

This thesis is dedicated to

Ody, Chinenye and Ada.

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CHARACTERIZATION OF MULTIPLE FORMS
OF γ -GLUTAMYL TRANSFERASE
IN HUMAN LIVER, BILE AND SERUM

A Thesis
submitted for the degree of
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by

Zeinep Omirdjanovna Echetebe, M. Sc.

Royal Postgraduate Medical School,
London

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ABSTRACT

In recent years the determination of the activity of γ -glutamyl transferase (EC 2.3.2.2) in human serum has become important in the investigation of diseases of the hepatobiliary system. The increased enzyme activity in serum is believed to originate from the liver, but the way in which release of the enzyme molecules into plasma takes place is not known. Many workers have shown γ -glutamyl transferase activity in both normal and pathological sera to be heterogeneous, with respect to both charge and size, although the causes of this heterogeneity and its possible pathological significance are unknown.

This thesis describes the characterization of the multiple forms of γ -glutamyl transferase in serum and bile, and from liver tissue, in an attempt to elucidate their origins and nature.

The experimental work falls into three main parts: (I) observations on the multiple forms of γ -glutamyl transferase in serum, bile and extracts of liver which are separable by size- and charge-dependent means, and their occurrence in serum specimens in health and disease; (II) studies of catalytic and other properties of the various forms thus identified, and of the enzyme partially purified from human liver, and (III) attempts to reproduce experimentally the various components identified in serum, by incubating human liver tissue in serum and other fluids.

The various fractions of γ -glutamyl transferase were found to be virtually indistinguishable in their catalytic properties. The differences between them in other respects seem consistent with the view that they reflect various states of aggregation, or complexing with non-enzymic components, of a single form of the enzyme.

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My thanks to Mrs. Katrine Whitaker and many other members of the Department of Chemical Pathology for useful advice and encouragement during the period of this project.

I wish to thank Mr. A. McPherson of the Department of Surgery, Hammersmith Hospital, for providing me with samples of human bile and Mr. O. Traynor of the same department for obtaining samples of blood for the liver metastases study.

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CHAPTER 1

GENERAL INTRODUCTION

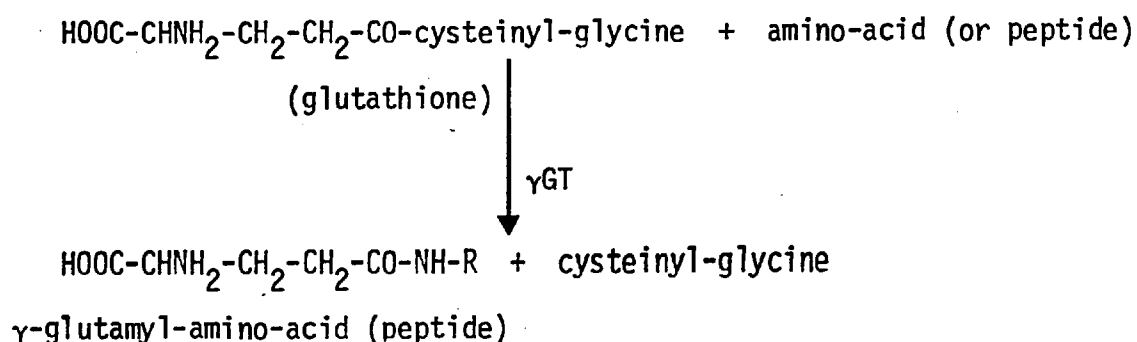
CHAPTER 1

GENERAL INTRODUCTION

γ -Glutamyl transferase [$(\gamma$ -glutamyl)-peptide:amino-acid γ -glutamyl transferase (EC 2.3.2.2)] was first described by Hanes, Hird and Isherwood in 1950. The enzyme has been widely referred to as γ -glutamyl transpeptidase although γ -glutamyl transferase is now the preferred trivial name. Clinical interest in this enzyme has grown particularly over the last decade. In 1960, Goldbarg et al. and Szewczuk and Orlowski demonstrated the presence of γ -glutamyl transferase activity in normal human serum and its changes in liver disease. The works of Orlowski and Meister (1963), Szasz (1969) and Rosalki and Rau (1972), led to the recognition of the importance of γ -glutamyl transferase in the investigation of liver disease and to the routine estimation of its activity in serum in laboratories all over the world.

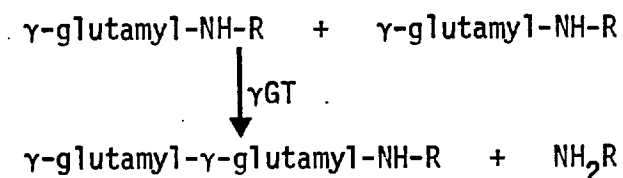
1.1 Catalytic action of γ -glutamyl transferase

An important reaction catalyzed by the enzyme is transfer of a glutamyl residue from its γ -linkage in glutathione or other γ -glutamyl peptides to L-amino acids or peptides to form a new γ -glutamyl peptide and cysteinyl glycine:



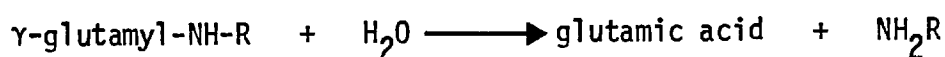
In most biological systems glutathione is the glutamyl donor, but other peptides or amino-acids as well as synthetic substrates with a glutamyl residue attached through its γ -carboxyl group can also serve as donor substrates for the enzyme, i.e., the enzyme is not very specific.

γ -Glutamyl transferase can also catalyze a reaction of auto-transpeptidation in which the γ -glutamyl group is transferred from one molecule of substrate to another molecule of it to form a γ -glutamyl- γ -glutamyl peptide:



The presence of an α -methyl group attached to the γ -glutamyl residue was shown to prevent enzymatic transfer of the L- α -methyl- γ -glutamyl residue to the amino group of the substrate (L- α -methyl- γ -glutamyl-L- α -aminobutyrate) itself, i.e. to eliminate the autotranspeptidation reaction (Karkowsky, Bergamini and Orlowski, 1976). Auto-transpeptidation was also precluded when D- γ -glutamyl-p-nitroanilide had been used as the γ -glutamyl donor (Thompson and Meister, 1977).

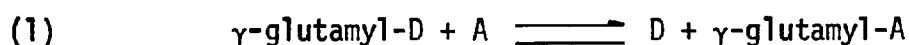
A third type of reaction catalyzed by γ -glutamyl transferase is transfer of a glutamyl residue to water (hydrolysis) with formation of free glutamic acid and the amino acid or peptide to which the γ -glutamyl residue was attached initially:



When suitable acceptors of the γ -glutamyl-group are added to the reaction mixture in increasing concentrations, the hydrolytic reaction is progressively inhibited in favour of transpeptidation (Hird and Springell, 1954).

