acids in normal urine and urine from a patient with Gyrate Atrophy, by ion-exchange chromatography. Hyperornithinuria is clearly demonstrable (peak 25).

Numbered peaks are defined as follows:

Peak 1, taurine; 2, aspartic acid; 3, threonine; 4, serine; 5, glutamic acid; 6, α-amino adipic acid; 7, glycine; 8, alanine; 9, citrulline; 10, α-amino-n-butyric acid; 11, valine; 12, cystine; 13, methionine; 14, L-cystathionine; 15, isoleucine; 16, leucine; 17, norleucine (internal standard); 18, tyrosine; 19, (not determined); 20, phenylalanine; 21, β-alanine; 22, DL-β-amino-isobutyric acid; 23, γ-amino-n-butyric acid; 24, ammonia; 25, ornithine; 26, lysine; 27, 1-methyl histidine; 28, histidine; 29, 3-methyl histidine; 30, tryptophan; 31, arginine; 32, phosphoserine; 33, glutamine.

HYPERORNITHINURIA



NORMAL URINE

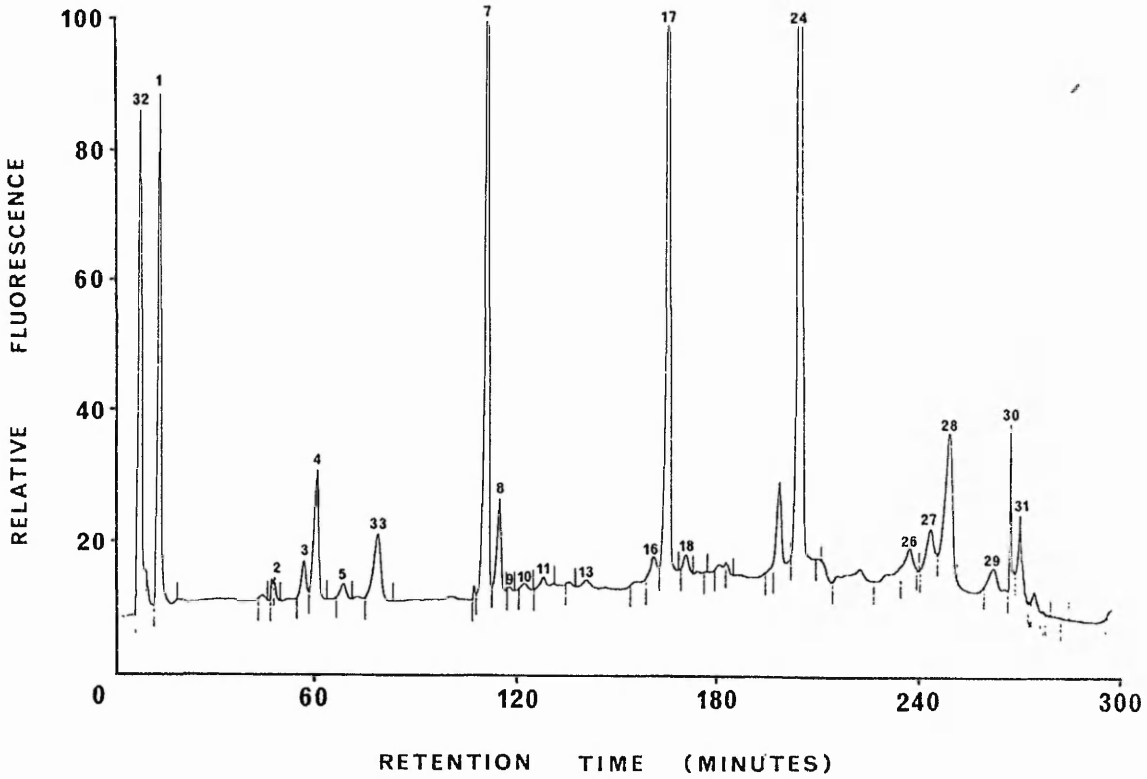


FIGURE 8.4 Reversed phase amino acid analysis of amino acid standards

Separation of a standard amino acid mixture (80 picomoles per component) by reversed phase HPLC as described in the text. The elution gradient is indicated as % methanol in the solvent mixture from 20-90% methanol with two isocratic stages.

Numbered peaks are defined as follows:

Peak 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, α-amino adipic acid; 5, asparagine; 6, serine; 7, glutamine; 8, histidine; 9, glycine; 10, threonine; 11, arginine; 12, taurine; 13, tyrosine; 14, tryptophan; 15, methionine; 16, phenylalanine; 17, isoleucine; 18, leucine; 19, 2,4-diaminobutyric acid (internal standard); 20, ornithine; 21, lysine; 22, ammonia (8 picomoles).

AMINO ACID STANDARDS

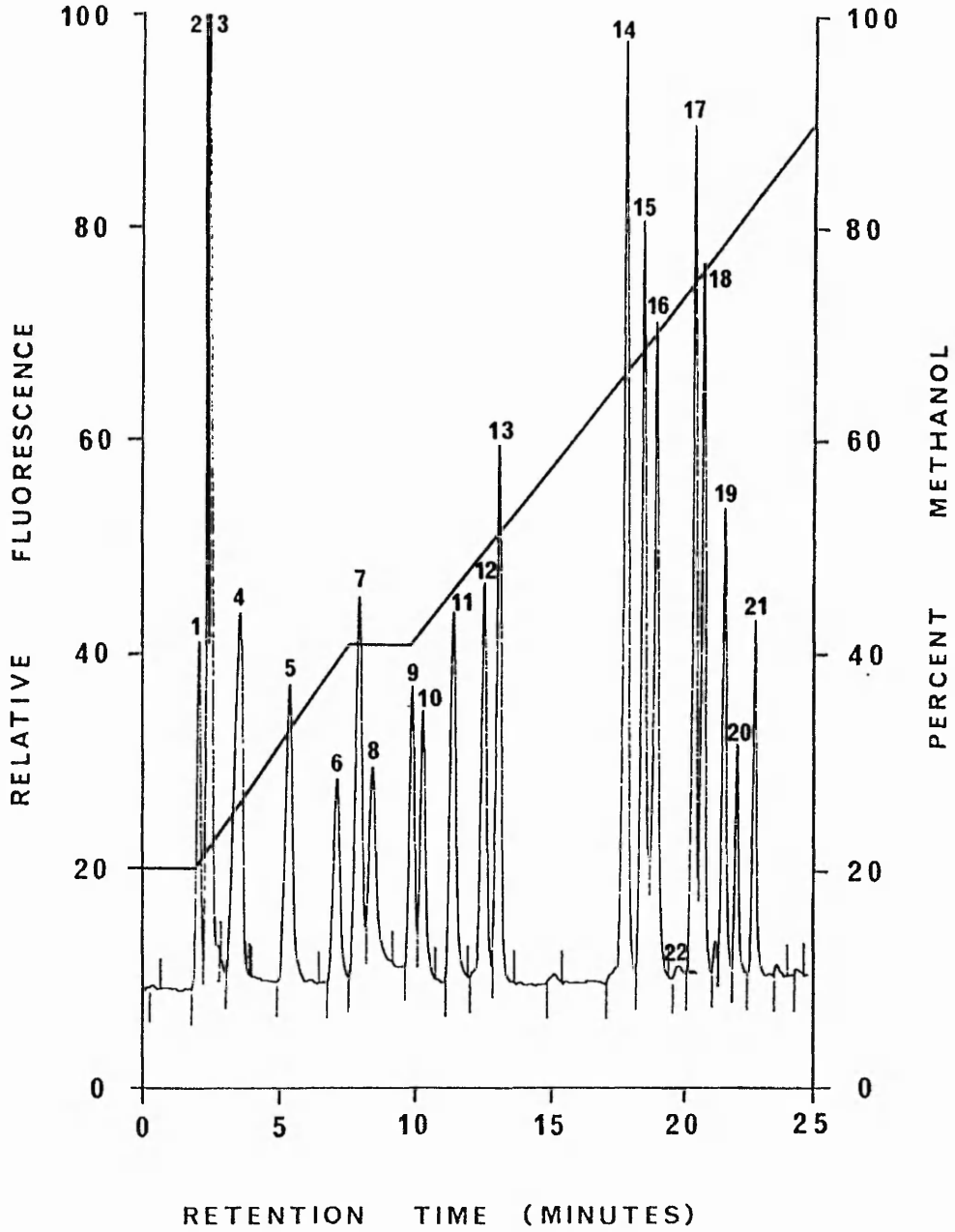
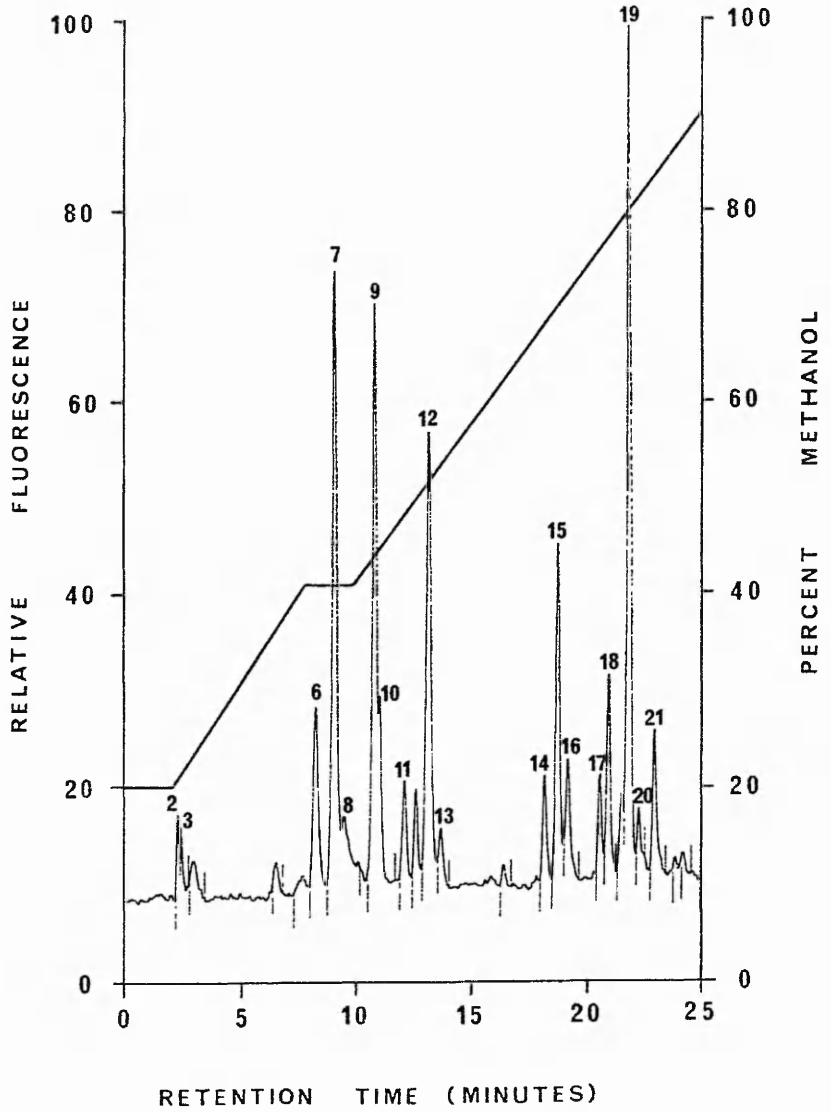


FIGURE 8.5 Reversed phase amino acid analysis of plasma samples

Separation of amino acids in normal plasma and plasma from a patient with Gyrate Atrophy, by reversed phase HPLC as described in the text. Hyperornithinaemia is clearly demonstrable (peak 20).
Numbered peaks are defined as follows:
Peak 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, α-amino adipic acid; 5, asparagine; 6, serine; 7, glutamine; 8, histidine; 9, glycine; 10, threonine; 11, arginine; 12, taurine[a]; 13, tyrosine; 14, tryptophan; 15, methionine; 16, phenylalanine; 17, isoleucine; 18, leucine; 19, 2,4-diaminobutyric acid (internal standard); 20, ornithine; 21, lysine; 22, ammonia.

[a] possible coelution with alanine

NORMAL PLASMA



HYPERORNITHINEMIA

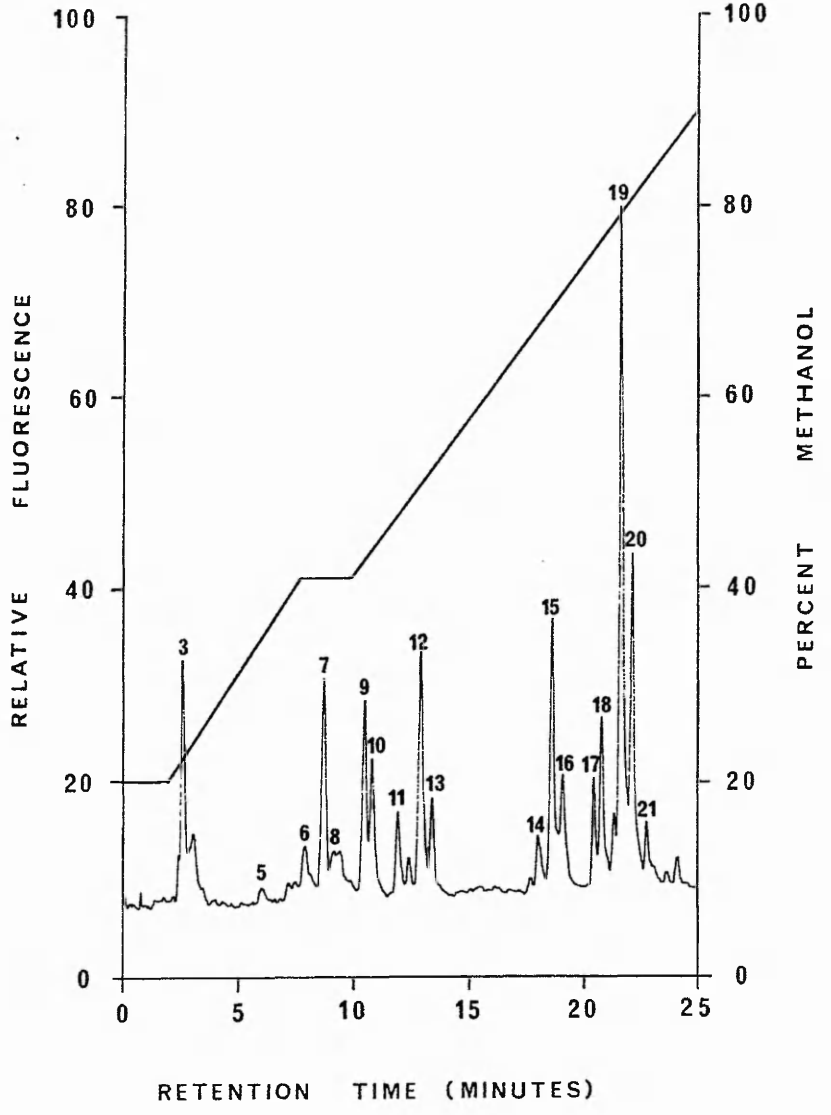
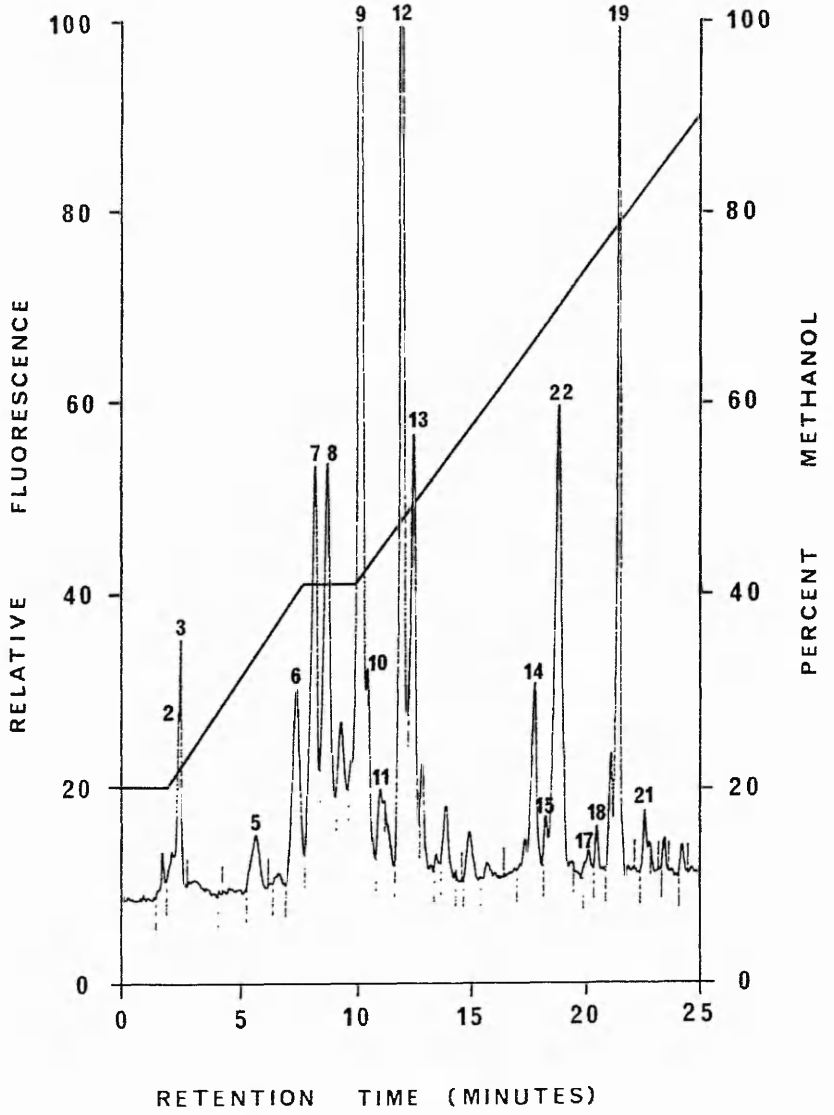


FIGURE 8.6 Reversed phase amino acid analysis of urine samples

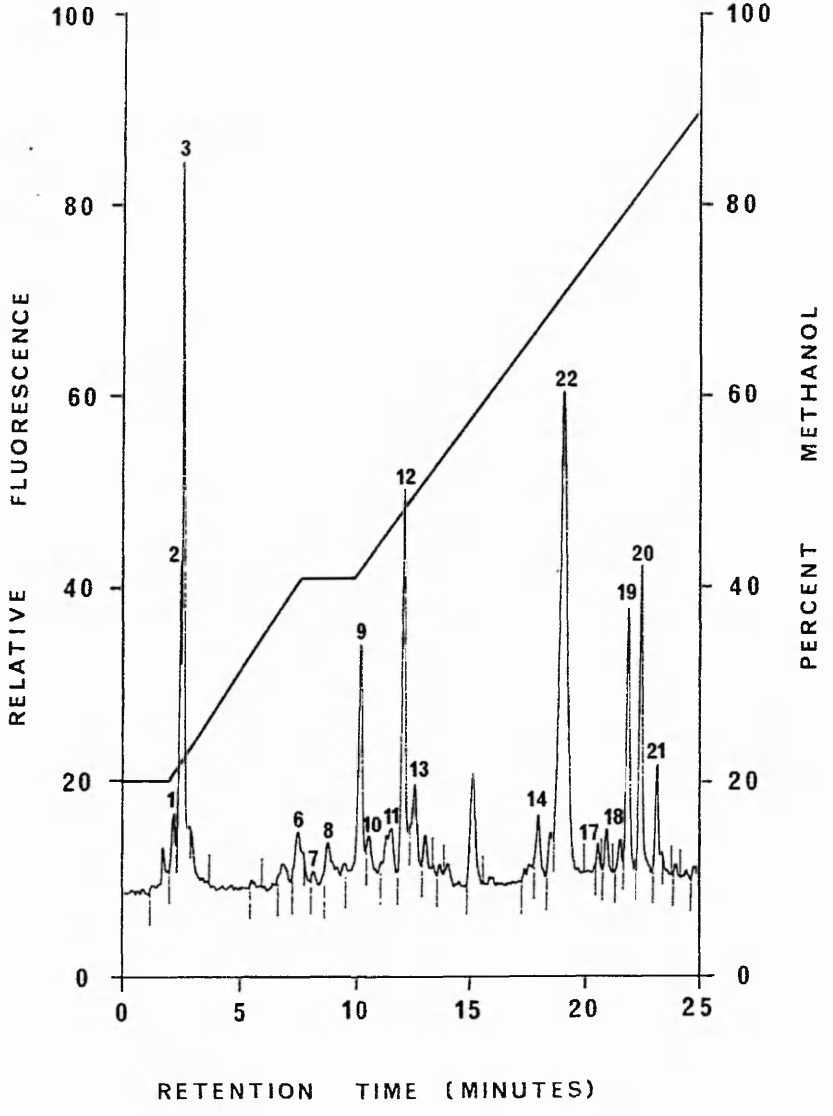
Separation of amino acids, in normal urine and urine from a patient with Gyrate Atrophy, by reversed phase HPLC as described in the text. Hyperornithinuria is clearly demonstrable (peak 20).
Numbered peaks are defined as follows:
Peak 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, α-amino adipic acid; 5, asparagine; 6, serine; 7, glutamine; 8, histidine; 9, glycine; 10, threonine; 11, arginine; 12, taurine[a]; 13, tyrosine; 14, tryptophan; 15, methionine; 16, phenylalanine; 17, isoleucine; 18, leucine; 19, 2,4-diaminobutyric acid (internal standard); 20, ornithine; 21, lysine; 22, ammonia.

[a] possible coelution with alanine

NORMAL URINE



HYPERORNITHINURIA



8.4 Discussion

A drawback of the reversed phase system is the coelution of some peaks in biological fluids containing a complex mixture of amino acids which can make quantitative analysis difficult. The unstable derivatives produced with OPA/2-ME reagent are a contributing factor in obtaining accurate determinations with both systems. This is more problematic with the reversed phase system since pre-column derivatization is performed manually and would therefore benefit from automation (Smith and Panico, 1985; Bruton, 1986).

Post column derivatization is automated on the ion-exchange system, OPA/2-ME reagent being pumped to a mixing manifold attached to the eluant outlet. Another disadvantage of OPA/2-ME reagent is that it will not react with secondary amines in spite of the sensitivity which is achieved. The ion-exchange system is more versatile in this respect since the OPA/2-ME reagent can be replaced by ninhydrin (with a consequent decrease in sensitivity) or secondary amines (proline and hydroxy-proline) could be reacted with sodium hypochlorite (Dong and DiCesare, 1982) prior to OPA/2-ME derivatization although this would necessitate an additional reagent pump connected to the system.

The ion-exchange system is subject to ammonia contamination which can be present in buffers, producing anomalous determinations of sample ammonia and amino acids eluting close to it.

Incorporation of a suitable 'ammonia trap' in the system would overcome problems of contamination and allow quantitative determination of ammonia and compounds with a similar retention time if so required. A consistent feature of urine and plasma samples stored frozen for a period of time is a large initial peak in the phosphoserine position upon ion-exchange chromatography. This may be a degradative product or an artifact produced during sample processing although this peak was

not present in standard amino acid mixture which underwent identical processing.

The problem of coelution which often occurs with reversed phase HPLC is usually a minor one which can in some cases be overcome by adjustment of elution conditions so that the system is ideally suited to sensitive and quantitative detection of specific abnormalities in biological fluids. A particular problem encountered during analysis of urine from Gyrate Atrophy patients was the detection of the δ-lactam of ornithine (the synthesis of which is described in section 9). The lactam was found to co-elute with ammonia, a major component of urine. Ammonia was removed from these urine samples by rotary evaporation and also by vacuum desiccation after the sample had been made slightly alkaline with sodium hydroxide. However, removal of ammonia by these methods also removes the ornithine lactam. No ornithine lactam could be detected in a plasma sample from a Gyrate Atrophy patient. This agrees with the findings of Valle et al. (1980), who were unable to detect plasma ornithine lactam in hyperornithinaemic patients.