The action of γ -glutamyl transferase is consistent with the "ping-pong bi-bi" kinetic mechanism. That this is the mechanism of action of the enzyme has been shown by several workers for γ -glutamyl transferase obtained from various sources (Tate and Meister, 1974a and 1974b; Elce and Broxmeyer, 1976; Karkowsky, Bergamini and Orlowski, 1976; Karkowsky and Orlowski, 1978; London et al., 1976; Strømme and Theodorsen, 1976; Thompson and Meister, 1977 and 1979; Curthoys and Hughey, 1979).

The overall reaction catalyzed by γ -glutamyl transferase is:



which is a two-substrate reaction, where D and A are the donor and acceptor substrates of γ -glutamyl group.

The kinetics of this enzyme are often complicated since the reaction of γ -glutamyl transfer (transpeptidation) can take place simultaneously with auto- γ -glutamyl transfer (autotranspeptidation) and hydrolysis.

In enzymic reactions with two substrates the binding of the substrates to the enzyme molecule can take place in different ways. Mechanisms where both the substrates must attach to the enzyme with the formation of a ternary complex before any products are released are called

"sequential". These mechanisms can be either "random", when either of the substrates bind to the enzyme non-selectively, or "ordered", if the substrates add in a definite sequence. In some two-substrate enzymic reactions no ternary complex is formed and one or more products are released before all substrates have bound to the enzyme: i.e., products and substrates alternate in their binding and release from the enzyme, and during the reaction, the enzyme will oscillate between two or more stable forms, acting as a transfer agent. Such mechanisms are known as "ping-pong" mechanisms. They can be further described by syllables Uni, Bi, Ter, Quad, etc., depending on the number of reactants involved in the reaction and the number of products released. Thus a reaction with two substrates and two products proceeding by the "ping-pong" mechanism, such as reaction (1) catalyzed by γ -glutamyl transferase, will be called "ping-pong bi-bi". The general rate equation of a two-substrate enzymic reaction of the double-displacement "ping-pong" type ($A + B \rightleftharpoons P + Q$) is:

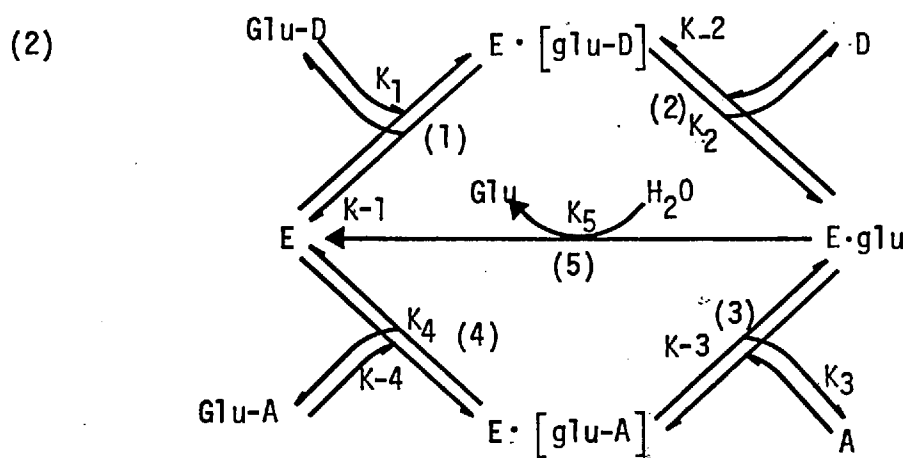
$$V_0 = \frac{V_{\max}}{1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]}}$$

where V_0 is the initial reaction velocity, $V_{\max}$ is the maximum velocity, $[A]$ and $[B]$ are concentrations of substrates A and B, respectively, and K_m^A and K_m^B are the Michaelis constants of these substrates.

On the basis of kinetic studies it has been postulated that an activated covalent γ -glutamyl-enzyme intermediate is formed in both hydrolysis and transfer reactions and that this intermediate reacts with

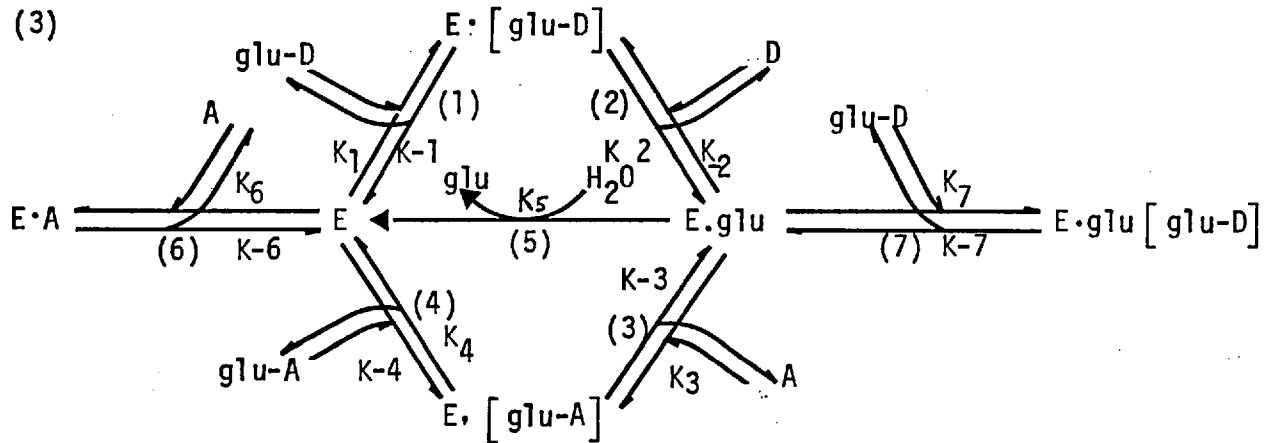
an amino acid (peptide) or water to yield the reaction products. The presence of the intermediate was chemically demonstrated (Elce and Broxmeyer, 1976) and later γ - $[^{14}\text{C}]$ glutamyl-enzyme intermediate has been isolated (Elce, 1978). The nature of the γ -glutamyl-enzyme bond and active site amino acid residues in rat kidney γ -glutamyl transferase have been recently investigated (Elce, 1980).

A scheme representing the proposed mechanism of action of γ -glutamyl transferase can be illustrated as follows:



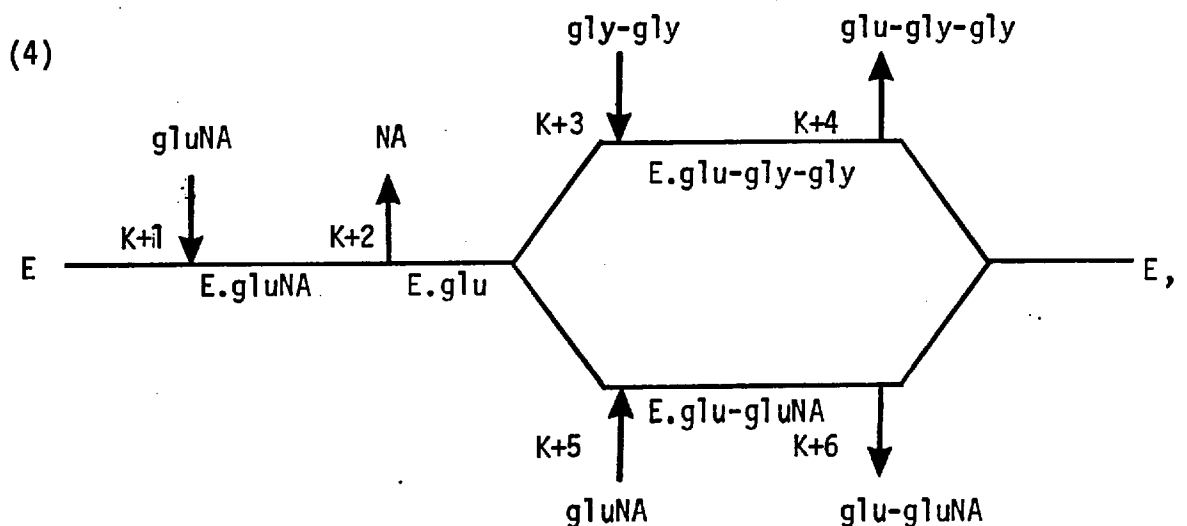
E is the enzyme, glu-D is the γ -glutamyl donor substrate, D is the first product of the reaction, E.glu is the γ -glutamyl-enzyme complex, A is the γ -glutamyl acceptor substrate, glu-A is the γ -glutamyl amino acid or peptide product and glu is glutamate formed in the hydrolytic reaction.

This scheme is consistent with the "ping-pong bi-bi" mechanism modified by a hydrolytic shunt. When γ -glutamyl-p-nitroanilide and glycylglycine are used as the donor and the acceptor of γ -glutamyl group, double competitive substrate inhibition is observed (Strømme and Theodorsen, 1976). Scheme (2) could probably be modified to include this double competitive substrate inhibition:



Scheme (3) takes into account the fact that the γ -glutamyl-donor substrate besides reacting with the free enzyme, may also combine with the γ -glutamyl-enzyme complex (reaction 7), and that the acceptor substrate besides reacting with such a complex may also react with the free enzyme E (reaction 6).

When γ -L-glutamyl-p-nitroanilide and glycylglycine are used as the donor and acceptor substrates for determination of γ -glutamyl transferase activity, the reaction may proceed by the following scheme (London et al., 1976):



where E is γ -glutamyl transferase, gluNA is γ -L-glutamyl-p-nitroanilide, gly-gly is glycylglycine, NA is p-nitroaniline.

γ -Glutamyl transferase has a low specificity for L- and D- γ -glutamyl donor compounds, at the same time it exhibits strict L-stereospecificity for the acceptor (Tate and Meister, 1974a; Thompson and Meister, 1976 and 1977; Goldberg, 1979). Partially and highly purified preparations of γ -glutamyl transferase from various sources exhibit similar specificities towards the γ -glutamyl donor and acceptor (Tate and Meister, 1975). When the heavy and light forms of the enzyme from rat kidney, which had virtually identical specificities, were incubated with 2.5 or 5 mmol/l γ -glutamyl donor and 20 mmol/l acceptor, the best amino acid acceptors were neutral amino acids, such as the L-isomers of glutamine, methionine, asparagine, serine and alanine. The branched-chain amino acids and threonine, aspartate and histidine were comparatively less active (Tate and Meister, 1974a and 1975). L-Proline, various D-amino acids, and α -substituted amino acids were shown to be inactive. When the sparingly soluble amino acids, L-tyrosine and L-cystine were studied as acceptors at about 0.5 mmol/l concentrations, L-tyrosine was only slightly active while L-cystine proved to be a very good acceptor of the γ -glutamyl group and was found to be more active than L-cysteine (Thompson and Meister, 1975). A number of dipeptides were active as acceptors, and several of these were shown to be more active than L-glutamine, e.g., glycylglycine, glycyl-L-alanine, L-methionyl-L-serine, L-glutamyl-L-glutamine, and L- α -aminobutyryl-glycine (Tate and Meister, 1974a). Peptides containing D-amino acids or more than two amino acid residues were only slightly active or inactive. The effects of amino acids and glycylglycine on γ -glutamyl transferase from rat kidney were rather similar to their effects on enzymes from other sources (Sobiech, Ziomek and Szewczuk, 1974;

Karkowsky, Bergamini and Orlowski, 1976; Miller, Awasthi and Srivastava, 1976; Danner et al., 1978; Shaw, London and Petersen, 1978; Jaken and Mason, 1979).

The specificity of the enzyme for γ -glutamyl donors is also rather broad. Compounds of γ -glutamyl with many amino acids were found to be active as γ -glutamyl donors, the most active being L- γ -glutamyl-L-glutamine and L- γ -glutamyl-L-methionine. Glutathione analogues, such as S-methylglutathione and ophthalmic acid (γ -glutamyl-2-aminobutyryl-glycine), and S-substituted glutathione derivatives were active as γ -glutamyl donors, and several of the S-substituted glutathiones were more active in transpeptidation than glutathione (Tate and Meister, 1974a). Synthetic γ -glutamyl substrates, upon which γ -glutamyl transferase acts similarly to glutathione have been used for assaying γ -glutamyl transferase activity and for histochemical studies (see Chapter 2).

1.2 Occurrence of γ -glutamyl transferase

γ -Glutamyl transferase has been purified from several sources including larvae of *Musca domestica* L. (Bodnaryk and Skillings, 1971), cow's milk (Sobiech, Ziomek and Szewczuk, 1974), rat kidney (Tate and Meister, 1974a and 1975; Elce and Broxmeyer, 1976; Hughey and Curthoys, 1976), normal and cancerous rat mammary tissue (Jaken and Mason, 1978 and 1979), rat hepatoma (Taniguchi, 1974; Szewczuk, Milnerowicz and Sobiech, 1978), rat seminal vesicles (De Lap, Tate and Meister, 1975), hog kidney (Orlowski and Meister, 1965; Leibach and Binkley, 1968), sheep kidney (Karkowsky et al., 1976), bovine kidney (Szewczuk and Baranowski, 1963), bovine colostrum (Yasumoto et al., 1978), bovine ciliary body, liver, spleen and brain (Das and Shichi, 1979), normal human kidney (Richter, 1969; Miller, Awasthi and Srivastava, 1976; Tate and Ross, 1977; Hada

et al., 1978), human renal carcinoma (Hada et al., 1978), human liver (Huseby, 1977; Shaw, London and Petersen, 1978), human bile (Indirani and Hill, 1977), human seminal fluid (Krisnaswamy, Tate and Meister, 1977).