The reversed phase HPLC system was also employed successfully to demonstrate other inborn errors of amino acid metabolism such as argininosuccinic aciduria which was demonstrable by a prominent argininosuccinic acid peak after 4 minutes. Urine from a patient with α-amino adipic aciduria produced an equally conspicuous peak in the α-amino adipic acid position (see Appendix B).

In conclusion, while the ion-exchange system is more reliable with respect to complete separation and quantitative analysis of an amino acid profile, the reversed phase HPLC system is preferable for rapid analysis of specific amino acids whilst still allowing analysis of a limited profile of amino acids.

9 AMINO ACID STUDIES

9.1 Introduction

Measurement of amino acids in humans and other mammals has been largely confined to free amino acids in physiological fluids, namely serum or plasma, urine, amniotic and cerebrospinal fluid. More recently, other directly available fluids have also been investigated, for example, amino acid levels in the vitreous space of rabbit eye have been reported (Gunnarson et al., 1987). Measurements of amino acids in homogenized tissue extracts have been reported employing rapid freezing of tissue in liquid nitrogen followed by complete subcellular disruption. Free amino acid pools have recently been measured in this way in rat liver and kidney tissue (Pastor-Anglada et al., 1986). Few attempts have been made at measuring subcellular amino acid pools, due to the problems associated with isolating cell organelles, diffusion of amino acids into the isolation medium and accurate measurement of organelle volume. Because of these problems, amino acid measurements are invariably expressed per gram of protein or wet weight of extract. Of the few reports of mitochondrial amino acid measurement (Matsuzawa, 1974), isolated mitochondria have been homogenized in acids prior to amino acid analysis. Wheatley et al. (1986) have studied the problem of intracellular amino acid pools in detail and extensively reviewed the literature on this topic. They have concluded that amino acids can move across membranes very rapidly even at $0^{o}C$ and that free amino acids exit organelles virtually immediately on washing. They have also postulated the existence of a number of pools of amino acids, some of which are acid extractable from membranes, others which are loosely adsorbed onto sites inside cells.

In the present study, amino acid measurements have been made on rat serum and rat liver cells fractionated into cytosolic and mitochondrial extracts. Attempts were made to determine which amino

acids are associated with mitochondrial membrane extracts. A brief study of the δ-lactam of ornithine was also made.

9.2 Materials And Methods

9.2.1 Synthesis and study of the δ-lactam of ornithine

The ornithine lactam was synthesized using the method described by Oberholzer and Briddon (1978). 500 mg ornithine hydrochloride (Sigma) was dissolved in distilled water and passed through 10 ml (swollen volume) of Amberlite CG-120 (H^+ form) resin (Sigma) equilibrated with distilled water. The resin was washed until the eluted fractions were free of chloride (determined by titration against silver nitrate). Ornithine base was then eluted with 2.5 M ammonium hydroxide. The eluates (ninhydrin positive fractions) were dried in a vacuum dessicator. The solid base was dissolved in absolute ethanol to produce a 1 %(w/v) solution which was boiled under reflux for 3 hours. The solution was dried in a vacuum desiccator to produce a waxy solid. The residue was dissolved in a solution of trichloromethane/methanol/5 M ammonium hydroxide (10:5:1 v/v) and passed through a column of silica gel. This procedure was performed in a fume cupboard. The ornithine lactam was eluted with the same solvent mixture and dried by vacuum desiccation. The final residue was dissolved in methanol prior to analysis by reversed phase HPLC (section 8.2.2).

The ornithine lactam was incubated at differing concentrations with fixed amounts of rat kidney OAT and assayed for OAT activity as described previously (section 2.2.1.2) to investigate any possible inhibitory effect upon the enzyme. To briefly assess the stability of the ornithine lactam *in vivo*, samples of normal fresh human serum and urine were incubated with the lactam at a concentration of 500 uM for 2 hours at 37°C in sterile containers. Other samples of serum and urine were incubated with ornithine hydrochloride at a concentration of 1 mM for 2 hours at 37°C in order to simulate the conditions of

hyperornithinaemia and hyperornithinuria produced in Gyrate Atrophy patients and determine whether the ornithine lactam was produced under these conditions. After the incubation period, serum and urine samples were analyzed by reversed phase HPLC to determine the concentrations of ornithine and ornithine lactam with respect to analyses prior to incubation.

9.2.2 Amino acid studies on intracellular compartments

9.2.2.1 Analysis of rat serum

Five male and five female adult Sprague-Dawley rats were used in this study and were fed ad libitum on a diet containing 20% protein. An aliquot of blood (1 ml) was removed from the neck artery of each freshly killed rat and allowed to clot for 2 hours at room temperature in a microcentrifuge tube (Eppendorf). The clotted blood was centrifuged at 7,500 x g for 5 minutes. Serum was collected and analyzed for amino acids by reversed phase HPLC as described previously (section 8.2.2). Samples were stored at -20^{o}C prior to analysis.

9.2.2.2 Rat liver cell fractionation

The liver of a freshly killed rat was perfused in situ with 0.9 %(w/v) saline by infusing 20-25 ml into the liver via the hepatic and hepatic portal veins. The resulting pale white liver was removed and weighed. All steps were performed on ice or with buffers cooled to 4^{o}C. The liver was then macerated and homogenized in buffered sucrose solution (0.25 M sucrose; 3 mM imidazole, pH 7.4) to produce a 20 %(w/v) homogenate. Homogenization was performed in a 50 ml homogenizing tube (Potter-Elvehjem) using five passes of a teflon pestle with a 0.2 mm clearance. The homogenate was centrifuged at 1000 x g for 10 minutes at 4^{o}C and the supernatant removed and stored on ice. At each stage, fractions were assayed for protein (Lowry, 1951). The pellet was resuspended in 10 ml of buffered sucrose solution by 5 passes of the homogenizer and again centrifuged at 1000 x g for 10 minutes.

Supernatants were combined and centrifuged at 16,000 x g for 3 minutes to produce a crude mitochondrial pellet. The supernatant was centrifuged at 100,000 x g for 30 minutes to produce a particle free supernatant which was stored at -20^{o}C. The mitochondrial pellet was resuspended in 25 ml of buffered sucrose solution by homogenization as described previously followed by centrifugation at 16,000 x g for 3 minutes. The pellet was washed a total of three times in this way. An aliquot of resuspended pellet containing 5 mg protein was pelleted as described above, and then homogenized in 1 ml methanol and sonicated at 10 kilocycles per second for 5 minutes. The precipitated protein was removed by centrifugation at 7,500 x g for 5 minutes. The particle-free supernatant was also deproteinized with methanol as described for serum prior to amino acid analysis by reversed phase HPLC (section 8.2.2). The remaining mitochondrial pellet was resuspended in 5 ml of buffered sucrose and sonicated at 10 kilocycles for 5 minutes. The sonicated extract was carefully pipetted onto a two-step sucrose density gradient prepared as follows: 10 ml of 1.5 M sucrose was pipetted into a 25 ml centrifuge tube which was carefully overlayed with 10 ml of 1.1 M sucrose (sucrose solutions were made up in distilled water). The sonicated extract was layered onto the sucrose and the tube finally filled to within 5 mm from the rim with 0.25 M sucrose. The tube was centrifuged at 24,000 r.p.m. in a Beckman ultracentrifuge, using a swing-out rotor, for 1 hour (the brake was left off to allow slow deceleration) at 4^{o}C. Bands of crude mitochondrial outer membrane (MOM) and inner membrane (MIM) were obtained as shown in figure 9.1. The MOM and MIM bands were collected by using a cut off needle and syringe. The MOM and MIM bands were mixed with 1 M sodium chloride to produce a 33 %(v/v) mixture of the two respective band extracts (this volume was dependent upon volume of sucrose solution required to remove each band). The two membrane

extracts were centrifuged at 100,000 x g for 30 minutes. The pelleted membrane extracts were resuspended in the minimum volume of distilled water to accurately remove an aliquot for protein assay (Lowry et al, 1951). Suitable aliquots of membrane were mixed with 1 ml of 10 %(w/v) trichloroacetic acid and sonicated at 10 kilocycles per second for 5 minutes prior to centrifugation at 7,500 x g for 5 minutes to remove precipitated membranes. Aliquots of the supernatant were mixed with 2,4-diaminobutyrate, also diluted appropriately in 10 %(w/v) trichloroacetic acid, so that a total of 72 picomoles of this internal standard was applied to the reversed phase HPLC system for amino acid analysis of the prepared membrane extracts (see section 8.2.2).

9.3 Results

9.3.1 Studies on the δ-lactam of ornithine

The synthesized ornithine lactam was found to be in a virtually pure form upon analysis by reversed phase HPLC (figure 9.2). Only very minor ornithine contamination was present. The OPA derivative of the lactam was poorly fluorescent (about 25% of the relative fluorescence of ornithine at an equivalent concentration and is comparable to the fluorescence of ammonia). The problem of coelution of the lactam with ammonia could not be resolved even with major alterations to the elution gradient. An isocratic elution at 50% methanol produced two discernible peaks for pure lactam preparations containing added ammonia, but were insufficiently separated to permit separate peak area determination.

The ornithine lactam had no inhibitory effect upon OAT under the conditions tested. Measurements of ornithine added to normal urine and serum samples incubated for 2 hours at 37^{o}C showed no significant changes in ornithine concentrations or formation of ornithine lactam, although this was difficult to assess precisely in urine due to coelution with ammonia upon HPLC analysis. Incubation of the ornithine

lactam with normal serum produced a decrease in the concentration of this compound from 491 ± 28 to 305 ± 21 (mean ± S.E., n=3). No change in ornithine concentration was produced. Ornithine lactam incubated with urine produced no observable change of either lactam or ornithine.

9.3.2 Analysis of amino acid pools in rat

9.3.2.1 Problems associated with sample analysis

Amino acid analysis of rat serum samples could be performed using the reversed phase HPLC system as described for human serum (section 8.2.2) to provide adequate amino acid separation (figure 9.3). Most subcellular fractionation methods use a buffer containing Tris and EDTA. Unfortunately, these compounds could not be used in this study since both contain primary amine groups which react with OPA and consequently would interfere with amino acid analysis. A suitable buffer component (imidazole) was therefore chosen for fractionation.

Problems of amino acid coelution upon HPLC analysis have been discussed previously (section 8.4) and the coelution of taurine with alanine and threonine with arginine may have affected quantitative estimations of these amino acids. A further problem concerned the presence of sucrose in samples, the viscosity of which made handling difficult although did not affect analysis since derivatization was apparently not impaired and this process diluted the sample. Figures 9.4 and 9.5 show examples of amino acid profiles from subcellular rat liver fractions. Where fractionated samples were too dilute for analysis, it was necessary to concentrate them by vacuum desiccation once the samples had been deproteinized and were in methanol. This enabled the addition of internal standard to the sample without overdiluting it. At least 2 mg of protein from whole mitochondrial extract was required to obtain amino acid profiles at the picomole level. Fractionated MOM and MIM required a minimum of 4 mg membrane protein to produce amino acid analyses above the quantitative detection

limit of the HPLC system. The deproteinization and concomitant extraction of amino acids with 10 %(w/v) trichloroacetic acid and its subsequent inclusion into the derivatization and HPLC system did not interfere with analysis. Since a 25 µl aliquot of sample was diluted to 275 µl by derivatization with reagent containing 0.4 M boric acid buffer (pH 9.4), OPA derivatives are still formed. Standard amino acid mixtures dissolved in 10 %(w/v) trichloroacetic acid produced comparable detection limits, peak areas and retention times to analyses of standards reconstituted in methanol.

9.3.2.2 Distribution of amino acid pools in rat serum and subcellular compartments

Estimated amino acid levels determined by HPLC analysis of rat serum, cytosolic and whole mitochondrial extracts are shown in Table 9.1. Amino acid levels in rat serum were comparable to normal human serum ranges (Table 8.3) with the exception of taurine due to its coelution with alanine, which in some cases has resulted in anomalously high estimations. This is also likely to be the case for threonine and arginine. Absolute concentrations of amino acids measured from isolated rat liver subcellular fractions are of little significance as will be discussed later, and results can only be analyzed in terms of relative levels. Threonine/arginine and taurine/arginine measurements for both cytosolic and mitochondrial extracts produced the highest concentrations with respect to other amino acids and these values reflect relative levels in serum, alanine being the most abundant amino acid in serum and cytosolic extracts. Glutamine and to a lesser extent glycine appeared to be at much lower levels in cytosol than in serum with respect to other amino acids in these samples. Glutamine, arginine (assuming no coelution with threonine), tyrosine and ornithine could not be detected in whole mitochondrial extracts. Relative levels of ornithine were comparable in serum and cytosolic extract.

Several 'acid extractable' amino acids could be detected upon disruption and separation of whole mitochondrial extracts into crude outer and inner membrane extracts (Table 9.2). Extraction of these amino acids during the deproteinization of samples with 10 %(w/v) trichloroacetic acid allowed analyses to be made at the quantitative detection limit of the HPLC system. A number of amino acid peaks could be detected but not accurately quantified since the level was below 1 pmole per mg protein in the original sample. Glutamic acid and lysine which are fully resolved by HPLC (and therefore reliably quantified) were predominantly associated with the MIM and to a lesser extent with the MOM extract. Glycine was also associated predominantly with the MIM extract. Methionine and histidine were associated at lower levels, predominantly with the MOM extract. The threonine/arginine and taurine/alanine estimations were associated with both MOM and MIM extracts in roughly equal distributions. No other amino acids could be detected in association with these mitochondrial membranes. Ornithine, therefore, was not detected in whole mitochondria, nor was this amino acid extractable from membrane extracts.

TABLE 9.1

Distribution of amino acid pools in rat serum and intracellular compartments. Values are expressed as mean ± S.E. (n=10).

AMINO ACID	SERUM (μm)	CYTOSOL[a] (μmol/g)[b]	MITOCHONDRIA (nmol/g)[b]
ASPARTIC ACID	13 ± 3	2.0 ± 0.4	9.8 ± 3.2
GLUTAMIC ACID	35 ± 7	2.4 ± 0.4	33 ± 12
GLUTAMINE	415 ± 34	3.0 ± 0.6	0
HISTIDINE	68 ± 11	9.2 ± 2.2	17 ± 4.1
GLYCINE	377 ± 23	12 ± 2.6	13 ± 3.6
THREONINE[c]	246 ± 20	34 ± 7.5	73 ± 21
ARGININE[c]	112 ± 16	9.5 ± 2.2	0
TAURINE[c]	465 ± 38	20 ± 3.5	26 ± 10
ALANINE[c]	195 ± 18	69 ± 12	48 ± 14
TYROSINE	30 ± 7	3.1 ± 0.5	0
TRYPTOPHAN	38 ± 7	3.6 ± 0.6	4.3 ± 1.5
METHIONINE	66 ± 10	8.7 ± 0.7	17 ± 4.5
PHENYLALANINE	28 ± 6	4.1 ± 0.5	6.5 ± 1.8
ISOLEUCINE	34 ± 5	5.9 ± 0.5	12 ± 2.4
LEUCINE	70 ± 8	12 ± 1.2	21 ± 3.8
ORNITHINE	36 ± 5	2.1 ± 0.5	0
LYSINE	142 ± 14	11 ± 1.8	16 ± 2.9

[a] Represented by the particle free supernatant (see text).

[b] Units expressed per gram of protein in sample.

[c] Results may be affected by coelution by HPLC.

TABLE 9.2

Measurement of 'acid extractable' amino acid pools associated with crude mitochondrial membrane extracts. Values are expressed as mean ± S.E. (n=10 for whole mitochondrial extracts and n=6 for membrane extracts).

AMINO ACID	MITOCHONDRIA (nmol/g)[a]	MOM (nmol/g)[a]	MIM (MOM + ER) (nmol/g)[a]
ASPARTIC ACID	9.8 ± 3.2	0	0
GLUTAMIC ACID	33 ± 12	3.5 ± 0.8	12 ± 3
GLUTAMINE	0	0	0
HISTIDINE	17 ± 4.1	2.7 ± 0.5	<1.0
GLYCINE	13 ± 3.6	<1.0	4 ± 1.3
THREONINE[b]	73 ± 21	8 ± 1.7	9 ± 2.7
ARGININE	0	0	0
TAURINE[b]	26 ± 10	4 ± 1.2	5 ± 0.9
ALANINE[b]	48 ± 14	6 ± 1.5	3 ± 0.5
TYROSINE	0	0	0
TRYPTOPHAN	4.3 ± 1.5	0	0
METHIONINE	17 ± 4.5	3 ± 0.7	<1.0
PHENYLALANINE	6.5 ± 1.8	0	0
ISOLEUCINE	12 ± 2.4	0	0
LEUCINE	21 ± 3.8	0	0
ORNITHINE	0	0	0
LYSINE	16 ± 2.9	<1.0	7 ± 1.5

[a] Units expressed per gram of protein in sample.

[b] Results may be affected by coelution by HPLC.

FIGURE 9.1 Separation of mitochondrial membranes by sucrose density gradient centrifugation

Membranes were separated as described in the text (section 9.2.2.2). Mitochondrial outer membrane (MOM) forms a band between the interface of the 1.1 M sucrose and the sucrose in the sample. Mitochondrial inner membrane (MIM), MOM and endoplasmic reticulum (ER) form a band at the interface of the 1.5 M and 1.1 M sucrose.

SUCROSE DENSITY

GRADIENT CENTRIFUGATION

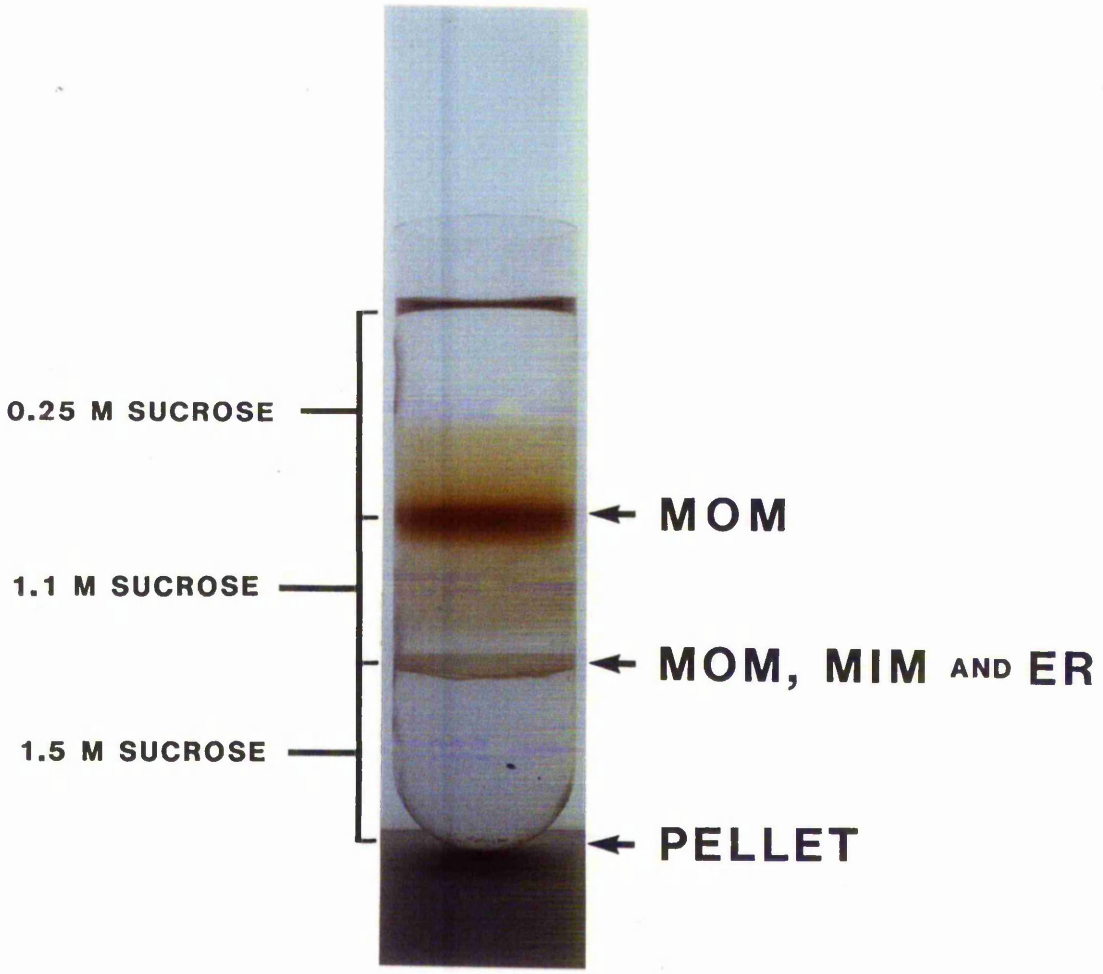


FIGURE 9.2 Reversed phase HPLC analysis of the ornithine δ-lactam

Assessment of the purity of the δ-lactam of ornithine (3-aminopiperid-2-one) synthesized as described in section 9.2.1. A major peak was observed after analysis by reversed phase HPLC using a linear gradient from 20% to 100% methanol for elution. The analytical system used was identical to that described in section 8.2.2 except that a Novapack C18 column (15 x 0.39 cm, Waters Associates) was used for separation.