(a) γ -Glutamyl transferase activity in various tissues

Of all human tissues, the highest γ -glutamyl transferase activity is found in kidney, then in decreasing order of concentration, in prostate gland, pancreas, liver, small intestine, testes, spleen and lungs. The proportions of γ -glutamyl transferase activity in aqueous extracts of human kidney, prostate, pancreas and liver are, according to Rosalki (1975), 100:30:15:10, respectively. The enzyme is also present in the lactating breast, brain, bowel, placenta and thyroid homogenates (Goldberg et al., 1960; Szczeklik, Orlowski and Szewczuk, 1961; Orlowski, 1963; Orlowski and Meister, 1970b; Rosalki and Rowe, 1973; Goldberg and Martin, 1975; Rosalki, 1975). The enzyme was first histochemically demonstrated in tissue sections of some animals and human beings with γ -L-glutamyl- α -naphthylamide as substrate (Albert, Orlowski and Szewczuk, 1961). The liberated α -naphthylamine was coupled with a diazonium salt, Fast Garnet GBC, to form an azo-dye.

γ -Glutamyl transferase is a glycoprotein which occurs in animal tissues mainly in a membrane-bound form. Szewczuk (1966) has shown that when human liver or kidney homogenates are centrifuged at 100,000 xg for 30 minutes, approximately 90% of the γ -glutamyl transferase activity is sedimented ("insoluble" form) and only about 10% is recovered in the supernatant ("soluble" form). To solubilize γ -glutamyl transferase from particulate fractions of tissue homogenates, the use of detergents, such

as deoxycholate, Triton X-100 and Lubrol WX, and n-butanol has been suggested (Binkley, 1961; Orlowski and Meister, 1965; Tate and Meister, 1975). The enzyme activity released from membranes by the action of detergents is usually present in "heavy" forms of over 200,000 molecular weight (DeLap, Tate and Meister, 1975; Tate and Meister, 1975; Miller, Awasthi and Srivastava, 1976; Hughey and Curthoys, 1976; Zelazo and Orlowski, 1976; Indirani and Hill, 1977; Elce, 1980). The detergent-extracted preparations of γ -glutamyl transferase are not easily soluble in the absence of detergents. Treatment of such high molecular weight forms with proteolytic enzymes produces smaller molecular weight (50,000-90,000) forms of the enzyme (Leibach and Binkley, 1968; Curthoys and Kuhlenschmidt, 1975; DeLap, Tate and Meister, 1975; Tate and Meister, 1975; Hughey and Curthoys, 1976; Miller, Awasthi and Srivastava, 1976; Zelazo and Orlowski, 1976; Huseby, 1977; Hada et al., 1978; Horiuchi, Inoue and Morino, 1978; Yasumoto et al., 1978; Jaken and Mason, 1979).

In the kidney, γ -glutamyl transferase has been located histochemically in the brush border of the epithelial cells that line the proximal convoluted tubules and in the lumen of the loops of Henle (Albert, Orlowski and Szewczuk, 1961; Albert et al., 1964; Wendel et al., 1978). Since the kidney is rich in γ -glutamyl transferase it is not surprising that most of the preparations of the enzyme have been made from renal tissue.

In lamb, pig and human liver, γ -glutamyl transferase has been located in the canaliculi of parenchyma, and the greatest concentration was found in the luminal borders of the epithelial cells lining the fine biliary ductules. Small amounts of activity were demonstrated in the

periportal hepatic cells (Glenner, Folk and McMillan, 1962; Albert et al., 1964; Aronsen et al., 1969; Naftalin et al., 1969b).

In rat liver, very low γ -glutamyl transferase activity was located in the walls of bile canaliculi and in single cells of the reticulo-endothelial system; in rabbits, the enzyme was found in the form of granules in the cytoplasm of liver cells mostly around the nucleus. In mouse and hamster liver, γ -glutamyl transferase activity was not detectable histochemically (Albert et al., 1964).

Although in normal human liver γ -glutamyl transferase activity is quite low, the enzyme activity is increased in hepatic tissue of patients with malignant tumour growth (Aronsen et al., 1969). It is also higher in intoxicated and pathologic states of the organ (Gibinski, Grzybek and Zmudzinski, 1963; Kokot et al., 1965; Schmidt and Schmidt, 1971; Takebayashi et al., 1972), as well as in foetal human liver (Albert, Rzucidlo and Starzuk, 1970) and after obstruction (Naftalin et al., 1969b; Aronsen, Hagerstrand and Norden, 1972). In foetal and regenerating rat liver, γ -glutamyl transferase was shown to be rich in sialic acid. This form of the enzyme is replaced by a sialic acid-poor enzyme in the adult rat liver (Köttgen, Reutter and Gerok, 1976 and 1978).

In human beings γ -glutamyl transferase has also been demonstrated histochemically in the pancreatic acini in the cells lining the pancreatic ducts, in the bile duct and common hepatic duct (Glenner, Folk and McMillan, 1962; Albert et al., 1964; Naftalin et al., 1969b), as well as in the intestine, with the highest activity in the jejunum, then, in decreasing order, in ileum and duodenum (Kokot, Kuska and Grzybek, 1965; Greenburg et al., 1967; Rosalki, 1975).

The enzyme activity has also been located in the epithelial lining cells of the following tissues: prostate gland, seminal vesicles, the ovary, Fallopian tubes, the endometrial glands of the uterus, glandular cells of the breast (Glenner, Folk and McMillan, 1962; Albert et al., 1964).

(b) γ -Glutamyl transferase in human body fluids

γ -Glutamyl transferase is normally found in human plasma, urine, cerebrospinal fluid, bile and seminal fluid.

γ -Glutamyl transferase in blood. An enzyme which catalyzes the hydrolysis of glutathione was found in blood serum by Woodward as early as 1939. Other authors described the presence in blood serum of an enzyme capable of hydrolyzing α -(N- γ -DL-glutamyl)-aminonitriles (Szewczuk and Orłowski, 1960); they suggested the use of this enzyme in the diagnosis of some pathological conditions in which its activity was raised.

The range of normal values of γ -glutamyl transferase in serum depends on the method used for the estimation of its activity. It has been found that the use of γ -L-glutamyl-p-nitroanilide as a substrate together with glycylglycine, which acts as an activator/acceptor for γ -glutamyl group and as a buffer at the same time, allows for the most easy, rapid and economical method (Dimov and Kulhanek, 1967). The normal values for serum γ -glutamyl transferase activity in men are on average about 1.5 times higher than those in women (Goldbarg et al., 1963; Rosalki, 1975). It has been suggested that the lower values in women may represent a hormonal effect (Orłowski, 1963) and that the higher

activities of γ -glutamyl transferase in the serum of men may arise from the high levels in the male reproductive tract (Rosalki and Rowe, 1973).

When γ -glutamyl transferase activity in serum is determined by the Scandinavian method (see "Materials and Methods"), the upper limit of normal for men is taken as 50 I.U./l and for women as 30 I.U./l. The method of Rosalki and Tarlow (1974) gives values which are higher by about 4% (Hopkins and Moss, 1979).