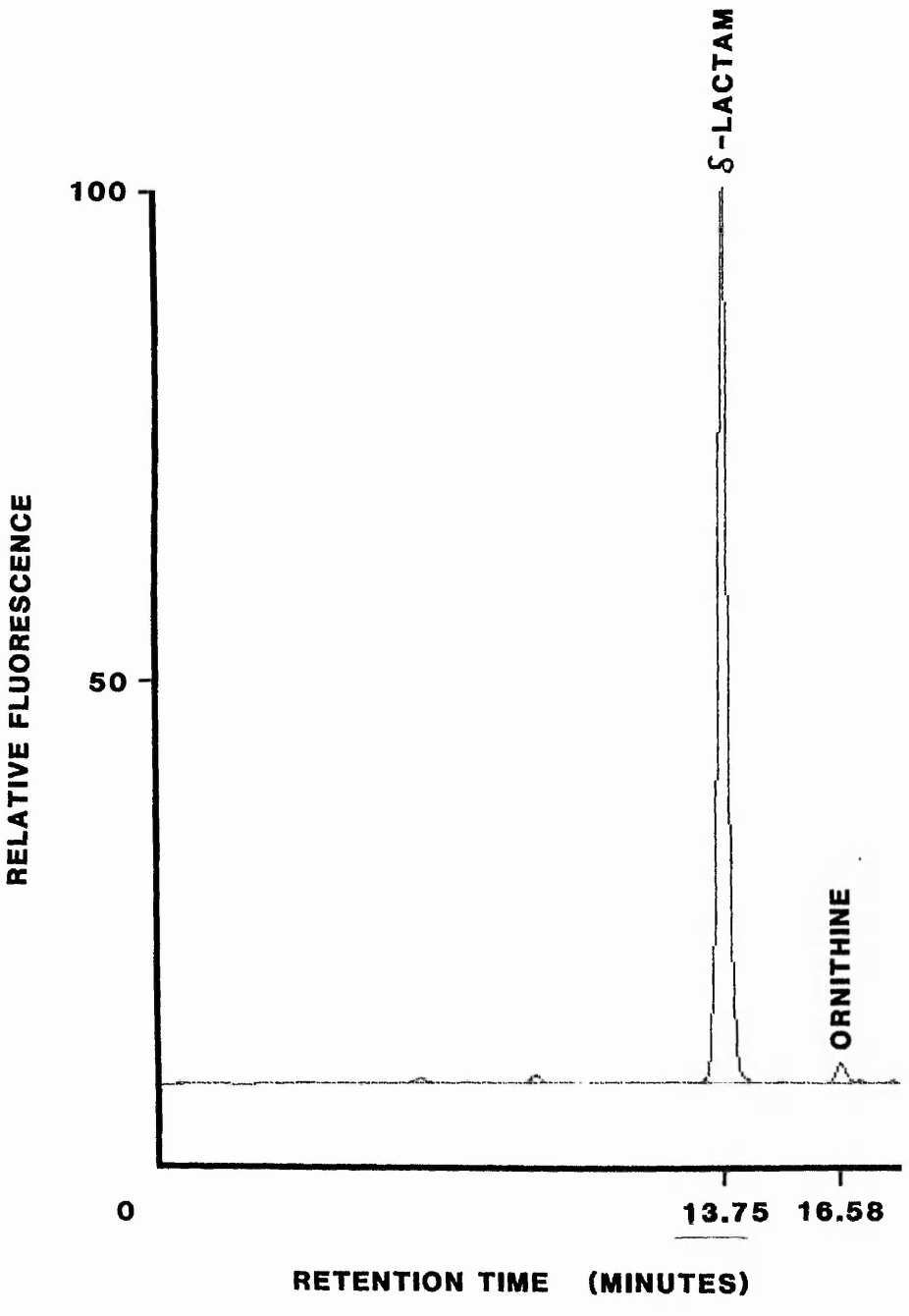

δ-LACTAM
100
50
0
RELATIVE FLUORESCENCE
ORNITHINE
13.75
16.58
RETENTION TIME (MINUTES)

FIGURE 9.3 Reversed phase amino acid analysis of rat serum

A typical elution profile for rat serum after reversed phase HPLC amino acid analysis. Elution conditions are described in section 8.2.2. Numbered peaks are defined as follows:
Peak 1, aspartic acid; 2, glutamic acid; 3, glutamine; 4, histidine; 5, glycine; 6, threonine[a]; 7, arginine[a]; 8, taurine[a]; 9, alanine[a]; 10, tyrosine; 11, tryptophan; 12, methionine; 13, phenylalanine; 14, isoleucine; 15, leucine; 16, 2,4-diaminobutyric acid (internal standard); 17, ornithine; 18, lysine.

[a] Results may be affected by coelution

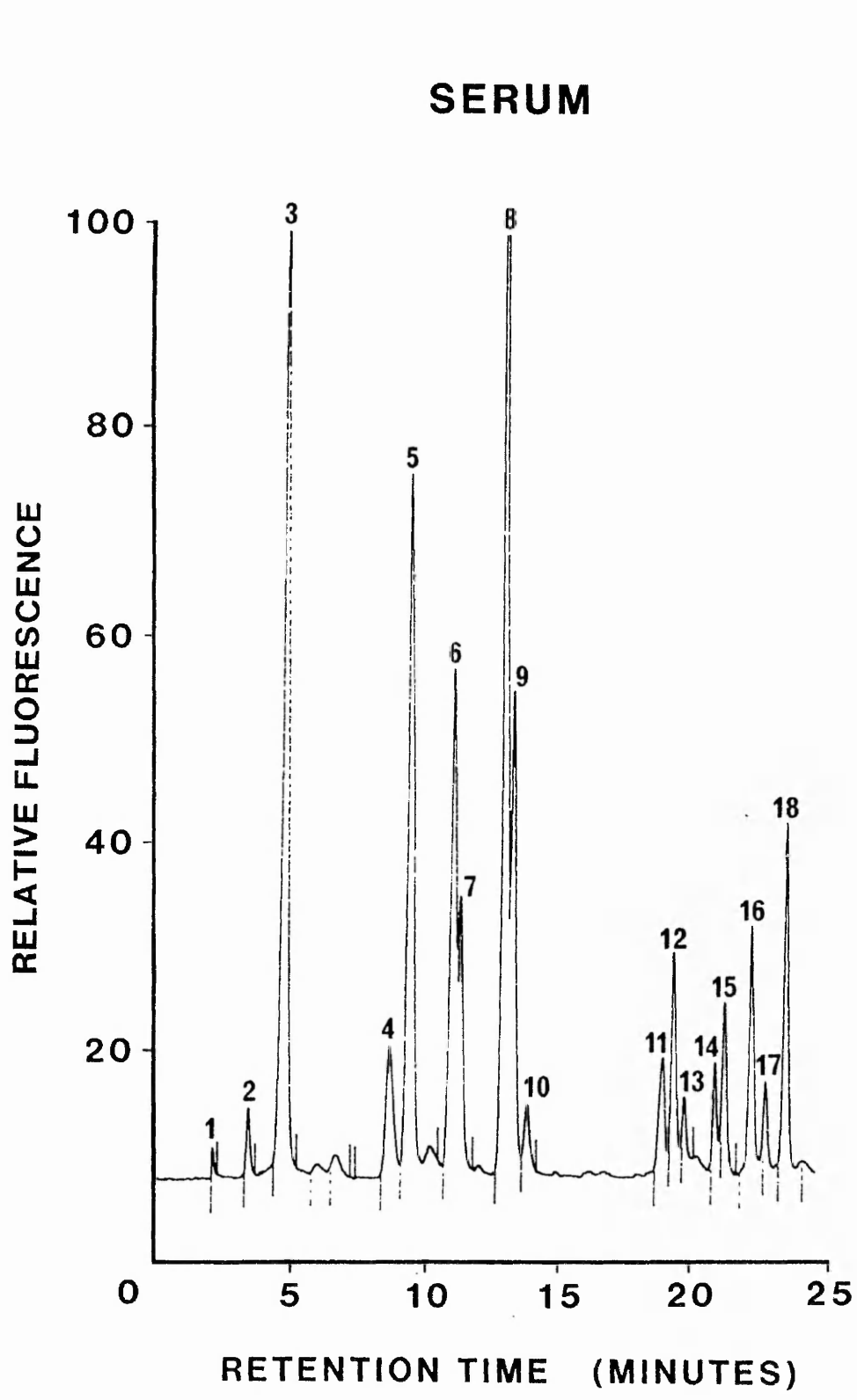

SERUM
RELATIVE FLUORESCENCE
100
80
60
40
20
0
5
10
15
20
25
RETENTION TIME (MINUTES)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

FIGURE 9.4 Reversed phase amino acid analysis of rat liver cytosolic extract

A typical elution profile for rat liver particle free supernatant (cytosolic fraction) prepared as described in section 9.2.2.2, after reversed phase HPLC analysis. Elution conditions are described in section 8.2.2.

Numbered peaks are defined as follows:

Peak 1, aspartic acid; 2, glutamic acid; 3, glutamine; 4, histidine; 5, glycine; 6, threonine[a]; 7, arginine[a]; 8, taurine[a]; 9, alanine[a]; 10, tyrosine; 11, tryptophan; 12, methionine; 13, phenylalanine; 14, isoleucine; 15, leucine; 16, 2,4-diaminobutyric acid (internal standard); 17, ornithine; 18, lysine.

[a] Results may be affected by coelution

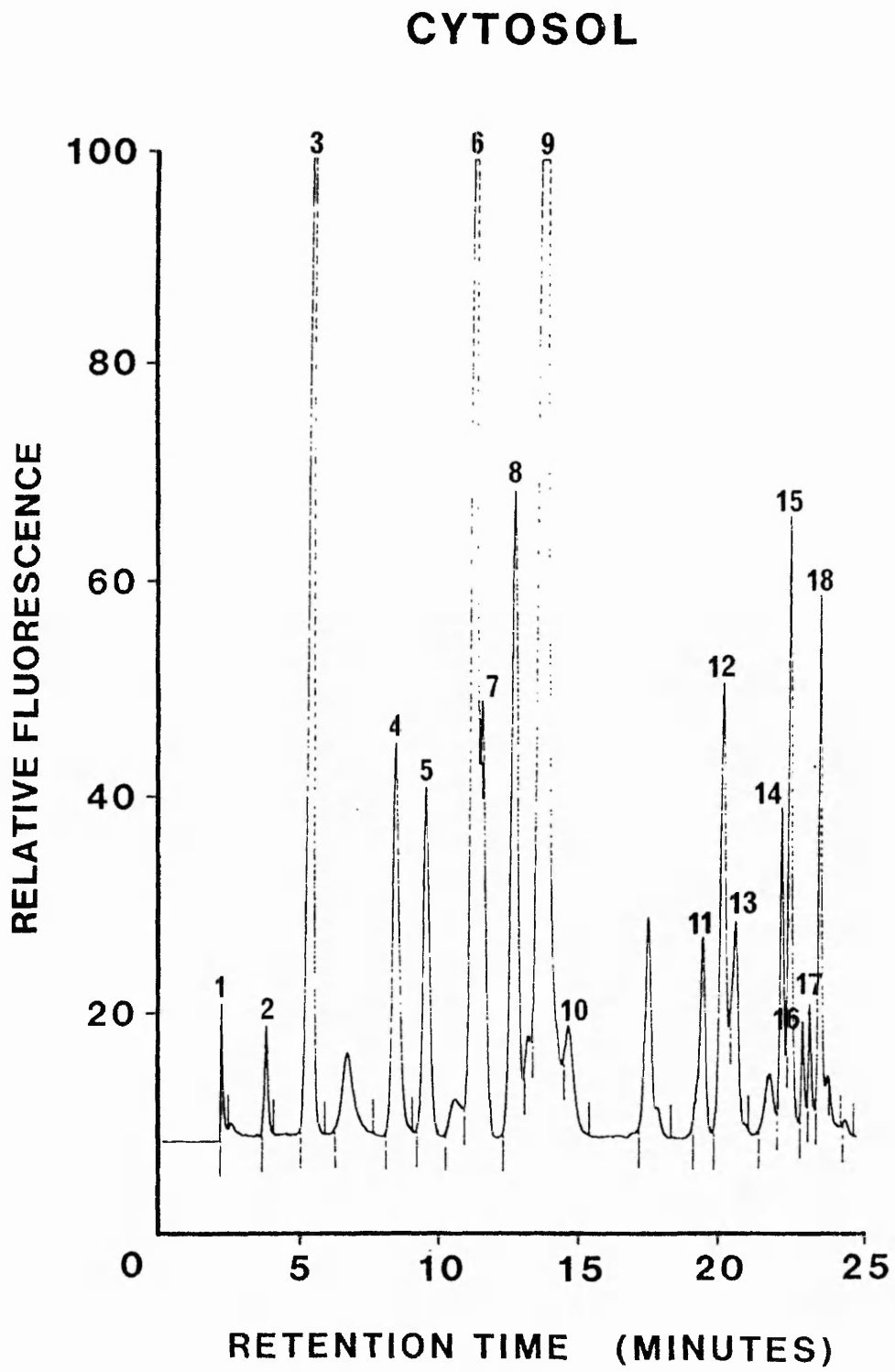
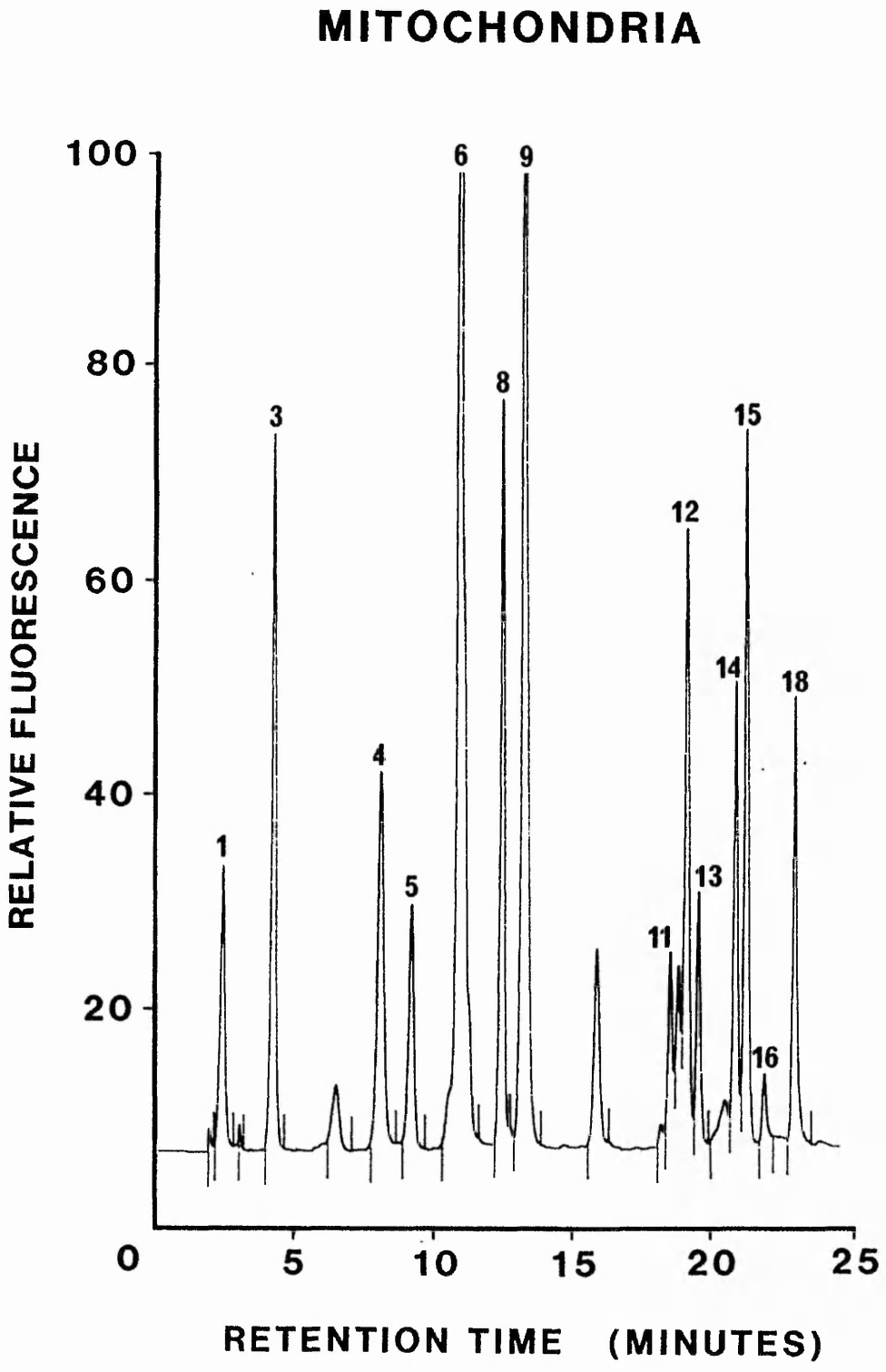

CYTOSOL
RELATIVE FLUORESCENCE
100
80
60
40
20
0
5
10
15
20
25
RETENTION TIME (MINUTES)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

FIGURE 9.5 Reversed phase amino acid analysis of rat liver mitochondrial extract

A typical elution profile for rat liver whole mitochondrial extract prepared as described in section 9.2.2.2, after reversed phase HPLC analysis. Elution conditions are described in section 8.2.2. Numbered peaks are defined as follows:
Peak 1, aspartic acid; 2, glutamic acid; 3, glutamine; 4, histidine; 5, glycine; 6, threonine[a]; 7, arginine[a]; 8, taurine[a]; 9, alanine[a]; 10, tyrosine; 11, tryptophan; 12, methionine; 13, phenylalanine; 14, isoleucine; 15, leucine; 16, 2,4-diaminobutyric acid (internal standard); 17, ornithine; 18, lysine.

[a] Results may be affected by coelution


MITOCHONDRIA
RELATIVE FLUORESCENCE
100
80
60
40
20
0
5
10
15
20
25
RETENTION TIME (MINUTES)
1
3
4
5
6
8
9
11
12
13
14
15
16
18

9.4 Discussion

9.4.1 Studies on the δ-lactam of ornithine

It was unfortunate that the reversed phase HPLC system was not able to separate the lactam from ammonia and that the ion-exchange system (using post-column OPA derivatization) was unable to detect the compound (section 8). Simple investigations into the possible conversion of ornithine to the ornithine lactam at a physiologically high concentration of ornithine indicated that this did not occur under the conditions used in this study. Ornithine hydrochloride was used to simulate hyperornithinaemic and hyperornithinuric samples and it may be that ornithine base can be converted to the lactam of ornithine.

Degradation of the ornithine lactam could be demonstrated in serum although there was no concomitant increase in ornithine levels. Fell and Pollitt (1978) have reported the conversion of ornithine lactam to ornithine by treatment with 6 M hydrochloric acid or 5 M sodium hydroxide for 30 minutes at room temperature or on standing for longer periods at a neutral pH. This may explain the difficulty of lactam detection by ion-exchange analysis where samples are deproteinized and initially bound to the column with acidic buffers. A minor ornithine peak was detected, but this was assumed to be a contaminant and was insufficient to account for the amount of lactam applied to the column. No attempt was made to investigate the methyl ester of ornithine, from which the ornithine lactam can spontaneously form.

The ornithine lactam in urine is formed as a consequence of hyperornithinaemia (Oberholzer and Briddon, 1978), independent of its primary cause. Since the formation of lactam in the presence of high ornithine concentrations could not be demonstrated under the conditions used here, it is possible that the lactam may be formed in tissues. Valle and Simell (1983) have suggested that the lactam may be produced in kidney as serum lactam is undetectable or at low levels. Incubation

of high ornithine concentrations with tissue homogenates with subsequent amino acid analysis may confirm this. The physiological mechanism of lactam formation and its precise role remain uncertain however.

9.4.2 The validity of intracellular amino acid measurements

The problem of coelution which affects measurement by HPLC has already been discussed. Upon isolation of subcellular components there is invariably cross-contamination of fractions, however many washes are performed on the extract. It is therefore common practice to assess the degree of contamination by assay of marker enzymes. Assessment of organelle integrity is necessary for quantitative analysis involving mitochondria, usually done by measurement of electron transport chain activity. However, assuming that isolated mitochondria could be washed until virtually contaminant free, any free or loosely membrane-associated amino acids would have diffused into the washing medium. The problem of measuring absolute concentrations of amino acids is compounded by the difficulty in accurately measuring the volume of mitochondria. Attempts have been made using radiochemical techniques to measure cytosolic cell volume and direct measurement of organelles from electron micrographs (stereology) has also been reported although the accuracy of these techniques is probably poor. Amino acid levels in cytosol and other subcellular compartments have therefore been expressed per mg of sample protein in this study and per gram wet weight by others (Pastor-Anglada *et al.*, 1986; Matsuzawa, 1974). It has already been suggested by Wheatley *et al.* (1986) that intracellular amino acid pools are largely dependent upon the extraction method used and expressing values per mg of protein or wet weight bases the measurements on the assumption that isolated protein concentrations are representative of the original state *in vivo*.

Furthermore, the recovery of amino acids are assumed to be the same for each component.

Having discussed the apparently insurmountable problems of direct intracellular amino acid measurement, the arbitrary measurements which can be made may still be of value provided the method of pool isolation is reproducible and more stringently performed than in the preliminary study described in this section. While amino acid levels may not be directly related to *in vivo* levels when isolated, they could represent an arbitrary set of values which could alter significantly in response to physiological changes such as diet, drug administration or disease. The most easily measurable amino acid pool associated with organelles would appear to be acid-extractable or membrane-associated pools described by Wheatley *et al.* (1986). Such measurements may be largely independent of organelle integrity, rapid loss of amino acids by diffusion and organelle volume, provided membrane-associated amino acids do not diffuse off the membrane significantly during tissue processing.

9.4.3 *Intracellular amino acid pools in rat liver*

In the light of previously discussed factors affecting intracellular pool measurement, the following conclusions can only be implied from the data obtained.

Measurements of ornithine in intra- and extracellular compartments suggest that negligible amounts, if any, of this amino acid are associated with mitochondrial membrane extracts. This implies that either ornithine is present at significant concentrations within the mitochondrial matrix but diffuses out completely upon tissue processing or is present at very low concentrations *in vivo*. Ornithine concentrations in rat serum are directly measurable and are not significantly different from reported values in humans (Ibott, 1974). Ornithine levels in a particle free supernatant of rat liver extracts

reflect the relative level in serum with respect to other amino acids isolated identically with this pool. This suggests that ornithine is readily diffusible across cell membranes to and from extracellular fluids. This also seems to be the case for lysine. It has been suggested previously (Valle and Simell, 1983) that it would take only a minor redistribution of intracellular ornithine to produce a major change in serum ornithine (see section 1.6.2.6) and this may also be true of lysine.

A number of amino acids had reduced relative levels in the particle-free supernatant compared to serum, particularly glutamine. Whether this is due to breakdown of this amino acid during sample processing or is physiologically significant is not known. No glutamine was detected in any of the mitochondrial extracts or isolated membrane extracts. Measurement of amino acid pools from whole mitochondrial extracts are likely to represent membrane associated pools although different patterns may emerge after acid extraction. Another possibility is that amino acid profiles from intact organelles reflect the relative rates of diffusion across membranes of these solutes, although Wheately et al. (1986) imply that diffusion of all free amino acids would be complete using present isolation methods.

None of the amino acids, namely glutamine, tyrosine and ornithine, which could not be detected in whole mitochondrial extracts could be detected in membrane extracts after acid extraction. Coeluted components cannot be regarded as reliably measured amino acids, although these components appear to be associated in significant quantities with both whole mitochondria and its membrane extracts. Matsuzawa (1974), who has expressed amino acid levels in isolated rat liver mitochondria in nmoles/g mitochondrial wet weight, was also unable to detect ornithine, arginine or tyrosine in rats killed at rest. However, glutamine levels were high with respect to other amino

acids which differs from the findings of this study possibly supporting the likliehood of degradation of this amino acid during processing. The measurements of Matsuzawa, which were made from acid-extracted mitochondria, recorded histidine and glutamic acid in highest relative concentrations whereas measurements in this study made after methanol extraction show only moderate relative levels. However, glutamic acid was acid-extractable in relatively high concentrations from crude mitochondrial membrane extracts, histidine also being detected.

Clearly from these studies, the significance of such measurements is unclear and without rigid isolation and detect methods, unreliable. However, these measurements at least suggest that some amino acids are 'acid-extractable' from membranes or are associated in some way with membranes to a greater extent than others. Further investigations into intracellular pools should be able to resolve the question of whether these measurements are a complete artefact of the isolation procedure or whether, in vivo, amino acids are associated with membranes and are of indirect significance when measured after isolation. It would seem sensible, from a metabolic and evolutionary viewpoint, to have amino acids effectively compartmented within organelles, much in the same way as enzymes, thereby making metabolic reactions more efficient by having substrate molecules pooled around the enzyme.

10 GENERAL DISCUSSION

The major topics covered in this study, together with the main achievements, will initially be summarized in this section The latter part of the section will discuss the wider significance of this work.

OAT has been purified and characterized from human and rat tissue and the physicochemical properties of OAT from these two sources have been shown to be quite similar. The property of OAT to self-associate and form higher molecular weight aggregates upon increasing enzyme concentration has been investigated and its physiological implications will be discussed later. Immunodiffusion analysis using polyclonal antibodies raised to OAT has shown that human and rat OAT are structurally similar. Polyclonal antibodies raised to OAT in rabbits have been affinity-purified and a low affinity antibody population used to develop a novel method of OAT purification from crude tissue extracts by immunoadsorption. This method has been successfully applied to human liver, rat kidney and rat blood platelets. It is possible that blood platelets may provide a suitable source of OAT from Gyrate Atrophy patients for biochemical study. A competitive ELISA for subfemtomole measurement of OAT protein has been developed and applied to several human and rat extracts. Levels of OAT protein measured in rat tissues by ELISA have been shown to be comparable to levels estimated from OAT activity measurement suggesting the absence of an inactive OAT pool within tissue extracts.

Development of a partly automated ion-exchange system and a reversed phase HPLC system for amino acid analysis has been achieved allowing comparison of the two systems. Both analytical systems have been applied to the analysis of physiological fluids frcm inborn errors of metabolism. The reversed phase HPLC system was selected for rapid and convenient analysis and used for investigating intracellular amino

acid pools isolated from rat liver. The significance of the findings made during study of these topics will now be discussed.

Preliminary studies on the characterization of OAT from rat and human sources has shown that this enzyme undergoes self-association with increasing enzyme concentration. This is consistent with previous reports (Morris et al., 1974; Boernke et al., 1981). While it could not be demonstrated in this study, Boernke and co-workers have shown that Km values of OAT for ornithine increase with increasing enzyme aggregation since substrate binding is sterically hindered. Native molecular weight studies on OAT using gel filtration in the absence and presence of detergent and the observation that OAT readily binds to the hydrophobic interaction resin, Octyl-Sepharose CL-4B, have suggested that changes in the hydrophobicity of OAT through aggregation could represent an important means of regulating OAT activity in vivo at the molecular level.

Recent studies by Cohen et al. (1987) suggest that ornithine is channelled between its transporter on the mitochondrial inner membrane and OCT. Furthermore they have proposed that OCT is in some way associated with the inner face of the inner mitochondrial membrane, in close proximity to the ornithine transporter. Since other soluble mitochondrial matrix enzymes have also been reported to be organized within the matrix (Robinson and Srere, 1985), it may also be true of OAT. Assuming that OAT was processed through the inner mitochondrial membrane in an aggregated form, it may be easier to move across the membrane in a hydrophobic state and would also tend to associate with the hydrophobic areas of the membrane. As aggregated OAT diffuses away from the inner membrane into the matrix space, it could dissociate into a monomeric state. This could then allow the ornithine pool for OCT to be effectively by-passed by OAT since Km values for aggregated OAT may be significantly higher than for OCT whereas the Km is lowered upon OAT

dissociation into a monomeric state. OAT could therefore be responsible for metabolizing a different intracellular ornithine pool, distinct from the pool available to OCT.

Amino acid studies have indicated that ornithine does not appear to be associated with mitochondrial membranes and cannot be detected in whole mitochondrial extracts. Other workers (Matsuzawa, 1974; Cohen et al., 1987) have also been unable to measure mitochondrial ornithine and have concluded that ornithine must be present at low levels within the matrix. However, this may well be due to the rapid diffusion of ornithine out of the organelle upon isolation. Since OCT may be associated with the ornithine transporter, the total amount of ornithine within the matrix may not have to be very high if OCT is able to metabolize a large proportion of ornithine entering the matrix as it diffuses off its membrane transporter. If ornithine transport into the matrix is the rate-limiting-step of ornithine metabolism within the matrix, then ornithine would not accumulate to measurable levels, but would remain in the vicinity of the inner membrane. OAT may be responsible for maintaining low matrix levels of ornithine by metabolizing ornithine which diffuses away from the relatively high concentration pool available to OCT. McGivan et al. (1977) has shown that intra- and extramitochondrial radiolabelled ornithine concentration are virtually identical when OAT activity is blocked with amino-oxyacetate. This would be expected if OAT controlled the overall concentration of ornithine in the matrix space since the ornithine pool in proximity to OCT may expand, unchecked by OAT, thereby raising ornithine levels throughout the matrix. It would be interesting to see whether inhibition of OCT resulted in ornithine accumulation in isolated mitochondria.

OAT has been shown to be regulated at the substrate level by substrate inhibition with both ornithine and 2-oxoglutarate. This

would provide another means of sparing a high concentration ornithine pool within the matrix. Furthermore, studies on aspartate aminotransferase have shown that this enzyme enters the mitochondrial matrix as an apo-enzyme (Sharma and Gehring, 1986). If this is also true for OAT, then an ornithine pool associated close to the inner membrane may be completely untouched by an inactive apo-form of aggregated OAT.

The physicochemical and particularly the structural similarity of human and rat OAT has enabled the development of an immunoadsorbent capable of purifying human OAT using IgG raised against rat OAT. The method has the advantage of enabling relatively small amounts of OAT protein to be efficiently purified from crude tissue extracts. This has provided the methodology to isolate any residual or abnormal OAT protein from Gyrate Atrophy patients. Unfortunately, no patients were available for study, but the feasibility of such studies has been demonstrated. Using rat tissue as a model for human study, it has been found that blood platelets contain levels of OAT suitable for isolation. It should therefore be possible to isolate OAT from human blood platelets to allow characterization. Further studies would provide the normal range of specific activities of platelet OAT and separation of platelet sub-populations may reveal a relatively abundant source of OAT in human tissue extracts. Removal of suitable volumes of blood from Gyrate Atrophy patients is feasible and can be repeated at intervals; this may be preferable to skin biopsy and tissue culture.

Studies by Ohura et al. (1984) suggest that the levels of OAT protein are reduced in the cultured skin fibroblasts from a patient with Gyrate Atrophy. This implies that OAT deficiency is not a result of normal levels of an abnormally synthesized OAT protein. However, the possible heterogeneity of this disease (discussed in section

1.6.2.2) may indicate several sites of OAT defect. However, Sipila and Simell (1981) have reported the presence of a mutant OAT enzyme in liver, although studies were performed using only activity measurements in crude extracts.

A number of sites exist within cells for the OAT defect to occur. The production of a specific inhibitory substance which inactivates normally-produced OAT or synthesis of an abnormal OAT protein are both possible. These two possibilities can easily be investigated by comparison of OAT activity measurements with OAT protein measurements by immunoassay, thereby demonstrating the presence of inactive OAT. Incubation of normal OAT with tissue extracts from Gyrate Atrophy patients would be expected to reduce OAT activity if an inhibitor was present. Western blots of tissue extracts detecting OAT in rat and human tissue have shown that precursor OAT is not detectable and suggest that either very low levels are present within cells or that the precursor is unstable. This is unfortunate since detection of precursor OAT could demonstrate a defect at the transport and maturation stage of OAT processing or abnormal precursor OAT synthesis. This would be determined by a Western blot in which any precursor OAT band (separated by SDS-PAGE) would be present. It may still be possible to detect precursor OAT if it accumulated in the cytosol to detectable levels in Gyrate Atrophy patients. However, the detection of a single OAT band by Western blotting demonstrates that the competitive ELISA is measuring only OAT protein and that cross-reaction with other proteins is not occurring. Since the ELISA developed in this study would not distinguish between the precursor and mature OAT, a Western blot would be necessary to confirm which was being detected. A pre-translational defect would result in a concomitant reduction of OAT protein and activity.

During the development of a competitive ELISA for OAT, the self-association of OAT was discussed as a possible cause of anomalous assay results prior to modifying the assay protocol. The self-association phenomenon of OAT may be exploited in future studies to purify OAT by immobilizing OAT monomers to produce an affinity column analogous to that originally reported for α-chymotrypsin (Chiancone and Gattoni, 1987).

Clearly the techniques developed in this study need to be applied to tissue from patients in order to fully assess their usefulness. The problems of obtaining human tissue and transfer of blood out of hospital establishments provides additional problems with such investigations.

The possibility that amino acids are associated with organelle and cell membranes and not completely free within organelles and cytosol is worth further investigation. If amino acids and possibly other metabolites are highly compartmentalized within organelles, then the efficiency of metabolic reactions is likely to be improved, and the regulation of metabolism at this level more complex than previously thought. Results obtained in this study can only provide a rough indication of membrane-association of amino acids and further studies using radiolabelled amino acids as well as different organelle isolation methods would be of value. However, as Wheatley *et al.* (1986) have pointed out, amino acid measurement of intracellular pools is at present largely dependent upon the isolation method used and probably not representative of intra-organelle amino acid pools *in vivo*.

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Appendix A

Technical data for the amino acid analyzer system

Construction of a semi-automated amino acid analyzer (outlined in Figure A) makes use of a laboratory's existing HPLC pumps, injector and detection equipment. The system as shown requires additional equipment consisting of airtight buffer reservoir bottles, a suitable solenoid switching valve consisting of 1 outlet and 6 inlet ports, a stainless steel column, a suitable high efficiency ion exchange resin which in our case was Dionex DC-4A and a water-tight glass column jacket connected to a suitable water circulating heater bath. The responsiveness of the water heater was 1 degree rise per minute. Dimensions of connector tubing are given in Figure A. The system shown uses post-column derivatisation of amino acids with OPA/2-ME followed by fluorescence detection.

A BBC model B microcomputer was chosen as an intelligent controller for the apparatus. This computer was chosen because of its versatility and common usage in manufacturing and research laboratories in the UK and Europe. The computer is an 8 bit 6502 based machine with an on board 6522 VIA (versatile interface adapter). The operating system (BBC operating system) calls allow access to this, as an on board 12 bit analogue-to-digital converter and an elapsed time clock. The latter two are also accessible to Basic users via Basic commands. The Basic interpreter is relatively fast and allows the use of user-defined procedures and function. The Basic program monitored column temperature and the clock of the computer. In response to these inputs the temperature and solvent selection valve were controlled.

Column temperature was monitored via a thermistor probe inserted into the glass heating jacket surrounding the column and connected to the

analogue port of the computer. This thermistor formed half of a potential-divider connected between the reference voltage output and ground; its junction with a fixed resistor being connected to analogue channel 1. The advantage of this simple system is that the measured temperature is essentially unaffected by reference voltage drift. The heater control algorithm has a $0.2^{o}C$ dead zone to reduce the relay activity. The heater and solvent selection switches were both driven from the computer's user port via an interface unit (Figure B).

A simplified flow chart for the control program is shown in Figure C. When entered, the program sets up a number of initial conditions, for example side B of the user VIA is set for output and the temperature probe calibration data is read into an array. From this point the program is controlled from a menu page. Run conditions can be set up from the keyboard, loaded from or saved to disk. The solvent selection switch can be incremented manually or a run can be started. Finally, exit from the program can be with an orderly return to normal. The program can support six temperature changes and six solvents. Before a run, the program enters an indefinite loop with a single controlled temperature for the purpose of equilibrating the column in solvent number one. The program returns to the equilibration loop after each run. A "key press" starts the run conditions by moving control to a second loop, during which the column temperature follows a pre-programmed sequence and the solvent can be changed at pre-set intervals. A plot on the computer VDU of temperature versus time (with solvent changes indicated) provides a visual check on the progress of the run.

Elution conditions optimised for separation of components in complex mixtures such as physiological fluids (shown in Table A) were found to

be adequate for continuous analyses every five hours, including column equilibration time.

TABLE A

Elution conditions programmed into the BBC computer for separation of amino acid components present in physiological fluids.

PICOBUFFER	pH	ELUTION TIME (min)	COLUMN TEMPERATURE (^{o}C)
1	2.0	38	40
2	2.08	30	40
3	2.1	22	40
3	2.1	16	60
4	2.25	38	60
5	2.25	68	60
LITHIUM HYDROXIDE (0.3 M)	12.25	30	60

FIGURE A

Schematic diagram showing the single-column amino acid analyser system described in the text. Sites under computer control are indicated by dashed lines. Tubing internal diameters are indicated as follows: A, 1.5 mm; B, 0.3 mm. Stainless steel tubing was used on the outlets of both pumps up to the mixing manifold. All other tubing was made of Teflon.

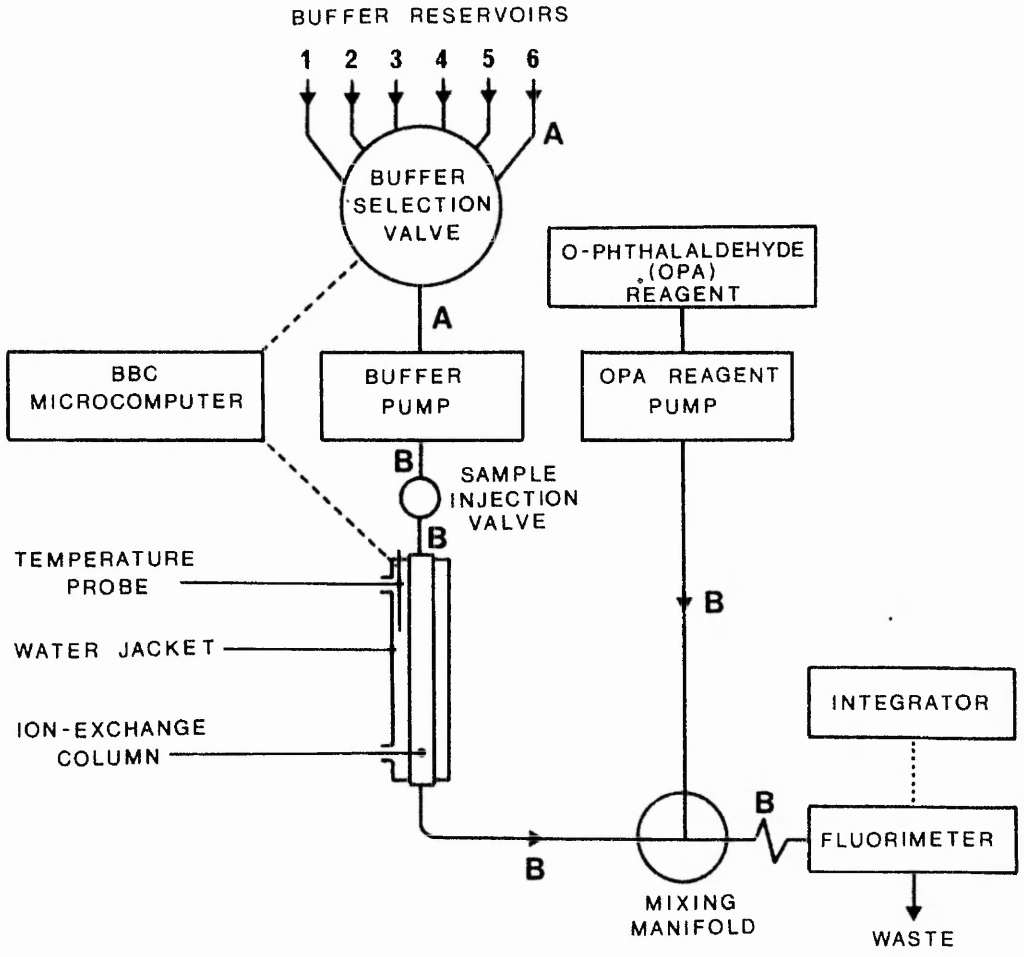

BUFFER RESERVOIRS
1
2
3
4
5
6
A
BUFFER SELECTION VALVE
O-PHTHALALDEHYDE (OPA) REAGENT
A
BBC MICROCOMPUTER
BUFFER PUMP
OPA REAGENT PUMP
B
SAMPLE INJECTION VALVE
B
TEMPERATURE PROBE
WATER JACKET
ION-EXCHANGE COLUMN
B
INTEGRATOR
B
B
FLUORIMETER
MIXING MANIFOLD
WASTE

FIGURE B

Schematic diagram of the interface unit which allows the BBC microcomputer to control the water jacket heater and solvent selection valve.

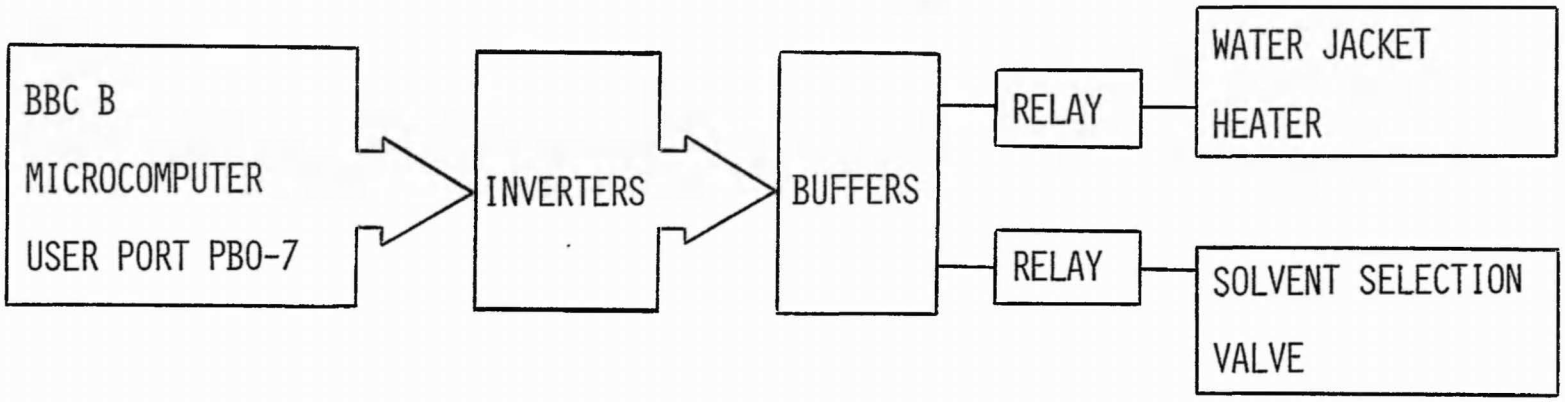
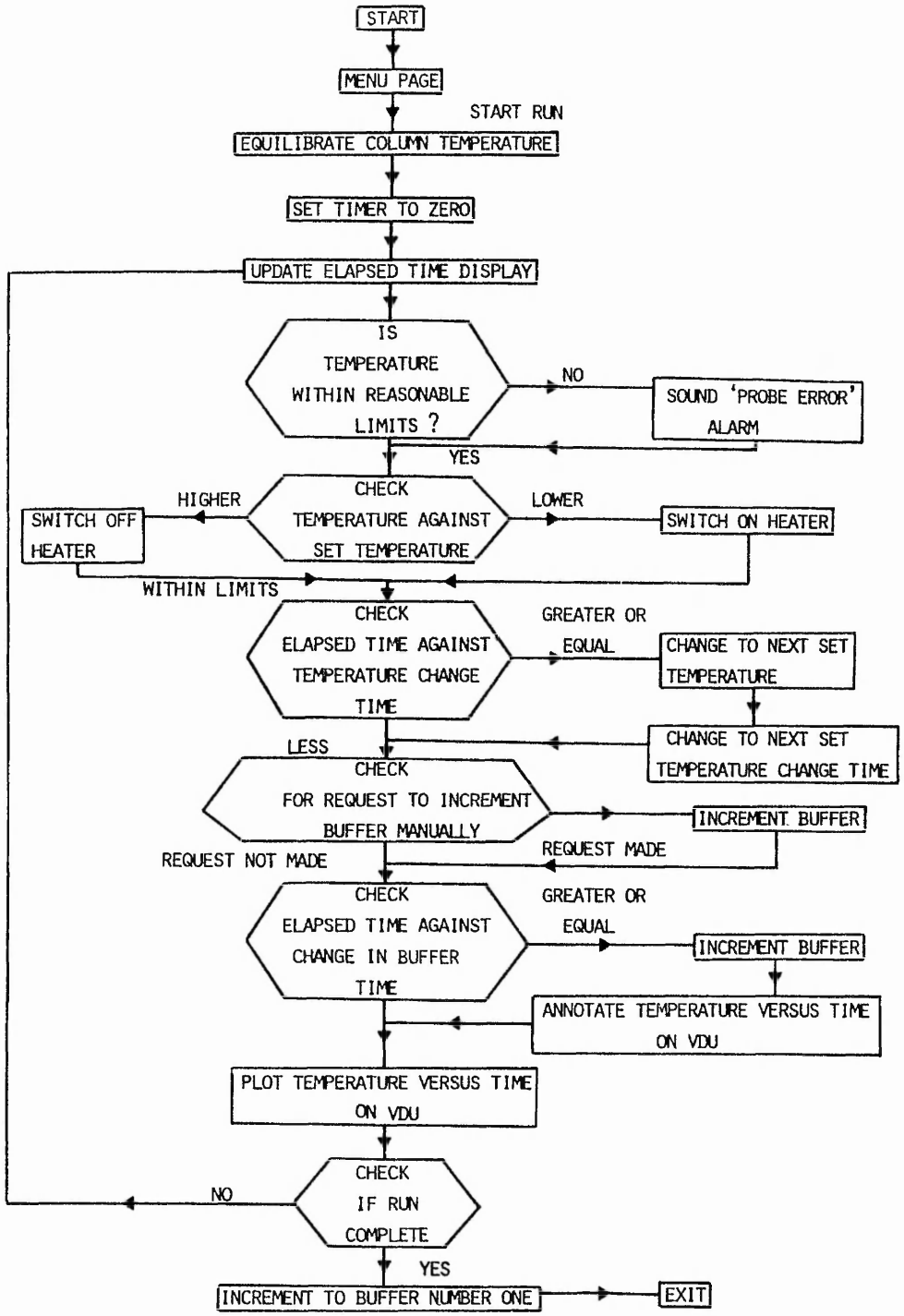

BBC B
MICROCOMPUTER
USER PORT PB0-7
INVERTERS
BUFFERS
RELAY
WATER JACKET
HEATER
RELAY
SOLVENT SELECTION
VALVE

FIGURE C

Flow diagram demonstrating how the computer software allows selection and subsequent control of sequential elution conditions of the analyser system.