γ -Glutamyl transferase activity in the cord blood and in the blood of newborn babies is about five times higher than the upper limit of normal for adults. The high values of the enzyme in newborns gradually, within the first year of life, fall to a level of activity that is about 25-50% lower than in adults (Rosalki, 1975). No sex difference is observed for γ -glutamyl transferase activity in the blood of children until they reach puberty.

Some authors reported normal serum values of the enzyme during pregnancy (Goldbarg et al., 1963; Lum and Gambino, 1972), some found a small progressive increase in γ -glutamyl transferase activity as pregnancy advanced (Jones, Williams and Prochazka, 1974), but others have shown that the enzyme levels in serum significantly decrease with advancing pregnancy (Rosalki, 1975).

γ -Glutamyl transferase in serum is quite stable. After storage at -20°C for one year only 0-5% of its activity was lost; it was equally stable after 30 days at 6°C and after a week at 20°C . However, exposure of serum to 65°C for 1 minute leads to complete loss of the enzymatic activity (Goldbarg et al., 1963; Szasz, 1969). γ -Glutamyl transferase

activities in serum and in heparinized plasma are not significantly different. EDTA does not inhibit the enzyme activity and haemolysis of blood samples does not affect it either, since there is virtually no γ -glutamyl transferase in red blood cells (Szewczuk and Orłowski, 1960; Orłowski, 1963; Goldberg et al., 1963). If the sample is very haemolyzed, however, it may be necessary to dilute it, so that its colour does not interfere with the enzyme activity measurements.

Since kidney, pancreas and liver are rich in γ -glutamyl transferase, the enzyme in serum could, in theory, originate from any or all of them. However, γ -glutamyl transferase activity does not change very much in the diseases of the kidney, and this makes the kidney a not very probable source of the serum enzyme. γ -Glutamyl transferase activity in serum is elevated in the diseases of the liver, biliary tract and the pancreas, with the greatest changes observed in liver disease and the smallest elevation of activity in acute pancreatic disease (Szczeklik, Orłowski and Szewczuk, 1961; Goldberg and Martin, 1975). These observations suggest that the liver is the main source of the enzyme in serum (Gibinski and Aleksandrowicz, 1965; Szewczuk, 1966; Rosalki, 1975).

γ -Glutamyl transferase in urine. The activity of γ -glutamyl transferase in urine is up to about six times higher than in serum (Orłowski and Szewczuk, 1962; Szasz, 1970; Rosalki, 1975). Orłowski and Szewczuk (1962) and Levy and Dubach (1972) found approximately equal γ -glutamyl transferase activity in the male and female urines. The enzyme in urine was found to be stable for about 24 hours at room temperature and longer at 4°C (Orłowski, 1963; Szasz, 1970; Beck and Sammons, 1975). But activity was lost rapidly when urine was frozen;

dialysis prior to freezing was found to prevent this loss, and the major factor responsible for the loss of urinary γ -glutamyl transferase activity is thought to be urea (Beck and Sammons, 1975).

γ -Glutamyl transferase activity levels in urine and plasma do not appear to be correlated (Orlowski, 1963; Lehman, Prentice and Rosalki, 1970; Szasz, 1970). The enzyme activity in urine is elevated in acute renal disease. Szewczuk (1966) found similarities in properties of the kidney and urine γ -glutamyl transferase, such as the occurrence of a soluble form of the enzyme in both the kidney and urine with molecular weight between 80,000 - 90,000, its electrophoretic behaviour before and after treatment with neuraminidase and inactivation of the soluble forms from both sources by inhibitors; this author therefore suggested that γ -glutamyl transferase in urine originates from kidney tissue. Furthermore, the electrophoretic pattern of the enzyme in urine was found to be different from that of the serum and liver enzymes (Szewczuk, 1966; Jacyszyn and Laursen, 1968; Kuska and Kokot, 1970). Gel-filtration (Sephadex G-200) patterns of serum and urine were also different (Jacyszyn and Laursen, 1968). Rosalki (1975) proposed that γ -glutamyl transferase in normal urine arises by the process of "wear and tear" breakdown of renal tract cells, rather than excretion of the plasma enzyme. In pathological urines, the presence of the "insoluble" (Szewczuk, 1966) high-molecular weight form of γ -glutamyl transferase may indicate cell destruction in the kidney (Jacyszyn and Laursen, 1968).

γ -Glutamyl transferase in cerebrospinal fluid. Very slight γ -glutamyl transferase activity was found in normal cerebrospinal fluid

using γ -glutamyl- α -naphthylamide and glycylglycine as the donor and acceptor substrates after prolonged incubation (up to 24 h); even in pathological cerebrospinal fluids the enzyme activity remained low (Swinnen, 1967). In a later study, no detectable γ -glutamyl transferase activity was found in the cerebrospinal fluids of 30 patients, 10 of whom had epilepsy with elevated activity of the enzyme in serum, 5 had other neurologic disease, and 15 of whom had non-neurologic disease (Ewen and Griffiths, 1973).

γ -Glutamyl transferase in bile. The presence of γ -glutamyl transferase activity in bile has been reported with levels which are up to 150 times higher than those found in normal serum (Orlowski, 1963; Kuska, 1965; Indirani and Hill, 1977). It has been suggested that biliary γ -glutamyl transferase originates in the cells of the liver and biliary tract by the process of wear and tear and breakdown of the cells (Szczeklik, Orlowski and Szewczuk, 1961; Orlowski, 1963; Rutenburg, Goldbarg and Pineda, 1963).

γ -Glutamyl transferase in seminal fluid. Rosalki and Rowe (1973) reported the presence of very high levels of γ -glutamyl transferase activity (up to 300 times the upper limit of normal for male blood serum) in human seminal fluid plasma. High levels of the enzyme were observed in washed sperm extracts, and sperm, rather than prostate, epididymus and seminal vesicles, were proposed to be possible sites of its origin (Rosalki, 1975; Krishnaswamy, Tate and Meister, 1977). γ -Glutamyl transferase from human seminal fluid plasma has been purified and its properties studied by Krishnaswamy, Tate and Meister (1977); these authors reported that the enzyme is present in seminal fluid plasma in a

particulate form. No correlation was found between the seminal plasma enzyme activity and fertility (Rosalki and Rowe, 1977).

1.3 Physiological role of γ -glutamyl transferase

The physical characterization of γ -glutamyl transferase purified from different sources indicates that it is an intrinsic membrane glycoprotein. The enzyme was shown to consist of two subunits (Tate and Meister, 1975 and 1976; Hughey and Curthoys, 1976; Huseby, 1977; Tate and Ross, 1977; Horiuchi, Inoue and Morino, 1978; Yasumoto et al., 1978), with the γ -glutamyl binding site located on the smaller subunit. The enzyme is believed to be located on the outer surface of a variety of cells (Novogrodsky, Tate and Meister, 1976; Inoue, Horiuchi and Morino, 1977; Hahn, Wendel and Flohe, 1978; Curthoys and Hughey, 1979). γ -Glutamyl transferase is found in membrane structures which are primarily involved in absorption or secretion, such as the epithelium of the pancreas, choroid plexus, testis, jejunal villi, ciliary body, bile duct and epididymus (Glenner, Folk and McMillan, 1962; Albert, Orłowski and Szewczuk, 1961; Greenburg et al., 1967; Ross et al., 1973).