START
MENU PAGE
START RUN
EQUILIBRATE COLUMN TEMPERATURE
SET TIMER TO ZERO
UPDATE ELAPSED TIME DISPLAY
IS TEMPERATURE WITHIN REASONABLE LIMITS ?
NO
SOUND 'PROBE ERROR' ALARM
YES
CHECK TEMPERATURE AGAINST SET TEMPERATURE
HIGHER
SWITCH OFF HEATER
LOWER
SWITCH ON HEATER
WITHIN LIMITS
CHECK ELAPSED TIME AGAINST TEMPERATURE CHANGE TIME
GREATER OR EQUAL
CHANGE TO NEXT SET TEMPERATURE
CHANGE TO NEXT SET TEMPERATURE CHANGE TIME
LESS
CHECK FOR REQUEST TO INCREMENT BUFFER MANUALLY
INCREMENT BUFFER
REQUEST MADE
REQUEST NOT MADE
CHECK ELAPSED TIME AGAINST CHANGE IN BUFFER TIME
GREATER OR EQUAL
INCREMENT BUFFER
ANNOTATE TEMPERATURE VERSUS TIME ON VDU
PLOT TEMPERATURE VERSUS TIME ON VDU
CHECK IF RUN COMPLETE
NO
YES
INCREMENT TO BUFFER NUMBER ONE
EXIT

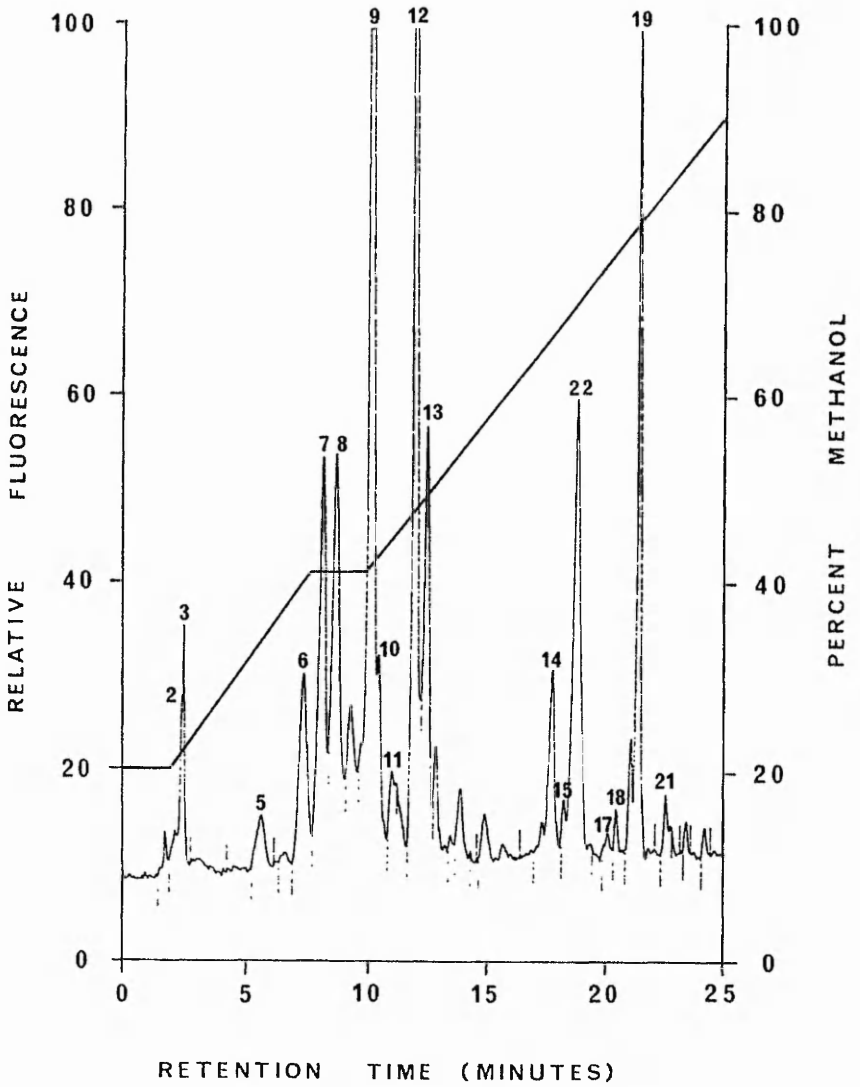
Appendix B

Separation of amino acids, in normal urine and urine from patients with various inborn errors of metabolism, by reversed phase HPLC as described in Section 8. Hyperornithinuria (peak 20) is clearly demonstrable in a patient with Gyrate Atrophy, argininosuccinic acid (peak 23) in a patient with argininosuccinic aciduria, α-amino-adipic acid (peak 24) in a patient with α-amino-adipic aciduria and N-acetylglucosaminyl-asparagine (peak 25) in a patient with aspartylglycosaminuria.

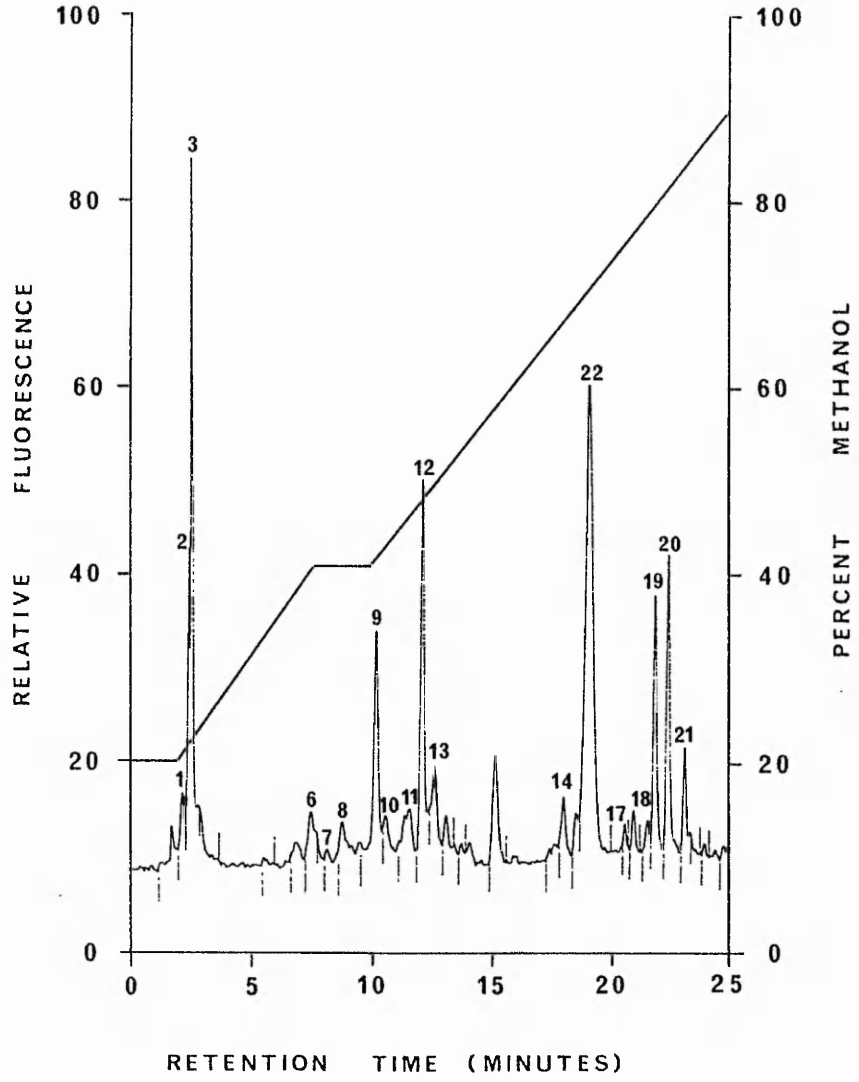
Numbered peaks are defined as follows:

Peak 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 5, asparagine; 6, serine; 7, glutamine; 8, histidine; 9, glycine; 10, threonine; 11, arginine; 12, taurine/alanine; 13, tyrosine; 14, tryptophan; 15, methionine; 16, phenylalanine; 17, isoleucine; 18, leucine; 19, 2,4-diaminobutyric acid (standard); 20, ornithine; 21, lysine; 22, ammonia; 23, argininosuccinic acid; 24, α-amino-adipic acid; 25, N-acetylglucosaminyl-asparagine.

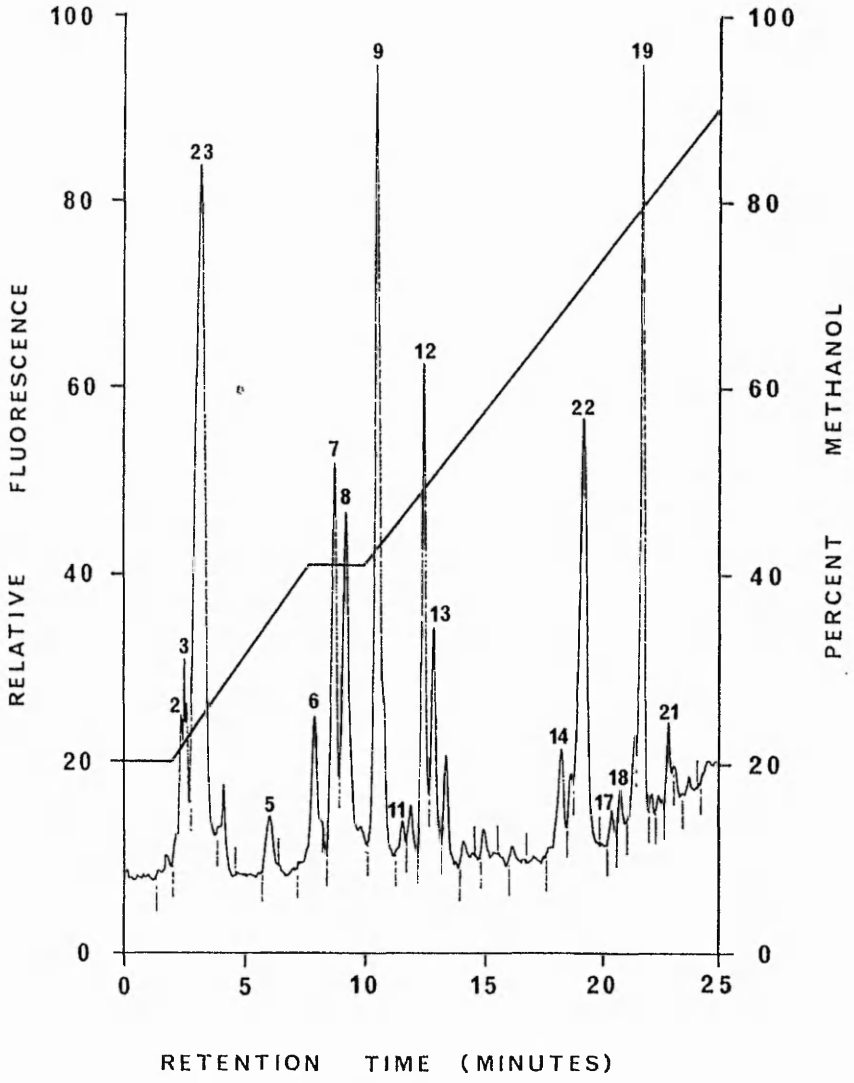
NORMAL URINE



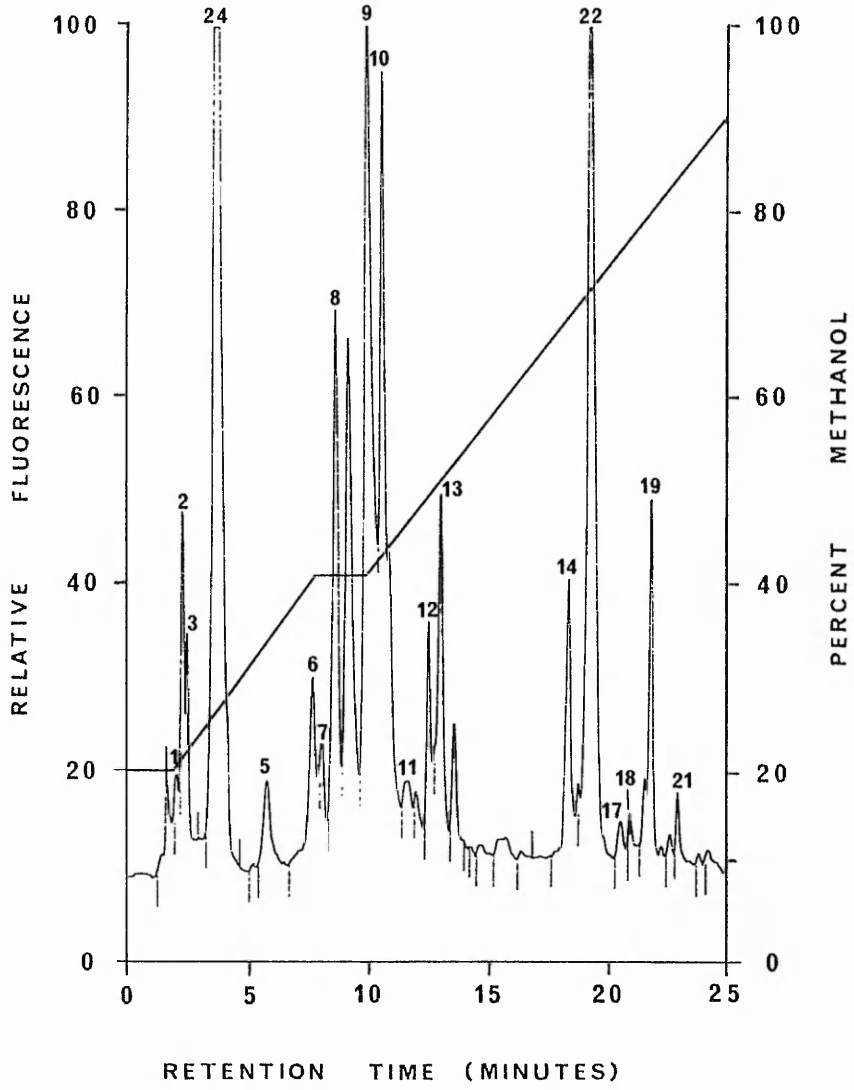
HYPERORNITHINURIA



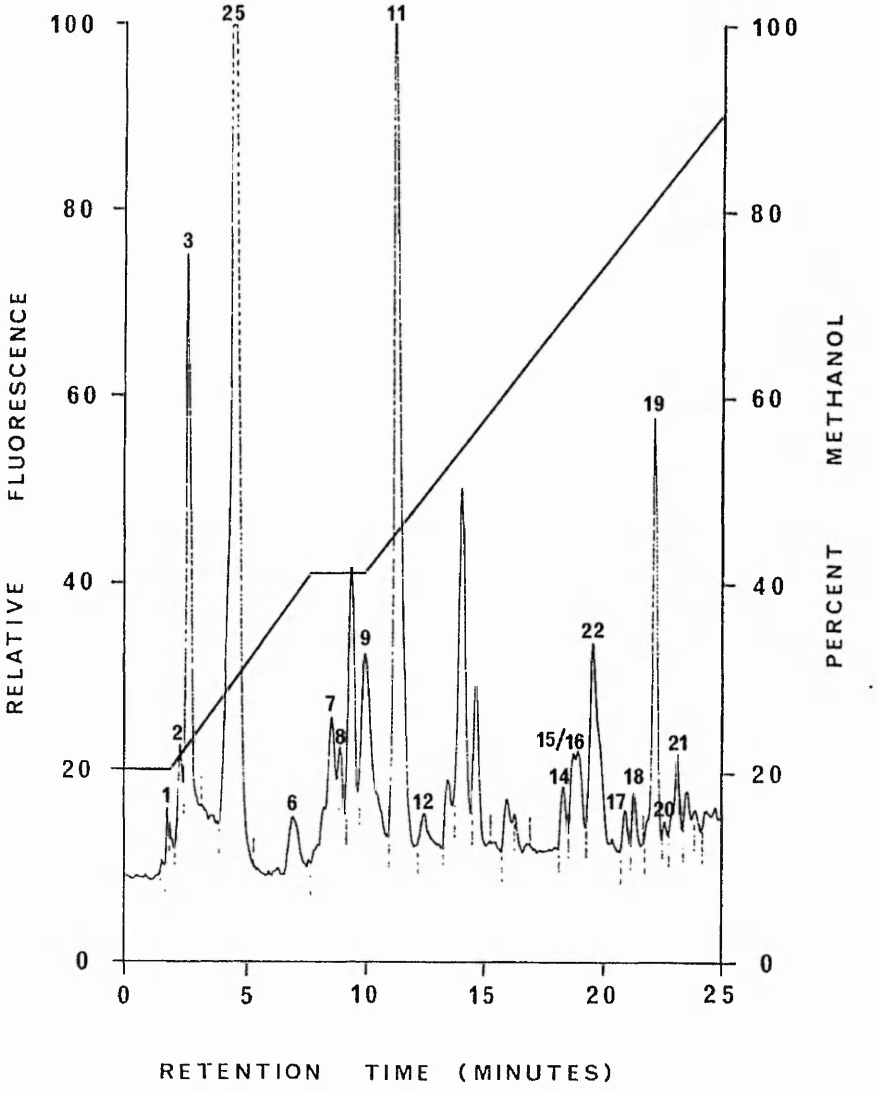
ARGININOSUCCINIC ACIDURIA



∝-AMINO-ADIPIC ACIDURIA



ASPARTYLGLYCOSAMINURIA



J. Inher. Metab. Dis. 9 Suppl. 2 (1986) 250–253

Short Communication

Urine Amino Acid Analysis by HPLC in the Investigation of Inborn Errors of Metabolism

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Reversed-phase high performance liquid chromatography (HPLC) of *o*-phthalaldehyde (OPA) derivatives provides a technique that is readily applicable to the estimation of free amino acids (Turnell and Cooper, 1982; Griffin *et al.*, 1982; Price *et al.*, 1984; Palmer, 1985). It has the advantage over classical ion-exchange chromatography of rapidity, increased sensitivity and the ability to handle small sample sizes, utilizing apparatus which is not dedicated to one technique. In previous reports we have described the application of the procedure to amino acid analysis of plasma specimens from normal individuals (Griffin *et al.*, 1982) and from patients with inborn errors of metabolism (Palmer, 1985). Here we describe its application to the screening of urine samples for suspected amino acid disorders.

PATIENTS AND METHODS

All patients with inborn errors of metabolism had previously been diagnosed by other procedures, including thin layer and/or ion exchange chromatography.

The HPLC system was essentially that described by Griffin *et al.* (1982). A Zorbax C_8 column was employed using a linear gradient from 20% to 90% methanol. Gradients were formed from two solvents: potassium acetate buffer (0.02 mol/l, pH 5.5) and methanol. Both solvents contained 1% (v/v) tetrahydrofuran. A flow-rate of 1.8 ml/min was employed, eluting most amino acids within 25 min.

The derivatization procedure was that described by Price *et al.* (1984), which gives greater sensitivity than that previously employed. Immediately before use, 250 μl of OPA reagent (60 mg/10 ml methanol) was added to 250 μl 0.4 mol/l boric acid buffer, pH 9.4, and 25 μl 2–mercaptoethanol; 250 μl of this mixture was vortex-mixed with 25 μl prepared sample for 2 min, and then a 10 μl aliquot removed and applied to the column. For urine amino acid analysis, 70–170 μl urine was mixed with 1 ml of methanol and centrifuged; 25 μl supernatant was then treated with reagent as described above.

RESULTS AND DISCUSSION

It was found that the technique which had been used for plasma amino acid analysis could be applied without modification for the analysis of amino acids in urine.

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Ammonia may first be removed from the urine, if required, by rotary evaporation or vacuum desiccation after the specimen has been made slightly alkaline with NaOH. However, the ammonia peak does not interfere with the analysis of any amino acid normally present in urine so that prior removal of ammonia is not essential; for simplicity this step was not incorporated into the procedure used here. The total analysis time, including derivatization, was less than 30 min even with the inclusion of two isocratic stages in the linear solvent gradient. These isocratic stages could be removed with only a slight deterioration in performance.

A series of urine specimens from patients with known disorders of amino acid metabolism all gave clearly abnormal results when analysed by this procedure. A specimen from a patient with argininosuccinic aciduria (ASAuria) (McKusick 20790) gave a large peak in the ASA position, retention time approximately 4 min, where no significant peak occurs in a normal urine (Figure 1). Urine from a patient with α-amino-adipic aciduria (McKusick 24513) had an equally conspicuous peak in the α-amino-adipic acid position, retention time about 6 min. The abnormal ornithine peak in a specimen from a patient who had hyperornithinaemia with gyrate atrophy (McKusick 25887) was not in itself so conspicuous, but the ornithine/lysine ratio was clearly far in excess of normal. A δ-lactam is present in the urine of patients with hyperornithinaemia, but this co-elutes with ammonia, so it is not easily detectable by this technique, especially since the procedure which may be used to remove ammonia also removes the δ-lactam.

A urine specimen from a patient with aspartylglycosaminuria (McKusick 20840) gave a conspicuous peak with a retention time of approximately 5 min, presumably *N*-acetylglucosaminyl-asparagine. The abnormalities in a specimen from a patient with hyperargininaemia (McKusick 20780) were not conspicuous, since the arginine concentrations found in this disorder are not particularly high in absolute terms, and arginine elutes in a crowded portion of the chromatogram. Nevertheless, a distinct arginine peak was detectable between threonine and taurine, and the glutamine and lysine peaks were relatively high in comparison to the other peaks.

Two urine specimens from patients with other disorders were also investigated. One from a patient with fumaric aciduria showed no obvious amino acid abnormalities; one from a patient who had xanthinuria with sulphite oxidase deficiency gave a conspicuous peak in the taurine position and another in the leucine region.

The value of this technique in screening for amino acid disorders is clearly apparent. It has a big advantage over paper or thin-layer chromatography in that it is fully quantitative, and its only significant disadvantage when compared to conventional ion exchange chromatography is its inability to detect the amino acids proline and hydroxyproline. Our preliminary studies suggested that the reagent might react with urea (Griffin *et al.*, 1982), but we have now established that this is not the case. It detects only substances with primary amino groups, so is more selective than conventional amino acid analysis, and may be complementary to this technique.

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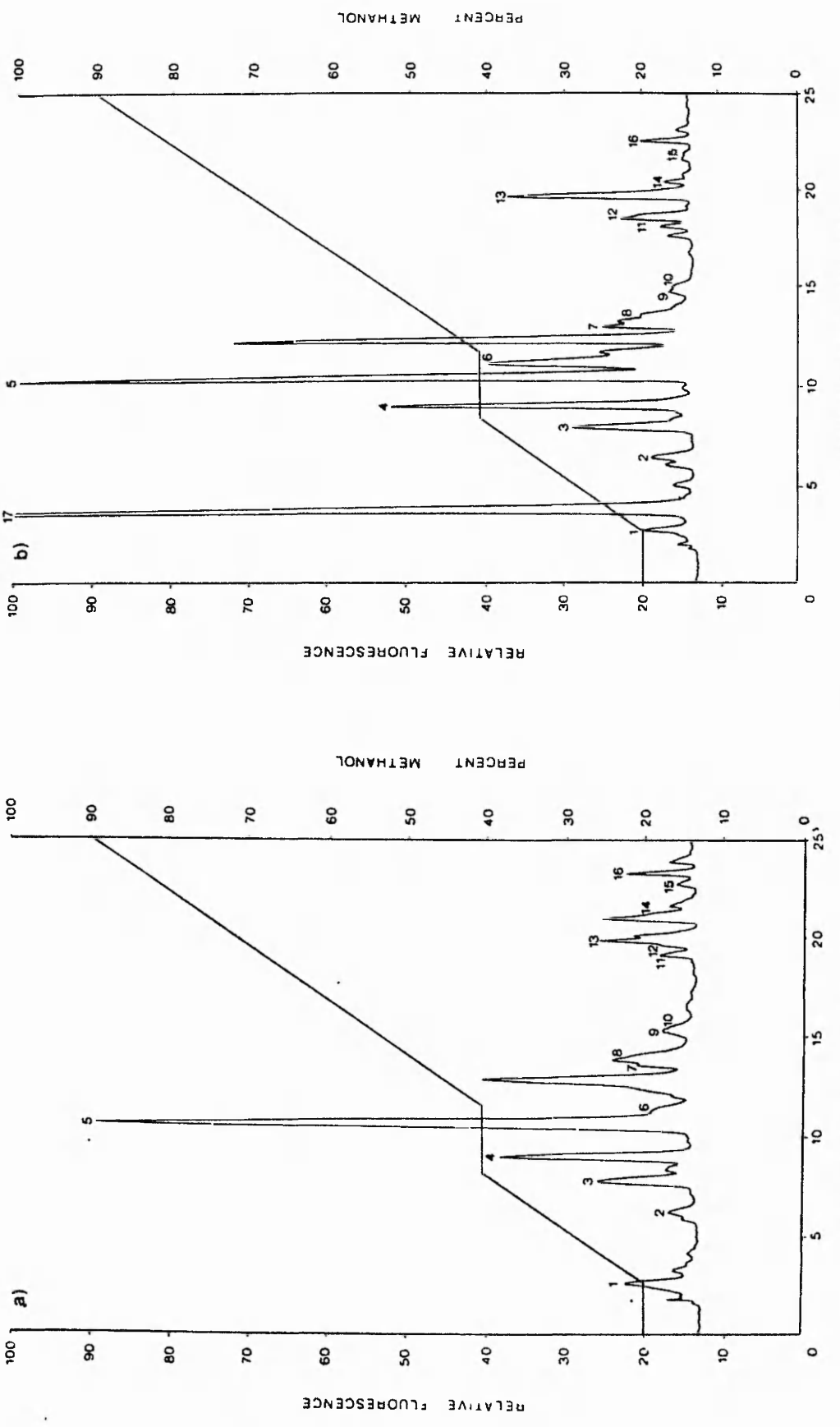

Figure 1 HPLC amino acid chromatograms, obtained using the method described in the text, from (a) a normal urine and (b) a urine specimen from a patient with ASAuria. Peak 1, aspartate; 2, asparagine; 3, serine; 4, glutamine; 5, glycine; 6, threonine; 7, alanine; 8, tyrosine; 9, β-amino-isobutyrate; 10, α-amino-*n*-butyrate; 11, valine; 12, phenylalanine; 13, ammonia; 14, leucine; 15, ornithine; 16, lysine; 17, argininosuccinate. The main peak between threonine and alanine is taurine.