γ -Glutamyl transferase is the only enzyme known to be capable of cleaving the γ -glutamyl bond of its putative natural substrate, glutathione. The biological role of γ -glutamyl transferase may therefore reflect the functions of glutathione.

The wide distribution of this enzyme together with its lack of substrate specificity led to proposals that γ -glutamyl transferase may participate in peptide-nitrogen storage and protein synthesis, and in the transport of amino acids across cellular membranes. A γ -glutamyl

cycle as a possible transport system for amino acids has been proposed by Orłowski and Meister (1970a). The ability of the enzyme to catalyze the transfer of the γ -glutamyl to water has suggested other possible biological functions of γ -glutamyl transferase, e.g., degradation of extra-cellular glutathione, with γ -glutamyl transferase acting as glutathionase (Elce and Broxmeyer, 1976; Hahn, Wendel and Flohe, 1978; Hughey and Curthoys, 1979), and biosynthesis of mercapturic acids (Boyland and Chasseaud, 1969; Hughey et al., 1978).

Peptide nitrogen storage and protein synthesis

Glutathione has been suggested to function as an intermediate between free amino acids and proteins, transferring amino acids or functioning as a regulating mechanism (Waelch and Rittenberg, 1941).

The cysteinyl-glycine linkage in glutathione is not hydrolyzed unless the γ -glutamyl group is first removed either by transfer or by hydrolysis (Binkley and Nakamura, 1948). The presence of the γ -glutamyl group is said to stabilize the cysteinyl-glycine linkage making the whole peptide resistant to the hydrolysis by peptidases. It was therefore proposed that glutathione may act as a stabilized reserve of α -aminoacyl peptide linkages and that when the γ -glutamyl group is removed, such linkages become available for the synthesis of other peptides and proteins (Hanes, Hird and Isherwood, 1952). The transfer of γ -glutamyl groups to other amino acids or peptides may start off yet another group of transpeptidation reactions (Hanes, Dixon and Connell, 1954). Thus, γ -glutamyl transferase may play a role in protein synthesis through its action on glutathione.

Amino acid transport; the γ -glutamyl cycle

The transport of amino acids across cellular membranes is generally thought to be a multistep process in which a membrane-bound component, probably a protein, interacts with the molecule to be transported. This step is followed by translocation in which the amino acid (or an amino acid-carrier complex) moves to the inside of the cell; then dissociation or release of the amino acid from the carrier occurs. It has been shown that active transport requires expenditure of energy (Stern et al., 1949; Christensen and Riggs, 1952; Jervis and Smyth, 1960; Tenenhouse and Quastel, 1960).

The kidney, the intestine and the brain are active in amino acid transport. The localization of γ -glutamyl transferase in these and some other tissues led to a proposal of the participation of the enzyme in the transport of amino acids across cellular membranes and absorption (Ball, Cooper and Clarke, 1953; Binkley, 1954; Ball, Revel and Cooper, 1956). In the kidney, where it is located in the brush border of epithelial cells which line the proximal convoluted tubules and loops of Henle (Albert, Orlowski and Szewczuk, 1961; Glenner, Folk and McMillan, 1962; Orlowski and Meister, 1970c), γ -glutamyl transferase may be responsible for absorption of amino acids from glomerular filtrate with the formation of γ -glutamyl peptides. Compared to the colon and the stomach, the levels of the enzyme are much higher in the intestine, where it is located in the surface epithelial cells (Goldbarg et al., 1960; Kokot, Kuska and Grzybek, 1965). Such distribution confirms the possibility that γ -glutamyl transferase may be involved in the transport and absorption of amino acids. Studies on γ -glutamyl transferase localization in the

central nervous system of man and different animal species have established the presence of the enzyme in the epithelium of choroid plexus (Albert et al., 1966). This again suggested that γ -glutamyl transferase may play some role in amino acid transport across the blood-brain barrier.

Orlowski and Meister (1970a) have introduced the concept of the γ -glutamyl cycle as a possible system for transport of amino acids, when they discovered the presence in rat kidney of four highly active enzymes that synthesize and utilize γ -glutamyl amino acids. Meister (1973) suggested that the γ -glutamyl cycle mediates the amino acid transport in mammalian kidney as well as in the brain and possibly in other tissues, and that a very important physiological function of glutathione is that of a donor of the γ -glutamyl group, which serves as a carrier in amino acid transport.

The γ -glutamyl cycle is a sequence of six enzyme-catalyzed reactions which degrade and resynthesize the tripeptide glutathione (Fig. 1.1).

The crucial step in the cycle takes place at the cell membrane where γ -glutamyl transferase is located. The amino acid outside the cell becomes coupled to the enzyme to which glutathione is also attached, i.e., a ternary complex is formed. It is postulated that the amino acid is then translocated into the cell by the mechanism outlined in Figure 1.2.

According to this scheme, the amino acid which is to be transported binds noncovalently to a site on the membrane (Fig. 1.2A). Group X on the membrane-bound γ -glutamyl transferase interacts with the γ -glutamyl moiety of glutathione producing γ -glutamyl-enzyme and releasing cysteinyl-glycine (Fig. 1.2B). The amino acid nitrogen then attacks the γ -carbon

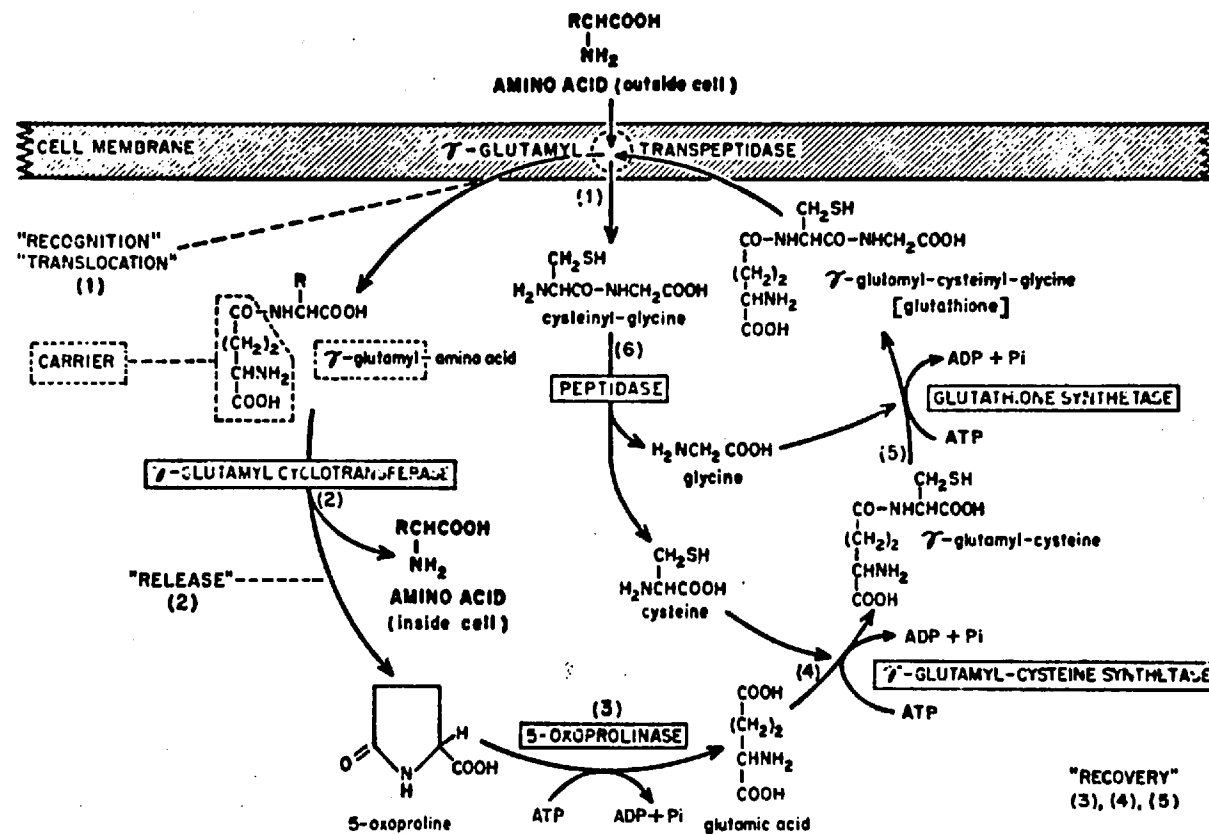


Fig. 1.1 The γ -glutamyl cycle (Meister, 1973)

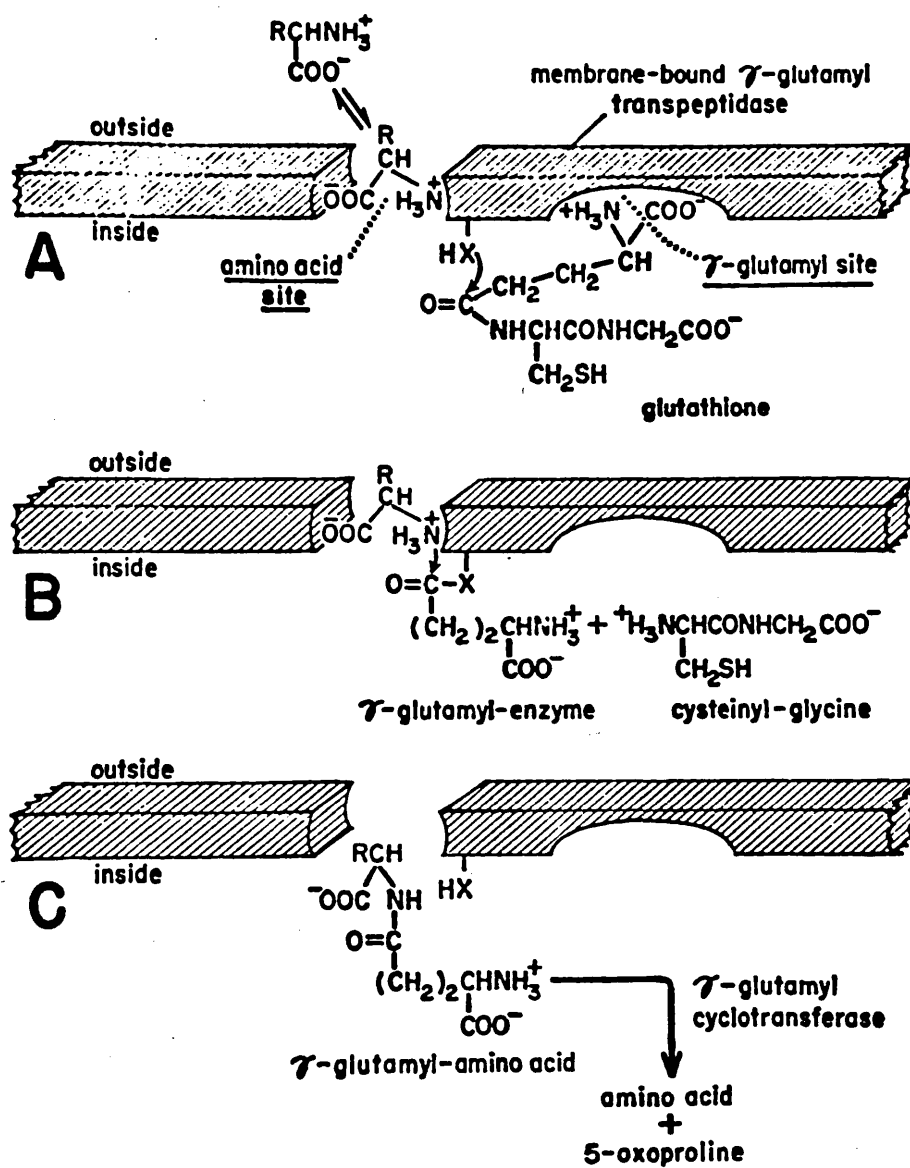


Fig. 1.2 Scheme for amino acid translocation (Meister, 1973)

atom of the γ -glutamyl-enzyme complex to yield the γ -glutamyl-amino acid (Fig. 1.2C). The formation of the γ -glutamyl-amino acid linkage leads to the translocation of the amino acid from the external to the internal surface of the cell membrane, either through a "pore" or by virtue of a conformational change in the membrane.

The γ -glutamyl-amino acid complex, which is now intracellularly located, becomes a substrate for γ -glutamyl cyclotransferase, a soluble intracellular enzyme; the γ -glutamyl residue is thus converted to 5-oxoproline and the amino acid is released (Fig. 1.1). The 5-oxoproline formed by this reaction and the cysteine and glycine formed by hydrolysis of cysteinylglycine go through three energy-requiring reactions leading to regeneration of the glutathione. Cysteinylglycine is cleaved by a specific peptidase, and 5-oxoproline is hydrolyzed to glutamic acid by the enzyme 5-oxoprolinase, the latter reaction requiring input of energy in the form of ATP. Cysteine and then glycine are re-attached to glutamic acid in two energy-requiring steps. At the end of the cycle, glutathione molecule has been regenerated and three molecules of ATP consumed. In a day, an estimated 0.5 moles of amino acids is reabsorbed from the glomerular filtrate (Goldberg, 1979), which is accompanied by the expenditure of 1.5 moles of ATP.