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Pages 629-634

A COMPARISON OF ORNITHINE AMINOTRANSFERASE FROM HUMAN AND RAT SOURCES

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SUMMARY: Ornithine aminotransferase was purified from human liver, rat liver and rat kidney. Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated a subunit molecular weight of 45,000 in all three cases. Estimations of the native molecular weights of ornithine aminotransferase were determined by Sephadex G-200 chromatography in the presence and absence of 0.1%(w/v) Triton X-100. Human and rat enzymes were tetrameric in the presence of detergent but the rat subunits aggregated further in its absence. Characterisation of ornithine aminotransferase from the two rat sources indicated that they were the same protein. The human and rat enzymes were similar but not identical.

INTRODUCTION

Ornithine aminotransferase(EC 2.6.1.13) is a pyridoxal phosphate dependent enzyme located in the mitochondrial matrix(1). Several studies have shown that ornithine aminotransferase reduces urea cycle activity by catabolising ornithine by amino transfer to 2-oxoglutarate to produce glutamate and pyrroline-5-carboxylate(2,3).

Estimations of the native molecular weight of rat liver ornithine aminotransferase have produced conflicting results: Katunuma et al(4) first estimated 115,000 and molecular weights of between 132,000 and 256,000 have since been reported(5-8) despite general agreement that the molecular weight of the identical monomers is about 45,000. It is now known that the molecular weight of ornithine aminotransferase is concentration dependent(9) and that the enzyme undergoes self-association of subunits into aggregates as concentration increases(10). Ohura et al(11) estimated that the native molecular weight of human liver ornithine aminotransferase was 177,000 and the enzyme appeared to be a tetramer.

Abbreviations used: SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Km,Michaelis constant.

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In view of the differences in the reported results, we have carried out a comparative study of ornithine aminotransferase from three sources: rat liver, human liver and rat kidney. Characterisation has included determination of Km values, pH optima, cofactor dependence and thermal stability.

MATERIALS AND METHODS

ENZYME ASSAY: Ornithine aminotransferase was assayed using the method of Herzfeld and Knox(12), the incubation mixture being buffered to pH 8.0; the product, pyrroline-5-carboxylate, was reacted with the chromogen, o-aminobenzaldehyde. Protein was assayed by the method of Lowry et al(13) using bovine serum albumin as a standard.

SDS-PAGE: Determination of enzyme subunit molecular weight was by electrophoresis using the method of Laemmli(14) in a 10% gel. Molecular weight markers were obtained from Sigma.

SEPHADEX G-200 CHROMATOGRAPHY: Estimations of native molecular weight of ornithine aminotransferase were made using Sephadex G-200(Pharmacia) with column dimensions 1.6 x 82 cm. Elution was performed using 50 mmol/l potassium phosphate buffer (pH 8.0)containing 0.15 mol/l sodium chloride and 2 mmol/l 2-oxoglutarate in both the presence and absence of 0.1%(w/v) Triton X-100. A flow-rate of 9 ml per hour was used. The column was calibrated with molecular weight markers(Pharmacia).

PURIFICATION OF ORNITHINE AMINOTRANSFERASE: Human liver obtained at autopsy was used for ornithine aminotransferase purification by the method of Ohura et al(11). The procedure involved ammonium sulphate fractionation, heat treatment and DEAE-cellulose (Sigma), octyl sepharose CL-4B(Pharmacia) and Sephadex G-200(Pharmacia) chromatography. Rat liver and kidney ornithine aminotransferase were purified using the method of Sanada et al(15) with slight modifications. Purification involved sonication to release the mitochondrial enzyme, heat treatment, ammonium sulphate fractionation and concentration by collodion bag(Sartorius) prior to crystallisation. Rat liver ornithine aminotransferase underwent additional purification by Sephadex G-200 chromatography. The enzyme was crystallised by the method of Matsuzawa et al(6) by addition of saturated ammonium sulphate to the purified enzyme until a very faint turbidity was observed. Crystallisation occurred within 48 hours at 4°C, after which the enzyme was dialysed against distilled water.

FORMATION OF APO-ENZYME: Aliquots of holoenzyme were dialysed against 0.2 mol/l hydroxylamine for 2 hours at 4°C, followed by dialysis against distilled water, as outlined by Katunuma et al(4).

RESULTS AND DISCUSSION

Ornithine aminotransferase purified from each of the three sources produced a single band after SDS-PAGE on a 10% gel. The subunit molecular weight of ornithine aminotransferase was shown to be virtually identical for human and rat(fig.1), corresponding to a value of 45,000. This is in close agreement with Ohura et al(11), who estimated a value of 44,000.

Observations made during purification of ornithine aminotransferase, such as the poor solubility of the enzyme in water and the binding to the hydrophobic interaction resin(octyl sepharose CL-4B), together with knowledge

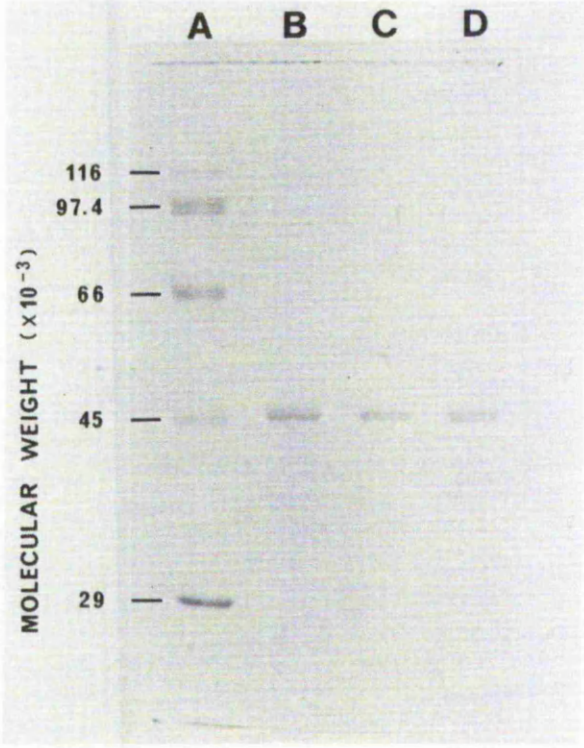

Fig.1. SDS-PAGE of ornithine aminotransferase from human liver(lane B), rat kidney(lane C) and rat liver(lane D) in a 10% gel.
Molecular weight markers(lane A) were as follows: carbonic anhydrase(29,000), egg albumin(45,000), bovine albumin(66,000), phosphorylase B(97,400) and beta-galactosidase(116,000).

of the self-association phenomenon, suggest an unusual degree of hydrophobicity for a matrix enzyme. Rat and human ornithine aminotransferase were assayed in the presence of a range of detergent concentrations up to 2% (w/v) Triton X-100 with no effect upon activity. Molecular weights of native ornithine aminotransferase were then estimated by Sephadex G-200 chromatography in the presence and absence of 0.1%(w/v) Triton X-100(fig.2). Absence of the detergent resulted in a value of 282,000$\pm$ 16,500 (mean$\pm$ standard deviation, n = 4) being obtained for both rat liver and kidney enzyme at a concentration of 10 mg/ml (Point D, Fig.2), in approximate agreement to the findings of Markovic-Housley et al (8). This estimation was reduced to 188,000 $\pm$ 13,000 (n = 4) in the presence of 0.1(w/v) Triton X-100 (Point C, Fig.2), indicating a tetrameric enzyme under these conditions. The molecular weight of human liver ornithine aminotransferase was estimated to be 178,000$\pm$ 12,500 (n = 4), both in the presence and absence of detergent (Point B, Fig.2);

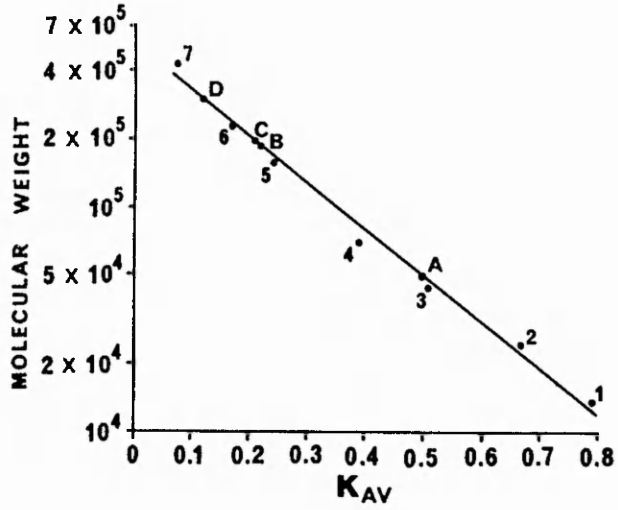

Fig.2. Estimation of the molecular weight of ornithine aminotransferase by Sephadex G-200 chromatography.
Kav = (Ve - Vo)/(Vt - Vo) where Ve = elution volume of protein(ml), Vo = void volume(45ml) and Vt = total bed volume(135 ml). Molecular weight markers are as follows: **1** ribonuclease; **2** chymotrypsin; **3** ovalbumin; **4** bovine serum albumin; **5** aldolase; **6** catalase; **7** ferritin. A line was fitted by the method of least squares. Ornithine aminotransferase samples (each point the mean value obtained) are: **A** sonicated rat kidney; **B** human liver with and without detergent present; **C** rat liver and kidney with detergent present; and **D** rat liver and kidney without detergent present. Calculated molecular weight values (mean $\pm$ standard deviation) are given in the text.

this also indicates a tetrameric enzyme, as suggested by Ohura et al(11). A value of 47,000 $\pm$ 2,800 (n = 4) was obtained for rat kidney ornithine aminotransferase after sonication of a 5 mg/ml solution for 5 minutes at 10 kilocycles per second (Point A, Fig.2). Since this value is in close agreement with molecular weight estimations made by SDS-PAGE it is likely that sonication dissociates the oligomeric enzyme into monomeric units. The specific activity of rat kidney ornithine aminotransferase before and after sonication was unchanged. However, it has been demonstrated that aggregation of the rat monomers sterically hinders substrate binding, since Km values are increased(10).

Recent studies(16) have demonstrated that extramitochondrial ornithine is channelled to ornithine transcarbamoylase(EC 2.1.3.3.) in the matrix and suggest that this enzyme could be in close proximity to the ornithine transporter protein on the inner face of the inner membrane. It may therefore be necessary for active ornithine aminotransferase to bypass this ornithine pool after processing through the inner membrane, by aggregating to a

relatively inactive form. This could dissociate as it was released into the matrix, allowing excess ornithine to be catabolised as the Km for ornithine was lowered. Such a mechanism would account for the undetectable levels of ornithine within the matrix space(16,17).

The characterisation of rat liver and kidney ornithine aminotransferase indicated that they are identical to each other but not to the human liver enzyme; this is consistent with previous reports (11,15). Human ornithine aminotransferase produced Km values approximately twice as high as rat enzyme for each of the two substrates, but a lower value for the coenzyme Km (Table I). Substrate inhibition was noted for both substrates at high concentrations, with 2-oxoglutarate inhibiting more strongly than ornithine under comparable conditions. Substrate inhibition would provide another means of avoiding catabolism of a high concentration ornithine pool within the matrix.

Human and rat ornithine aminotransferase pH-activity curves were virtually identical, both showing an optimal pH of 8.0. Cofactor dependence was demonstrated by loss of activity after apo-enzyme formation following dialysis of holoenzyme against hydroxylamine. Both human and rat ornithine aminotransferase activity could be restored within 30 minutes upon incubation with pyridoxal phosphate. The enzyme purified from all three sources showed a high degree of thermal stability in the presence of 0.02 mmol/l pyridoxal phosphate and 5 mmol/l 2-oxoglutarate. The human enzyme could withstand a 60°C incubation for one minute with negligible loss of activity; both rat ornithine aminotransferase samples retained 95% activity under these

TABLE I: Km values for human and rat ornithine aminotransferase. Fixed concentrations, where appropriate, were: 35 mmol/l ornithine; 5 mmol/l 2-oxoglutarate; and 0.055 mmol/l pyridoxal phosphate. Pyridoxal phosphate was pre-incubated for 30 minutes with apo-enzyme prior to assay.

Variable component	Km value(mmol/l)		
	rat liver	rat kidney	human liver
ornithine	1.1	1.25	2.3
2-oxoglutarate	1.1	1.15	2.5
pyridoxal phosphate	2.0	4.0	1.0

conditions. In all cases, the addition of 35 mmol/l ornithine to the enzyme incubated with cofactor and substrate as described above caused a destabilisation of the enzyme within 5 hours at 4°C with up to 60% loss of activity. A 60°C incubation in the presence of ornithine resulted in 100% loss of activity in one minute.

The characterisation of rat liver and kidney ornithine aminotransferase in this study and by previous workers suggests that the same protein is involved, rather than two isoenzymes. The close similarity of molecular weight and physicochemical data for human and rat ornithine aminotransferase suggest that a close structural relationship exists between them. This is supported by Ohura et al(11) who have shown the amino acid composition of human and rat enzyme to be very similar. We have confirmed this immunologically, and are currently carrying out more work in this area.

ACKNOWLEDGEMENT

We wish to thank Prof.R.J.Mayer(Nottingham) for his assistance in obtaining human liver tissue at autopsy.

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ANALYTICAL BIOCHEMISTRY 170, 495–501 (1988)

Purification of Ornithine Aminotransferase by Immunoadsorption

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Ornithine aminotransferase was purified by conventional biochemical methods from rat kidney, rat liver, and human liver. Affinity-purified antibodies raised to the rat kidney enzyme were used to produce an immunoadsorbent enabling a one-step purification of ornithine aminotransferase to be made from crude human liver extracts. The harsh chemical conditions often required to desorb immunoadsorbents were avoided by isolating antibodies with low functional affinity and employing an electrophoretic desorption method which allowed the enzyme activity to be retained. The close structural similarity between human and rat ornithine aminotransferase was demonstrated by immunodiffusion reactions. It was therefore possible to purify the enzyme from human liver using immobilized antibodies raised against rat kidney ornithine aminotransferase. Furthermore, desorption was more readily achieved due to the lower affinity for the human enzyme.

KEY WORDS: immunoadsorbent; protein purification; immobilized enzymes; immunochemical methods; electrophoresis; immunoprecipitation.

OAT[1] (EC 2.6.1.13) is a mitochondrial matrix enzyme thought to regulate urea cycle activity by ornithine catabolism (1). The abundance of OAT in rat kidney has enabled relatively simple purification (2). Crystallization of rat liver OAT has necessitated further purification steps (3,4). Purification of human liver OAT, a complex procedure involving heat treatment and several chromatographies prior to crystallization (5), has enabled the structural and physicochemical similarity of human and rat liver OAT to be demonstrated (6). Several studies have suggested that rat liver and rat kidney OAT are the same protein but under different hormonal control (7,8).

The use of immunoadsorption for affinity purification of specific antibodies is an established procedure (9) but has limited application to antigen purification since deforming agents usually required for desorption often denature and inactivate labile proteins. Low affinity antibodies require less severe desorption conditions however and electrophoretic desorption (10,11) avoids the use of chemical desorption altogether.

In this study, the close structural identity of human and rat OAT has been exploited to produce an OAT-specific cross-reacting immunoadsorbent. Relatively high recovery purification of human liver OAT was achieved by employing electrophoretic desorption of the immunoadsorbent.

MATERIALS AND METHODS

Assays. OAT was assayed by a modified method of Herzfeld and Knox (12). The reaction mixture contained 35 mM ornithine, 55 μM pyridoxal phosphate, 5 mM 2-oxoglutarate, 5 mM *o*-aminobenzaldehyde, and 50 mM potassium phosphate buffer (pH 8.0) in a total volume of 2.0 ml. The reaction was stopped by addition of 10% trichloroacetic acid after a 20-min incubation at 37°C. A millimolar extinction coefficient of 2.71 was

[1] Abbreviations used: OAT, ornithine aminotransferase; ELISA, enzyme-linked immunoadsorbent assay; IgG, immunoglobulin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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used to determine the amount of product formation. One unit of enzyme activity was defined as 1 μmol of pyrroline-5-carboxylate formed at 37°C per minute. Protein was assayed by the method of Lowry *et al.* (13) using bovine serum albumin as a standard. Pure protein extracts were determined spectrophotometrically at 280 and 260 nm.

Antibody titer was monitored using a noncompetitive ELISA (9). OAT was immobilized onto microtiter plates (Flow Laboratories, Herts, UK) at 1 μg/50 μl of 0.15 M PBS (pH 7.4) by incubation overnight at 4°C. Wells were aspirated and washed with blocking agent, 2 g of dried milk powder ("Marvel," Cadbury, Birmingham, UK) dissolved in 100 ml of 0.15 M PBS (pH 7.4). After two washes, 300 μl of blocking agent per well was incubated for 2 h at 20°C. Wells were washed out with 0.15 M PBS (pH 7.4) containing 0.05 g/100 ml Tween 80 (Sigma Chemical Co., Poole, UK) twice followed by two washes with distilled water. An anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) was used to indirectly detect specific IgG bound to immobilized OAT. The conjugate was diluted 1000-fold in blocking agent and incubated at 50 μl per well for 2 h at 20°C. Wells were washed out with 0.15 M PBS (pH 7.4)/Tween 80 solution as before, followed by two washes with distilled water. Wells were aspirated and incubated with 100 μl of substrate/chromogen solution made up just prior to use as follows: 150 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co.) dissolved at 10 mg/ml in dimethyl sulfoxide, was added to 20 ml of 0.1 M sodium acetate buffer (pH 6.0) containing 25 μl of hydrogen peroxide (3 g/100 ml). The reaction was stopped after a suitable reaction time (10–20 min) by addition of 50 μl of 2.5 M sulfuric acid per well. Absorbance was read at 450 nm using an ELISA reader (Titertek Multiskan, Flow Laboratories).

SDS–PAGE. Electrophoresis was performed on a 10% gel as described by Laemmli (15). Protein markers (Sigma Chemical Co.) were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin(66 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

Preliminary purification of OAT. Human liver OAT was purified using the method of Ohura *et al.* (5). Liver extract was subjected to 60°C heat treatment, ammonium sulfate fractionation, and chromatographies on DEAE-cellulose (Sigma Chemical Co.), Octyl-Sepharose CL-4B (Pharmacia Ltd., Bucks, UK), and Sephadex G-200 (Pharmacia Ltd.). Rat liver and kidney OAT were purified by the method of Sanada *et al.* (2). Purification involved 60°C heat treatment, ammonium sulfate fractionation, and extraction with distilled water. Rat liver OAT required further purification by Sephadex G-200 chromatography as described for human liver OAT purification (5). OAT was crystallized by the method of Matsuzawa *et al.* (3) followed by dialysis against distilled water.

Production of antibodies. Adult male white New Zealand rabbits were immunized with 250 μg of OAT purified from the three respective sources. OAT was dissolved in 0.15 M PBS (pH 7.4) emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.) in a total volume of 1 ml, administered subcutaneously as described by Mayer and Walker (11). A further three booster doses of 250 μg of OAT emulsified in Freund's incomplete adjuvant were administered at 3-week intervals. Test bleeds from the marginal ear vein were used to monitor serum antibody titer by ELISA. Control serum was taken prior to immunization.

Immunodiffusion. Double immunodiffusion was performed as described by Hudson and Hay (9).

Affinity purification of IgG. IgG was affinity-purified from rabbit serum using rat kidney OAT immobilized to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.) using the method of Dalchau and Fabre (14). Twenty milligrams of lyophilized rat kidney OAT was dissolved in 4 ml of "coupling" buffer (pH 8.3), containing 0.1 M

sodium carbonate dissolved in 0.5 M hydrochloric acid. Preswollen cyanogen bromide-activated Sepharose 4B equilibrated with coupling buffer was mixed with OAT (5 mg protein/ml of gel) overnight at 4°C by end over end rotation. This mixture was transferred to a scintered glass funnel and sucked dry under vacuum. The fluid was recovered and assayed for protein to assess coupling efficiency. The gel was washed thoroughly with coupling buffer and mixed as before, for 2 h with 1 M ethanolamine (pH 8.0). The gel was sucked dry and washed with coupling buffer. Protein noncovalently adsorbed to the gel was removed by washing the gel with alternating buffers of low (0.1 M sodium acetate, 1 M sodium chloride, pH 4.0) and high pH (0.025 M Tris, 1 M sodium chloride, pH 8.2). The OAT-linked Sepharose was washed with 0.15 M PBS (pH 7.4) which contained 0.1 M sodium azide during storage at 4°C.

A quantitative precipitin test was performed as described by Hudson and Hay (9) on rat kidney OAT antiserum to determine IgG content. Eight milliliters of antiserum was mixed with the OAT-linked Sepharose 4B prepared as described, by end over end rotation for 1 h at 4°C. The gel was transferred to a mini-column and washed with 100 ml of 0.15 M PBS (pH 7.4) until collected fractions gave negligible readings at 280 nm. A batch of IgG was eluted with 20 ml of 0.1 M glycine–hydrochloric acid buffer (pH 2.5). A further batch of higher affinity[2] IgG was eluted with 0.1 M glycine–hydrochloric acid (pH 2.5) containing 10 g/100 ml dioxane (BDH Ltd., Dorset, UK). All fractions (5-ml aliquots) were immediately adjusted to pH 8.0 with solid Tris. The two populations of IgG fractions were pooled separately and concentrated by collodion bag filtration (Sartorius Instruments Ltd., Surrey, UK) prior to dialysis against 5000 ml of 0.15 M PBS (pH 7.4).

[2] The term "affinity" used in the text refers to the functional affinity (avidity) as polyclonal antibodies are used.

Four milliliters of the lower affinity IgG was dialyzed against the coupling buffer (pH 8.3), adjusted to 5 mg/ml protein, and mixed with 4 ml of swollen cyanogen bromide-activated Sepharose 4B. The IgG was coupled by the method described for OAT coupling.

Immunoadsorption of OAT from human liver. Frozen human liver tissue was thawed, minced, and homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 5 mM 2-oxoglutarate and 20 μM pyridoxal phosphate using a Waring blender at top speed for 30 s. All steps were performed at 4°C. This produced a 20% homogenate which was centrifuged at 18,000*g* for 20 min. The supernatant was heated to 60°C for 1 min and rapidly cooled in ice. Precipitated protein was removed by centrifugation at 18,000*g* for 10 min. The supernatant was adjusted to pH 7.4 and mixed with Sepharose 4B at 1 ml/10 ml of supernatant. Mixing by end over end rotation for 1 h removed any proteins likely to adsorb nonspecifically to the immunoadsorbent. The supernatant fluid was isolated by vacuum filtration through scintered glass and mixed with 1.5 ml of the prepared immunoadsorbent preequilibrated with 50 mM potassium phosphate buffer (pH 7.4) by end over end rotation at 4°C overnight. The mixture was transferred to a vacuum filter to remove unbound protein and washed with 150 ml of 10 mM potassium phosphate buffer (pH 7.4). The immunoadsorbent was transferred to the eluting column of a preparative gel electrophoresis system (Bethesda Research Laboratories Ltd., UK) which was set up as shown (Fig. 1). Both reservoirs, eluting column, and collecting chamber contained 10 mM potassium phosphate buffer (pH 7.4). Dialysis membrane covered the base of the collecting chamber and the top of the eluting column. Once the immunoadsorbent was packed, a current of 10 mA with variable voltage was applied across the two reservoirs for 40 min at 4°C. After desorption the current was switched off, the top of the reservoir removed, and buffer decanted above the immunoadsorbent. The desorbed protein was

removed through a valve in the collecting chamber by syringe. The protein was dialyzed against 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and 20 μM pyridoxal phosphate.

RESULTS

Preliminary Studies

Antisera raised to OAT from rat liver and kidney gave heavy precipitin lines to both rat and human OAT (Fig. 2). The immunological identity of both rat enzymes and partial identity (spur formation) to human OAT were observed.

The coupling of rat kidney OAT to cyanogen bromide-activated Sepharose 4B was 93% efficient, producing OAT-linked Sepharose 4B at 4.65 mg of OAT/ml of gel. The IgG content of rat kidney OAT antiserum was estimated at 5.1 mg/ml from a quantitative precipitin curve. The binding capacity of the OAT-linked Sepharose 4B for anti-rat kidney OAT antibody was determined at 11.25 mg IgG/ml of gel.

The recovery of IgG desorbed from the OAT-Sepharose 4B by 0.1 M glycine–hydrochloric acid buffer (pH 2.5) was 73% (batch 1) and a further 15% (batch 2) was recovered by elution with the same buffer containing 10 g/100 ml dioxane. After concentration and dialysis of the lower affinity IgG (batch 1), recovery was reduced to 61%.

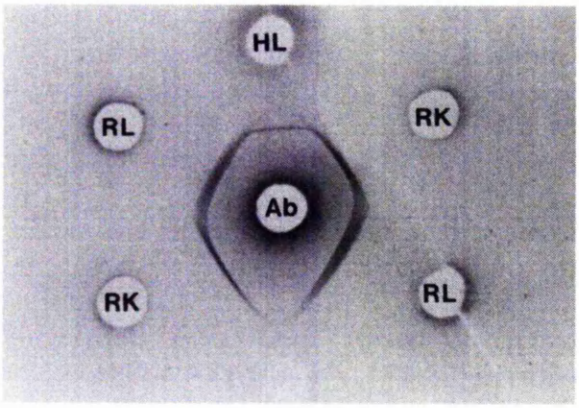

FIG. 2. Double immunodiffusion analysis in 2% agar made up in 0.15 M PBS (pH 7.4). Wells each contained 20 μl sample as follows: HL, human liver OAT (20 μg); RL, rat liver OAT (40 μg); RK, rat kidney OAT (40 μg); Ab, antisera raised to rat kidney OAT.

The coupling of IgG (batch 1) to activated Sepharose 4B was 84% efficient, producing IgG-linked Sepharose at 4.2 mg IgG/ml of gel. This immunoadsorbent was used for OAT purification.

Assessment of Antibody Affinity

In order to assess antibody affinity, a noncompetitive ELISA was performed on serial dilutions of respective antibody batches. IgG concentration was determined at 50% saturation of the antigen and this value used as an indicator of apparent antibody affinity in each case. The lower the IgG concentration needed for 50% saturation, the higher the affinity of the antibody for the antigen. Results for antibody titrated against rat OAT antigen were as follows[3]: anti-rat OAT antiserum, 0.2 nM; IgG (batch 1), 0.36 nM; IgG (batch 2),

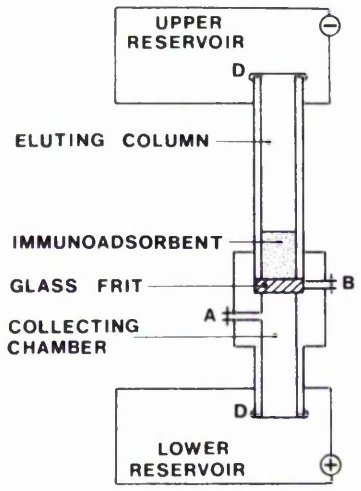

FIG. 1. Diagrammatic representation of the electrophoretic desorption system described in the text. Dialysis membrane (D) was held in position by rubber "O" rings on the top of the eluting column and on the base of the collecting chamber. Desorbed protein was removed via valve A after the immunosorbent was drained via valve B.

[3] These data provide only an indication of the apparent affinity since several epitopes on the antigen are involved. The assay is more valid for monoclonal antibodies (16).

0.01 nM. This indicates that IgG (batch 1) has a 20-fold lower affinity than IgG (batch 2) for rat OAT. IgG (batch 1) titrated against human OAT produced a value of 0.56 nM indicating a lower affinity for human OAT than rat OAT.

Immunoadsorption of OAT from Human Liver

Purification of human liver OAT by immunoadsorption (Table 1) produced a specific activity of 28.13 units/mg for the desorbed enzyme and a yield of 56%. Assessment of OAT purity (Fig. 3, lane C) by SDS–PAGE shows a single band with very minor contaminants desorbed from the immunoadsorbent. The extent of purification can be seen by comparison with crude extracts (Fig. 3, lanes A and B). Purification of human liver OAT by conventional methods (Table 2) produced a pure enzyme with a specific activity of 34.3 units/mg and a yield of only 14.5%.

The maximum binding capacity of the immunoadsorbent was determined by mixing 250-μl aliquots of gel with 1-ml aliquots of rat kidney OAT at 1, 5, and 10 mg/ml, respectively, overnight at 4°C. The immunoadsorbent contained 1 mg of immobilized antibody/250 μl and the maximum binding of OAT was 0.42 mg at 10 mg/ml. The effect of chemical desorption was briefly assessed by incubating unbound rat kidney OAT with 1 M propionic acid (pH 2.6) and 50 mM diethylamine (pH 11.5), respectively, both containing 0.5 g/100 ml Triton X-100. A 20-min incubation followed by dialysis against 50 mM potassium phosphate buffer (pH 8.0) containing 5 mM 2-oxoglutarate and 20 μM pyridoxal phosphate resulted in total loss of activity under acidic conditions and 40% loss under alkaline conditions.

DISCUSSION

The conventional methods needed to purify human liver OAT require the use of a series of different processes which can be costly and time-consuming. Each additional step in purification usually results in loss of yield and often necessitates concentration and dialysis before work can continue. As a result, low yields of pure protein are obtained and large volumes of crude extract often have to be processed in order to obtain sufficient end product. When an immunoadsorbent is used, however, most of these problems can be overcome.

The specific adsorption of human liver OAT onto the immunoadsorbent used in this study has relied on the close structural similarity between human and rat OAT, the latter being easily purified from rat kidney. It was therefore possible to purify human OAT using anti-rat OAT antibodies for immunoadsorption. Initial purification of human OAT by more complex methods can thus be avoided and would not be required for antibody production or affinity purification of specific IgG. The advantage of being able to use IgG raised to rat kidney OAT is that the affinity for the human source is likely to be

TABLE 1

PURIFICATION OF HUMAN LIVER OAT BY IMMUNOADSORPTION

Fraction	Volume (ml)	Total protein (mg)	Percentage yield	Specific activity (units[a]/mg)	Purification factor
Homogenate	100	3080	100	0.0104	1
60°C supernatant	81	1758	103[b]	0.0188	1.81
Desorbed protein	4	0.64	56	28.13	2705

[a] Units defined in text.

[b] Yield increases due to mitochondrial release of sequestered OAT.

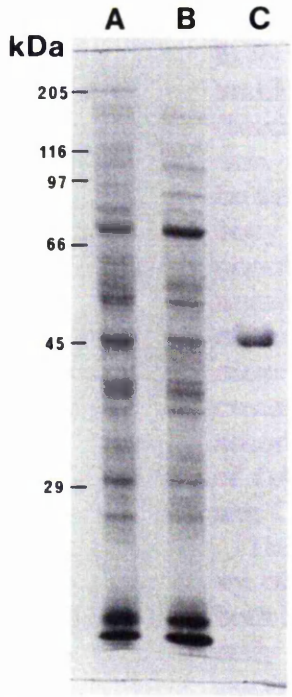

FIG. 3. SDS–PAGE of human liver fractions described in the text. Samples were run on a 10% gel as follows: lane A, homogenate (60 μg); lane B, 60°C supernatant (60 μg); lane C, desorbed protein (6 μg). Protein markers are described in the text.

lower and therefore more easily desorbed from an immunoadsorbent.

Assessment of apparent antibody affinity by ELISA suggests that the IgG used for immunoadsorption (batch 1) was of lower affinity than the original antiserum and that a small population of much higher affinity IgG (batch 2) was removed prior to immobilization. However, such measurements cannot be taken as a definitive assessment of antibody affinity since the respective antibody populations may have a different average number of binding sites. Scatchard analysis of the antibody populations would provide more valid data (17). This effectively increases the useful shelf life of the immunoadsorbent by preventing irreversible binding of OAT onto high affinity IgG after successive OAT purifications.

The maximum theoretical binding capacity of the immunoadsorbent occurs when both binding sites of every immobilized antibody molecule bind a molecule of OAT. Assuming the molecular weights of OAT and IgG are 45,000 and 160,000 respectively, 1 mg of immobilized IgG could bind 0.563 mg of OAT. The capacity of the immunoadsorbent used in this study (4.2 mg IgG bound/ml of Sepharose 4B) was estimated at 0.42 mg of OAT bound/mg of immobilized IgG (75% of the theoretical maximum). This estimation enables the immunoadsorbent to be used at a submaximal capacity with tissue extracts. However, OAT-linked Sepharose 4B has a higher binding capacity for IgG

TABLE 2

PURIFICATION OF HUMAN LIVER OAT BY CONVENTIONAL BIOCHEMICAL TECHNIQUES[a]

Fraction	Volume (ml)	Total protein (g)	Percentage yield	Specific activity (units[b]/mg)	Purification factor
Homogenate	4630	125.93	100	0.00787	1
60°C supernatant	3615	70.85	99	0.0139	1.76
Ammonium sulfate[c]	180	14.3	96.5	0.066	8.5
DEAE-cellulose[d]	220	1.82	76	0.414	53
Octyl-Sepharose[d]	28	0.12	68	5.6	714
G-200 Sephadex[e]	3	0.0042	14.5	34.3	4357

[a] Human liver (954 g).
[b] Units defined in text.
[c] Saturation 50%, resuspended pellet.
[d] Pooled fractions.
[e] Peak fraction.

since a number of IgG molecules can bind to the OAT molecule depending upon antigenic valency. The valency of OAT for IgG raised to rat kidney OAT was estimated at 4, calculated from quantitative precipitin data (9).

The binding of OAT from large volumes of crude tissue extract onto small volumes of immunoadsorbent appears to be very efficient and effectively concentrates the enzyme. Dean *et al.* (10) were able to purify ferritin by a factor of 50,000 from 1000 ml of plasma using 1.5 ml of immunoadsorbent. Nonspecific adsorption of human liver proteins was prevented by preincubation of the extract with Sepharose 4B which effectively removed these proteins prior to immunoadsorption.

The use of the electrophoretic desorption apparatus was modified to produce conditions similar to those described previously (10). The desorbed protein could be concentrated in the collecting chamber (Fig. 2) by reducing the chamber volume, although Mayer and Walker (11) have noted the use of a sample concentrator (ISCO Model 1750) which can elute and concentrate the antigen simultaneously. Where a specific immunoadsorbent cannot be produced it may be possible to combine the technique of preparative gel electrophoresis with desorption by overlaying an acrylamide gel with immunoadsorbent, for example. Desorbed proteins could then be separated through the gel and collected as fractions. By avoiding the use of chemical desorbing agents, the useful life of the immunoadsorbent and the recovery of desorbed native protein should be greatly improved. Electrophoretic desorption of higher affinity adsorbents may however not be as effective and could result in buildup of tightly bound antigen. This may necessitate chemical desorption in order to restore binding capacity after several uses. Immunoadsorbents are stable for several years if properly kept (14) and may be of commercial importance by replacing more costly puripurification methods. The versatility of immunoadsorption should enable scaling up of pilot purifications, although quality control would be necessary to avoid variable purity of antigen due to batch variability of IgG. If a low affinity monoclonal antibody can be made to the antigen of interest, immunoadsorbents of a consistent quality can be made available.

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CONSTRUCTION OF AN ION-EXCHANGE AMINO ACID ANALYSER KIT FOR USE WITH HIGH-PERFORMANCE LIQUID. CHROMATOGRAPHY APPARATUS

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SUMMARY

A conventional ion-exchange amino acid analyser kit has been simply and economically constructed for use with exiting high-performance liquid chromatography (HPLC) apparatus. Sequential elution conditions are fully programmable by virtue of a 32K BBC microcomputer interfaced with an elution buffer selection valve and a thermostatically controlled column. Post-column derivatization with *o*-phthalaldehyde–2-mercaptoethanol reagent enables fluorimetric detection at the pcomole level. The system enables sensitive amino acid analysis of complex mixtures to be carried out by clinical and research laboratories who already possess HPLC apparatus but whose sample turnover does not merit purchase of a dedicated fully automated analyser.

INTRODUCTION

A question which confronts many laboratories when in the process of either updating or purchasing for the first time an instrument for the quantitative analysis of amino acis, is whether to purchase a fully dedicated instrument or one which has the versatility to be used for other purposes. The relative merits of these have been reviewed previously [1,2].

Such a choice is now available because of the recent developments in amino acid analysis using reversed-phase high-performance liquid chromatography (HPLC) as an alternative to the more conventional cation-exchange systems [3].

Since amino acids are polar compounds of diverse charge their interaction with the hydrophobic stationary phase has to be promoted in some way to allow effective resolution of complex mixtures. One technique of increasing amino acid retention is to use ion-pair systems [4] and methods for the separation of amino

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acids using this technique for reversed-phase HPLC analysis of amino acids involves the use of precolumn derivatisation. In this method amino acids are reacted to yield a hydrophobic derivative which not only provides adequate interaction with the column stationary phase but also provides a means of detection.

A number of methods combining precolumn derivatisation and reversed-phase HPLC for the separation of amino acids have now been reported. Reagents used include dansyl chloride [6], phenylthioisocyanate [7], 9-fluorenylmethyl chloroformate [8] and *o*-phthalaldehyde–2-mercaptoethanol (OPA–2-ME) [9–11]. Each system has its advantages and disadvantages but in general derivatisation of amino acids with OPA–2-ME is probably the most versatile when considering ease of derivatisation, method of detection and complexity of columns and gradients required for efficient separation of amino acids.

However, although reversed-phase systems have the ability to provide rapidity of separation and sensitivities in the femtomole range they are limmited in the number of components which can be separated when dealing with complex mixtures.

For the analysis of complex mixtures such as physiological fluids, a dedicated ion-exchange system is generally required. This system is very costly, has little versatility and in most laboratories can only be justified if a large number of samples on a continuous basis are to be analysed.

The need is therefore for a versatile system which can be used for both reversed-phase and ion-exchange separation of amino acids and peptides. We report here a system which for a small capital outlay to existing HPLC users, provides the means of using both reversed-phase and ion-exchange chromatography for the analysis of amino acids.

EXPERIMENTAL

Reagents

Amino acid standards, OPA, 2-ME and Brij 35 were purchased from Sigma (London, U.K.). Physiological standards, DL-norleucine, lithium citrate diluent buffer (pH 2.2) and lithium citrate Picobuffer system IV, were purchased from Pierce (Chester, U.K.). Ion-exchange resin DC-4A (sulphonated polystyrene 8% cross-linked with divinylbenzene) supplied in the sodum form from Dionex (Hampshire, U.K.) was converted to the lithium form as instructed by Benson [12]. Analar grade methanol, boric acid and potassium hydroxide were purchased from BDH (Poole, U.K.).

OPA-2-ME reagent used for derivatisation of amino acids was made up as follows: 50 g of boric acid, 44 g of potassium hydroxide and 3.5 ml of Brij 35 were dissolved in double-distilled water, adjusted to pH 10.4 and made up to 1000 ml. A 600-mg amount of OPA was dissolved in 7.5 ml of methanol and 5.0 ml of 2-ME and the solution was added to the boric acid buffer. The reagent was vacuum-filtered through 0.22-μm Durapore filters (Waters Assoc.), degassed and stored in a dark bottle under nitrogen during use. Picobuffers were degassed and filtered as instructed by the suppliers and stored under nitrogen during use.

When the system was converted to amino acid detection by reaction with ninhydrin, the reagent was made up by the method of Moore and Stein [13] and contained in a total volume of 1000 ml: 750 ml methyl cellosolve, 250 ml of 4.0 *M* sodium acetate buffer (pH 5.5), 20 g of ninhydrin and 0.4 g of stannous chloride.

Chromatography

An M-45 pump (Waters Assoc) was employed to pump OPA–2-ME reagent to a post-column low-volume mixing manifold (Waters Assoc.) at a flow-rate of 0.2 ml/min from which a 2-min reaction time was permitted prior to fluorescence detection. A Perkin-Elmer 1000 m fluorimeter fitted with a 25-μl flow cell, 339-nm interference filter and an emission wavelength of 455 nm was used for detection. Peak areas were determined using a Spectra-Physics SP 4270 integrator. The buffer pump used at a flow-rate of 0.2 ml/min was a Waters Type 501, fitted with a Rheodyne 7125 injector and 100-μl sample loop. A stainless-steel column with an I.D. of 0.3 cm was used to support the DC 4A ion-exchange resin (final packed dimensions 35 cm $\times$ 0.3 I.D.). A purpose built glass jacket surrounded the column to which water was supplied by a Churchill circular (Chemlab Instruments, Essex, U.K.).

Elution buffers were stored in stoppered air-tight glass containers kept under a nitrogen pressure of 0.35 bar. Buffer reservoirs were connected to a Rheodyne pressure-regulated selection valve using PTFE tubing (Anachem) connected by flanged connectors (Pierce).

A BBC Model B microcomputer was used fitted with disk drive (Acorn) and interface as described in the text.

Preparation of physiological samples for amino acid analysis

Preparation methods were those described by Benson [12]. Deproteinisatioin was performed by vortex-mixing 0.8 ml of urine with 0.2 ml of sulphosalicylic acid (10 g per 100 ml) followed by centrifugation at 11 600 *g* for 5 min to remove precipitated protein. The supernatant was adjusted to pH 2.0–2.2 with 0.3 *M* sodium hydroxide (approximately 0.2 ml) producing a dilution factor of 1.5. The sample was diluted a further ten-fold with 0.2 *M* lithium citrate diluent buffer (pH 2.2) before analysis.

Deproteinisation of serum samples was performed by first drying 0.1 ml of 12.5% (w/v) sulphosalicylic acid in 95% ethanol in a microfuge tube to which 0.1 ml of serum was then added. The dried residue was mixed with the serum sample and then centrifuged as described for urine to remove the resulting protein precipitate. The serum supernatant was removed and diluted five-fold in 0.2 *M* lithium citrate diluent buffer (pH 2.2) and applied to the analyser.

RESULTS AND DISCUSSION

Construction of a semi-automated amino acid analyser (outlined in Fig. 1) makes use of a laboratory's existing HPLC pumps, injector and detection equipment. The system as shown requires additional equipment consisting of air-tight buffer reservoir bottles, a suitable solenoid switching valve consisting of one out-

let and six inlet ports, a stainles-steel column, a suitable high-efficiency ion - exchange resin which in our case was Dionex DC-4A and a water-tight glass column jacket connected to a suitable water circulating heater bath. The responsiveness of the water heater was 1 °C/min. Dimensions of connector tubing are given in Fig. 1. The system shown uses post-column derivatisation of amino acids with OPA–2-ME followed by fluorescence detection.

An alternative system which we have found equally effective is the use of post-column derivatisation of amino acids with ninhydrin which requires a fixed- or variable-wavelength LC spectrophotometer capable of detection at or around 570 or 440 nm. For detection of amino acids with ninhydrin a reaction coil consisting of stainless-steel tubing (35 m × 0.3 mm) immersed in a suitable oil bath (Chemlab Instruments) and kept at a temperature of 100 °C was utilized. This gave a dwell time of approximately 6 min when using a combined flow-rate from the buffer pump and ninhydrin reagent pump of 0.4 ml/min. An additional 2 m of tubing (0.3 mm I.D.) was connected to the detector outlet which acted as a bubble suppressor. The detector used in our case was an LC Waters M-440 (dual channel) fitted with 436- and 546-nm filters.

A BBC Model B microcomputer was chosen as an intelligent controller for the apparatus. This computer was chosen because of its versatility and common usage in manufacturing and research laboratories in the U.K. and Europe. The computer is an 8-bit 6502 based machine with an on board 6522 VIA (versatile interface adapter). The operating system (BBC operating system) calls allow access

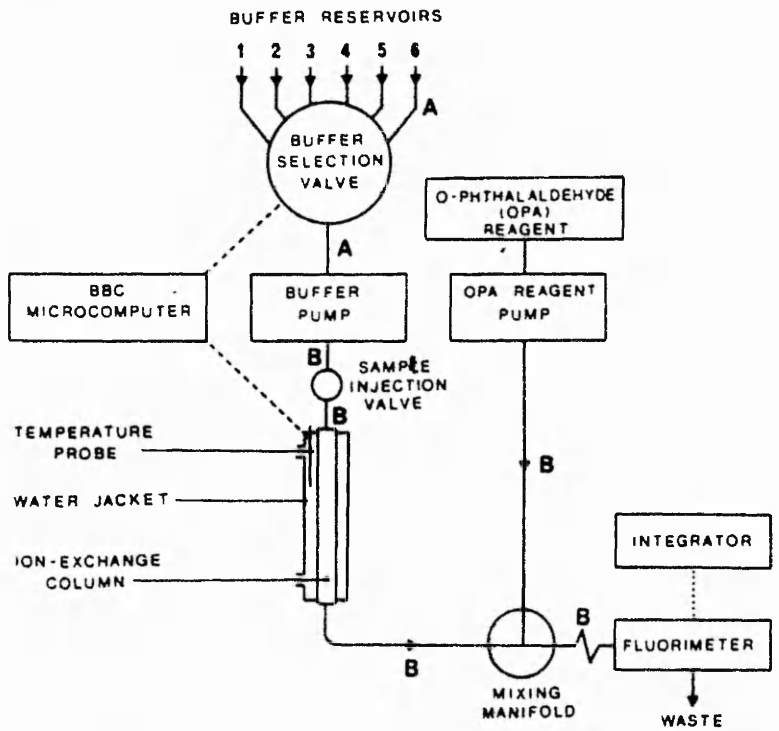

Fig. 1. Schematic diagram showing the single-column amino acid analyser system described in the text. Sites under computer control are indicated by dashed lines. Tubing internal diameters are indicated as follows: A, 1.5 mm; B, 0.3 mm. Stainless-steel tubing was used on the outlets of both pumps up to the mixing manifold. All other tubing was made of PTFE.

to this, as an on board 12-bit analogue-to-digital converter and an elapsed time clock. The latter two are also accessible to Basic users via Basic commands. The Basic interpreter is relatively fast and allows the use of user-defined procedures and function. The Basic program monitored column temperature and the clock of the computer. In response to these inputs the temperature and solvent selection valve were controlled.

Column temperature was monitored via a thermistor probe inserted into the glass heating jacket surrounding the column and connected to the analog port of the computer. This thermistor formed half of a potential-divider connected between the reference voltage output and ground, its junction with a fixed resistor being connected to analog channel 1. The advantage of this simple system is that the measured temperature is essentially unaffected by reference voltage drift. The heater control algorithm has a 0.2°C dead zone to reduce the relay activity. The heater and solvent selection switches were both drive from the computer's user port via an interface unit (Fig. 2).

A simplified flow chart for the control program is shown in Fig. 3. When entered, the program sets up a number of initial conditions, e.g. side B of the user VIA is set for output and the temperature probe calibration data is read into an array. From this point the program is controlled from a menu page. Run conditons can be set up from the keyboard, loaded from or saved to disk. The solvent selection switch can be incremented manually or a run can be started. Finally, exit from the program can be with an orderly return to normal. The program can support six temperature changes and six solvents. Before a run, the program enters an indefinite loop with a single controlled temperature for the purpose of equilibrating the column in solvent number 1. The program returns to the equilibration loop after each run. A "key press" starts the run conditions by moving control to a second loop, during which the column temperature follows a preprogrammed sequence and the solvent can be changed at pre-set intervals. A plot on the computer VDU of temperature versus time (with solvent changes indicated) provies a visual check on the progress of the run.

Elution conditions optimised for separation of components in complex mixtures such as physiological fluids (shown in Table I) were found to be adequate for continuous analyses every 5 h, including column equilibration time. Analysis of a mixture of physiological standards (Fig. 4) shows good separation of nearly all amino acids enabling quantitative analysis of over thirty components. Proline, which is not shown in Fig. 4, was found to elute just prior to glycine and may be detected by post-column reaction with ninhydrin and spectrophotometric detection at 436 nm.

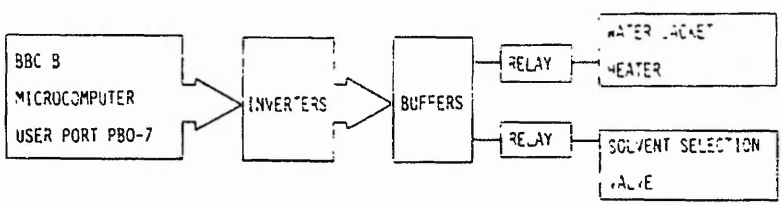

Fig. 2. Schematic diagram of the interface unit which allows the BBC microcomputer to control the water jacket heater and solvent selection valve.

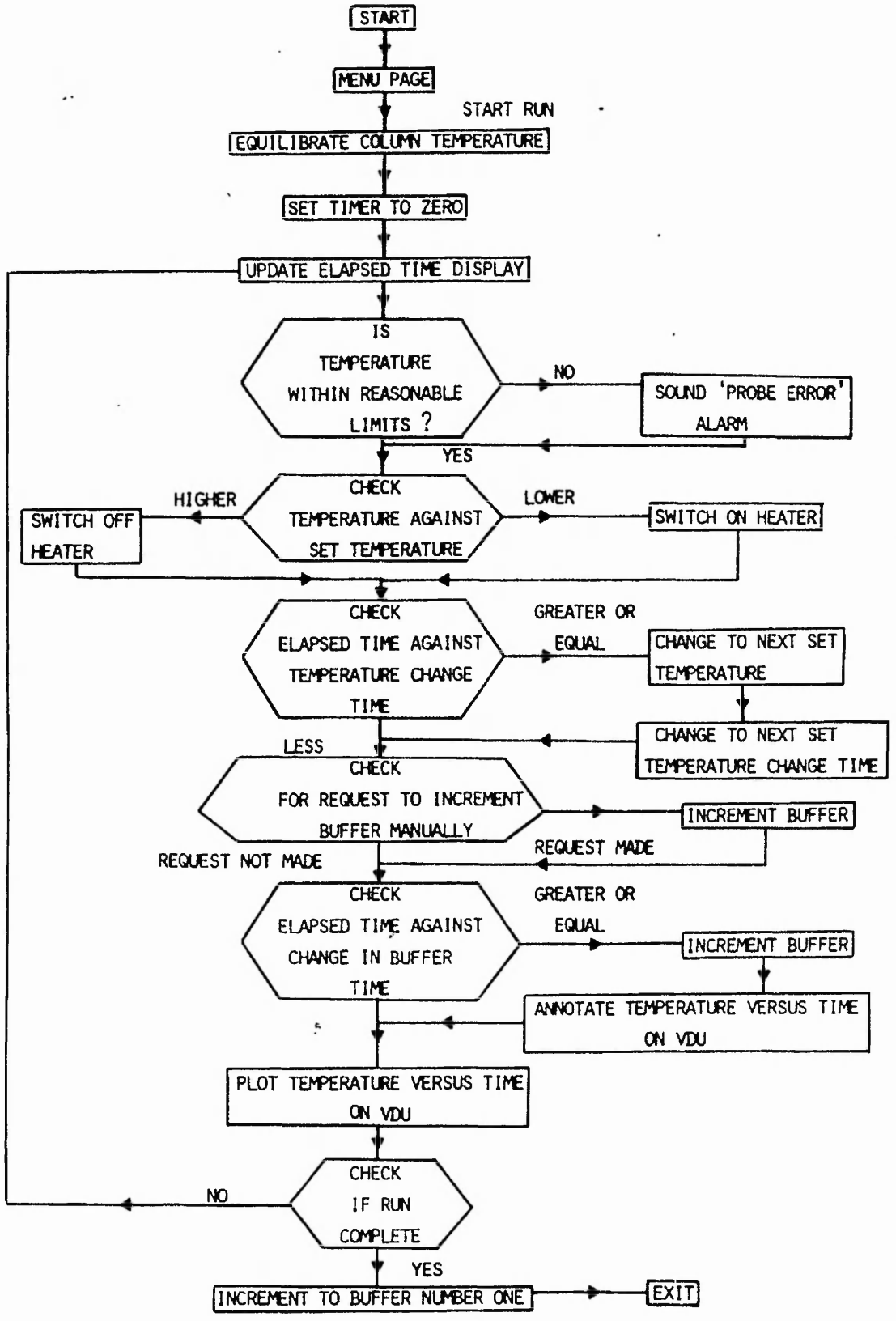

Fig. 3. Flow diagram demonstrating how the computer software allows selection and subsequent control of sequential elution conditions of the analyser system.

TABLE I

ELUTION CONDITIONS PROGRAMMED INTO THE BBC COMPUTER FOR SEPARATION OF AMINO ACI COMPONENTS PRESENT IN PHYSIOLOGICAL FLUIDS

Picobuffer	pH	Elution time (min)	Column temperature (°C)
1	2.0	38	40
2	2.08	30	40
3	2.1	22	40
4	2.25	16	60
5	2.25	38	60
	12.5	68	60
Lithium hydroxide (0.3 *M*)	12.5	30	60

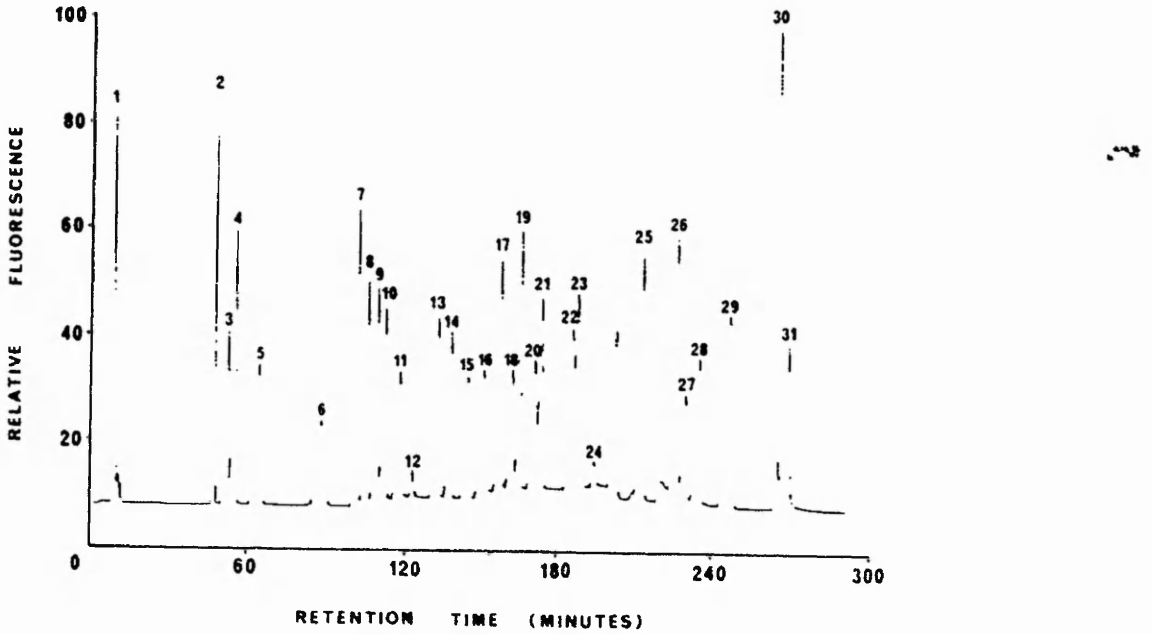

Fig. 4. Chromatogram showing typical separation of a standard physiological miture containing 10 nmol of each component in an injection volume of 100 μl. Elution conditions were those shown in Table I. Amino acid components were as follows: 1 = taurine; 2 = aspartate; 3 = threonine; 4 = serine; 5 = glutamate; 6 = α-aminoadipic acid; 7 = glycine; 8 = alanine; 9 = citrulline; 10 = γ-amino-N-butyrate; 11 = valine; 12 = cystine; 13 = methionine; 14 = L-cystathionine; 15 = isoleucine; 16 = leucine; 17 = norleucine; 18 = tyrosine; 19 = not determined; 20 = phenylalanine; 21 = β-alanine; 22 = DL-γ-amino-isobutyrate; 23 = γ-amino-N-butyrate; 24 = ammonia; 25 = ornithine; 26 = lysine; 27 = 1-methylhistidine; 28 = histidine; 29 = 3-methylhistidine; 30 = tryptophan; 31 = arginine; 32 = phosphoserine; 33 = glutamine.

Assessment of reproducibilities of column retention times and peak areas (Table II) was performed by applyng a series of physiological standards to the column. The coefficients of variation (C.V.) for retention times ranged from 0.3 to 4.0%, the majority of components having a C.V. of about 2.0%. Peak areas were more variable, the majority producing a C.V. of between 1 and 5%. Components

TABLE II

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS FOR SEPARATION OF AMINO ACIDS

The data represent the mean values from five separate experiments. Elution conditions were those described in Table I. Norleucine was used as an internal standard for calculation of peak areas.

Amino acid component	Retention time		Peak area
	Mean (min)	C.V. (%)	C.V. (%)
Phosphoserine	6.8	3.9	0.7
Taurine	11.3	2.0	1.4
Phosphoethanolamine	13.6	2.1	1.8
Aspartic acid	39.0	2.2	6.0
Threonine	51.5	1.9	5.5
Serine	54.7	1.7	5.2
Glutamic acid	59.8	0.6	4.9
α-Aminoadipic acid	88.4	3.7	7.3
Glycine	100.0	1.3	6.5
Alanine	103.6	2.6	4.8
Citrulline	106.3	2.3	2.8
Valine	116.5	2.8	4.8
Cystine	120.9	2.3	3.7
Methionine	129.7	2.4	2.3
Isoleucine	142.5	2.7	2.0
Leucine	148.7	2.4	3.3
Norleucine	153.9	1.7	-
Tyrosine	158.4	1.7	3.8
β-Alanine	168.1	1.6	2.3
Phenylalanine	170.1	1.9	3.3
Ammonia	192.9	1.0	6.7
Hydroxylysine	198.4	1.6	4.8
Ornithine	208.9	1.7	4.5
Lysine	222.5	1.8	3.2
1-Methylhistidine	227.5	1.8	5.6
Histidine	232.7	1.9	3.2
3-Methylhistidine	244.6	1.8	4.1
Tryptophan	253.9	0.29	4.9
Carnosine	255.2	0.47	1.3
Arginine	258.6	0.46	3.8

giving a notably high C.V. were α-aminoadipic acid and ammonia. Amino acid standars were used to assess the correlation between peak-area ratio and concentration. A chosen concentration range of between 1 and 12.5 nmol of each amino acid per 100-μl injection produced a linear relationship to peak-area ratio, for all amino acids tested. The quantitative detection limit of the system was 10 pmol, below which baseline noise interferes with poorly fluorescent derivatives. These results are consistent with other reports which have also demonstrated accurate detection down to these levels [14,15].

Chromatograms shown in Fig. 5A and 5B demonstrate the application of the

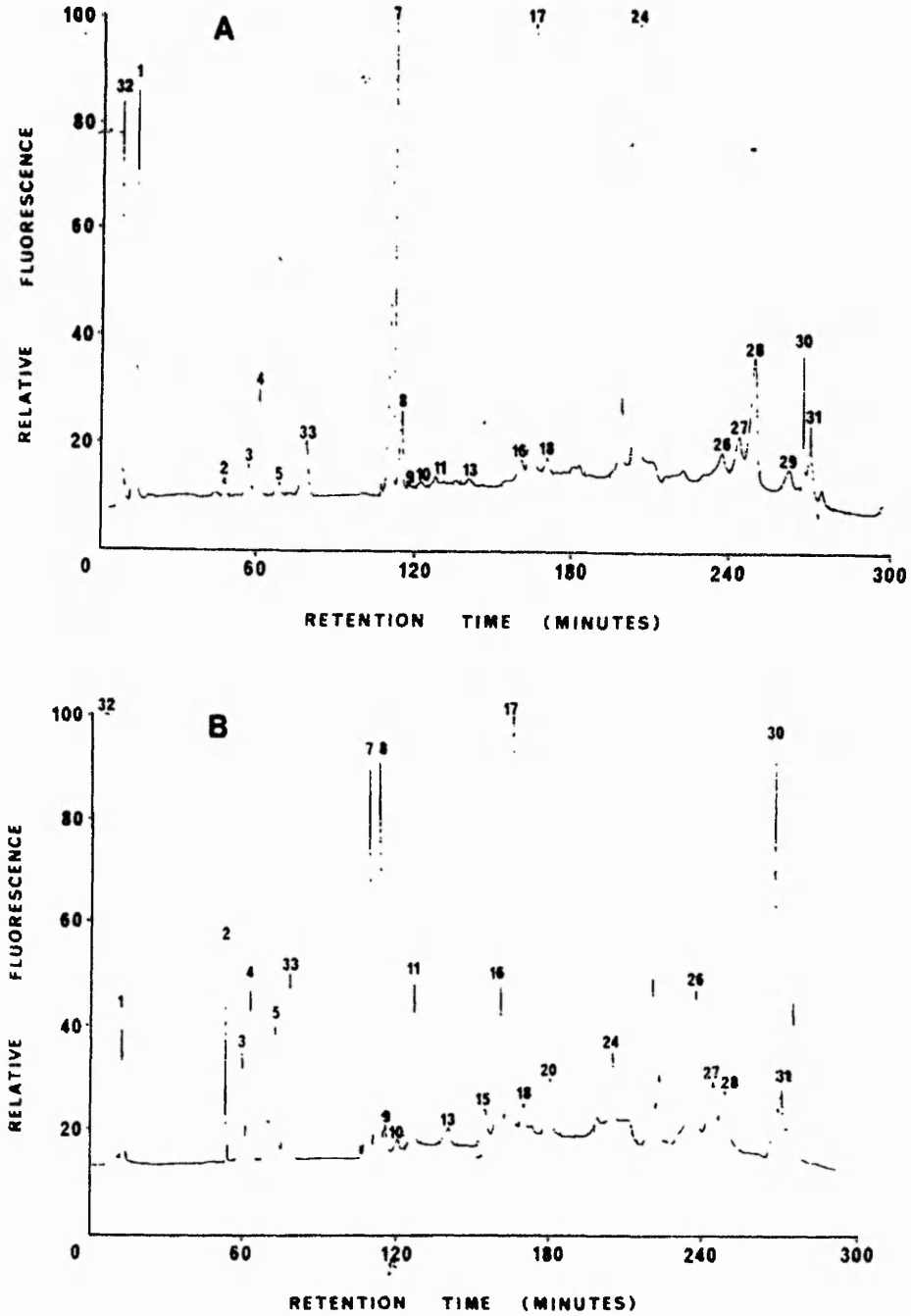

Fig. 5. Chromatogram showing separation of (A) normal human urine sample and (B) normal human serum sample. Fluorimeter sensitivity, injection volume and peak definitions are the same as those defined in the legend to Fig. 3. Urine amino acid levels ranged from 14 pmol (citrulline, peak 9) to 57 nmol (ammonia, peak 24).

system to analysis of human urine and serum, respectively. Comparison of the serum amino acid levels calculated from Fig. 5B with thosed quoted in the litea-ture (Table III) indicate good comparability thus demonstrating the reliability of this sytem. Only glutamine was found to be present at lower levels than those quoted which may be explained by the inherent instability of this amino acid in stored plasma [16]. A prominant ammonia peak found in urine is the most strik-ing difference between the two biological fluids. However, ammonia determina-

TABLE III

ASSESSMENT OF MEASUREMENT RELIABILITY

Comparison of serum amino acid levels calculated from Fig. 5B with those previously reported. Data after Ibbott [17].

Amino acid component	Calculated serum values (μM)	Normal serum ranges (μM)
Taurine	51	35–140
Aspartic acid	42	11– 54
Threonine	78	75–250
Serine	90	61–190
Glutamic acid	108	0–120
Glycine	240	130–490
Alanine	246	170–500
Citrulline	15	12– 55
γ-Amino-N-butyric acid	8	8– 35
Valine	123	120–330
Methionine	36	13– 39
Isoleucine	36	35–100
Leucine	72	69–160
Tyrosine	60	32– 87
Phenylalanine	63	34–120
Lysine	150	90–260
Histidine	60	56–120
Arginine	49	46–150
Glutamine	310	420–760

tion can only be assessed on a semi-quantitative basis in the present system due to buffer contamination which is evident in Fig. 5A where a broad raised plateau surrounds the sharper sample peak (peak 24). Incorporation of a suitable 'ammonia trap' in the system would overcome problems of contamination and allow quantitative determination of ammonia and compounds with a similar retention time if so required. A consistent feature or urine and plasma samples stored frozen for a period of time is a lar ge initial peak in the phosphoserine position. This may be a degradative product or an artifact produced during sample processing although this peak was not present in standard amino acid mixtures which underwent identical processing.

The system developed here is ideally suited to clinical and research laboratories whose sample turnover does not merit purchase of a ready-made fully automated ion-exchange analyser but who already possess HPLC equipment. Post-column derivatisation with OPA–2-ME reagent offers ease of derivatisation, increased sensitivity and selectivity of component detection compared to other methods. The main disadvantage of a fluorimetric detection system is that only primary amines can be analysed so that components such as proline, hydroxyproline and urea remain undetected. Proline and hydroxyproline may also be detected with OPA–2-ME if the column eluent is reacted with hypochlorite [14] prior to deri-

vatisation, although this would necessitate the use of a further pump. However, this system is equally adaptable to post-column derivatisation with ninhydrin if detection of these components is required.

In conclusion, the ion-exchange system for the analysis of amino acids which has been described can be simply and economically constructed for use with existing HPLC equipment. The versatility of the system is such that it will facilitate the rapid alteration of elution conditions for the analyses of complex mixtures or specific components which are presently difficult to analyse using existing reversed-phase HPLC methods.

NOTE

The program for the computer software described in this report may be obtained on request.

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SOCIETY FOR THE STUDY OF INBORN ERRORS OF METABOLISM

23rd ANNUAL SYMPOSIUM

LIVERPOOL, 4th to 6th SEPTEMBER 1985

ABSTRACTS OF FREE COMMUNICATIONS

ORAL PRESENTATIONS

POSTER DEMONSTRATIONS

URINE AMINO ACID ANALYSIS BY HPLC IN THE INVESTIGATION OF INBORN ERRORS OF METABOLISM

Jonathan M.Leah,Trevor Palmer,Martin Griffin,Anthony Briddon and Victor G. Oberholzer, Department of Life Sciences,Trent Polytechnic,Nottingham and Clinical Biochemistry Department,Queen Elizabeth Hospital for Children, London.

A method of amino acid analysis by reversed-phase HPLC using pre-column derivatization with o-phthalaldehyde/2-mercaptoethanol reagent has been applied to urine. A Zorbax C8 column was employed using a linear gradient from 20% to 90% methanol,including 2 isocratic stages. Gradients were formed from 2 solvents: potassium acetate buffer(0.02mol/l,pH 5.5)and methanol. Both solvents contained 1%(v/v) tetrahydrofuran. A flow-rate of 1.8ml/min was employed,eluting most amino acids within 25min. Immediately before use,250µl of o-phthalaldehyde reagent(60mg/10ml methanol) was added to 250µl 0.4mol/l boric acid buffer,pH 9.4,and 25µl 2-mercaptoethanol; 250µl of this mixture was vortex-mixed with 25µl prepared sample for 2 min.,and then a 10µl aliquot removed and applied to the column. For urine amino acid analysis,70-170µl urine was mixed with 1ml of methanol and centrifuged; 25µl supernatant was then treated with reagent as described above. Urine specimens from patients with a variety of inborn errors were analysed. These included hyperornithinaemia, hyperargininaemia, α-amino adipic aciduria, aspartyl glycosaminuria, argininosuccinic aciduria and xanthinuria with sulphite oxidase deficiency. Some specimens were from patients with hyperornithinaemia where an ornithine lactam(3-aminopiperid-2-one) had been detected previously by ion exchange chromatography. The lactam has,however, been found to co-elute with ammonia on the HPLC system. The procedure detects only substances with primary amino groups, so is more selective than conventional urine amino acid analysis employing post-column derivatization with ninhydrin. Hence the two techniques may be complementary. Elution patterns produced so far show that the reversed-phase HPLC method provides a rapid and convenient means of demonstrating abnormalites in urinary amino acids.

THE SOCIETY FOR THE STUDY OF INBORN ERRORS OF METABOLISM

SSIEM

ABSTRACTS OF THE 25th SSIEM ANNUAL SYMPOSIUM, 1987

held at Sheffield, 22–25 September

FREE COMMUNICATIONS

QUANTITATIVE AMINO ACID ANALYSIS: REVERSED-PHASE HPLC COMPARED TO IEC

Jonathan M.Leah, Trevor Palmer and Martin Griffin, Department of Life Sciences, Trent Polytechnic, Nottingham.

Quantitative amino acid analysis was carried out on standard solutions, as well as on plasma and urine specimens from normal individuals and patients with inborn errors of metabolism, by reversed-phase high performance liquid chromatography(HPLC) and ion exchange chromatography(IEC). The reversed-phase method employed pre-column derivatization with o-phthalaldehyde/2-mercaptoethanol(OPA/2-ME). Separation was carried out on a Zorbax C8 column(0.46 x 25 cm, 7 µm particle size) with a two-solvent system, based on potassium acetate buffer and methanol, employing a gradient from 20% to 90% methanol. The IEC system employed post-column derivatization with OPA/2-ME. Separation was carried out on a PC-4A cross-linked sulphonated polystyrene resin in a stainless steel column(0.3 x 35 cm), with sequential elution by five discrete lithium Picobuffers operating from 40°C to 60°C, programmed by means of a 32K BBC microcomputer interfaced with a buffer selection valve and a thermostatically controlled water jacket surrounding the column. Both systems gave coefficients of variation for retention time and peak area of under 5%, with very few exceptions. The reversed-phase method had the advantage of rapidity(25 minutes compared to 5 hours analysis time) and sensitivity(1 picomole compared to 10 picomole quantitative detection limit), but because of co-elution of some components was less suited than IEC to the separation and quantitative determination of amino acids in complex biological fluids.