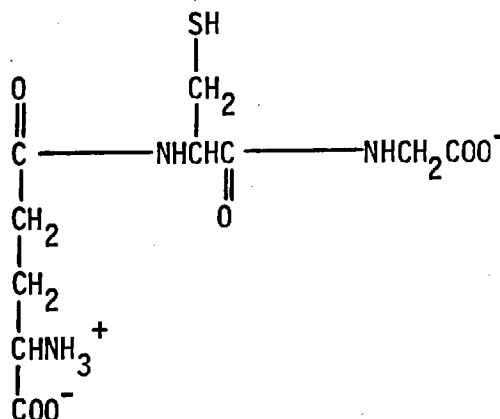
Meister (1973) proposed that the γ -glutamyl cycle functions not only in the kidney but in other mammalian tissues as well. The enzymes of this cycle together with a number of γ -glutamyl-amino acids have been found in the brain (Kakimoto et al., 1964; Kanazawa et al., 1965; Albert et al., 1966; Orlowski, Richman and Meister, 1969; Orlowski and Meister, 1970a and 1971a,b; Reichelt, 1970). γ -Glutamyl cyclotransferase

has been found in the intestine (Meister, 1973). High concentrations of the enzymes of the γ -glutamyl cycle have been found in the ciliary body of the eye, and all of the enzymes except 5-oxoprolinase, have been found in the lens (Ross et al., 1973). There are probably a number of amino acid transport systems, and the γ -glutamyl cycle's contribution to transport may vary from tissue to tissue (Meister, 1974).

Recent studies in several laboratories, however, have proposed that the physiological function of γ -glutamyl transferase is to catalyze the first step in the breakdown of glutathione or its S-substituted conjugates to produce L-glutamate and L-cysteinylglycine or S-substituted L-cysteinyl-glycine (Elce and Broxmeyer, 1976; Wendell, Heinle and Silbernagl, 1977; Hahn, Wendel and Flohe, 1978).

Degradation of extracellular glutathione

Glutathione is a tripeptide (below) that is found in virtually all living cells:



Glutathione (L- γ -glutamyl-L-cysteinyl-glycine)

Glutathione is widely distributed in microorganisms, in plant

and animal cells. It is found predominantly intracellularly, in rather high concentrations of about 0.5 - 10 mmol/l, whereas the level of extracellular glutathione is in the micromolar range (Griffith, Bridges and Meister, 1978). The most important characteristic of glutathione is the presence of a sulfhydryl (SH) and a γ -glutamyl group. Various investigations suggest that glutathione may play an important role by participating in such functions as (i) maintenance of the SH groups of proteins and other biomolecules; (ii) destruction of free radicals, hydrogen peroxide and other peroxides; (iii) catalysis of disulfide exchange reactions; (iv) acting as a coenzyme for certain enzymes (e.g. glyoxalase); (v) detoxification of foreign compounds by, for example the mercapturic acid pathway; (vi) translocation of amino acids (and maybe peptides and amines as well) across cellular membranes; (vii) protein synthesis (Zehavi-Willner et al., 1970; Kosower and Kosower, 1973; Meister and Tate, 1976).

A number of investigations suggest that glutathione protects cell membranes and proteins by maintaining essential SH groups and interacting with peroxides and free radicals.

Since γ -glutamyl transferase is the only known enzyme that can cleave the γ -glutamyl bond of glutathione, it suggests the involvement of the enzyme in the regulation of tissue glutathione levels (Rosalki, 1975). Goodman, Mace and Pollak (1971) observed high plasma and urine glutathione levels in a patient with no detectable γ -glutamyl transferase activity. Marked glutathionuria and glutathionemia observed in a patient who lacked γ -glutamyl transferase in his tissues provides some support for the inter-organ transport of glutathione and indicates the importance of γ -glutamyl

transferase in the hydrolysis and conservation of glutathione in man (Schulman et al., 1975).

While glutathione occurs mostly intracellularly, most of the γ -glutamyl transferase activity present in tissues is bound to cell membranes. It has been suggested that the enzyme is bound to the outer surface of the membrane with a hydrophobic sequence of amino acids acting as an anchor (Maack et al., 1974; Kuhlenschmidt and Curthoys, 1975; Hughey and Curthoys, 1976; Novogrodsky, Tate and Meister, 1976; Horiuchi, Haase et al., 1978; Hahn, Wendel and Flohe, 1978; Inoue and Morino, 1978; Hughey, Coyle and Curthoys, 1979). Curthoys and Hughey (1979) proposed that the hydrophilic portion of γ -glutamyl transferase (light subunit) which contains the active site (Tate and Meister, 1977) may be free to move in aqueous environment, but that it is unlikely that the active site can diffuse across the membrane or act inside the membrane because of the high content of carbohydrate in it and its solubility in aqueous buffers. The renal intracellular glutathione was shown to translocate to the membrane-bound γ -glutamyl transferase as a discrete step in the γ -glutamyl cycle (Griffith and Meister, 1979). These authors found considerable decrease in blood plasma glutathione after inhibition of glutathione synthesis by administration of prothionine sulfoximine, and increase in plasma glutathione levels following inhibition of γ -glutamyl transferase by, for example, L-serine plus borate, 6-diazo-5-oxo-L-norleucine and L- and D- γ -glutamyl-(o-carboxy)phenylhydrazide. They therefore suggested that glutathione synthesized in one organ (probably largely by the liver) may be transported via the plasma to another organ (probably mainly the kidney), where it can be used by transpeptidase in hydrolytic or transpeptidation reactions.

Kuhlenschmidt and Curthoys (1975) have shown that a large proportion of γ -glutamyl transferase activity was solubilized by papain treatment of intact kidney cells and ^{that} this was not accompanied by the release of intracellular enzymes. Experiments by Hahn, Wendel and Flohe (1978) in which $[U-^{14}C]$ glycine-labelled oxidized and reduced forms of glutathione and its analogue ophthalmic acid were administered to rats intravenously, have shown that these compounds were rapidly removed from the blood. The authors suggested that the liver does not play a significant role in removing γ -glutamyl tripeptides from the blood; that the hydrolysis of the tripeptides most probably takes place in the extracellular space of the renal tissue and that γ -glutamyl transferase which is located in the brush-border membrane of the kidney acts as a glutathionase.

That the active site of the enzyme is on the outer surface of the cells, and that the enzyme activity is therefore extracellular, is supported by some other experiments as well. Novogrodsky, Tate and Meister (1976) have shown that γ -glutamyl transferase of human lymphoid cells interacts with γ -glutamyl donor substrates which do not enter the cells (S-acetophenone-glutathione). Inoue, Horiuchi and Morino (1977) in experiments with rat ascites hepatoma cells found that glutathione conjugated to high molecular weight dextran (S-acetyldextran, M.W. 215,000) which is believed to be unable to penetrate cell membranes, inhibited the activity of γ -glutamyl transferase, from which they concluded that the catalytic site of the enzyme is localized on the outer surface of the tumour cells to permit a direct interaction with the derivative of glutathione. Experiments with antibodies to γ -glutamyl transferase in

human lymphoid cells provided further evidence for the cell-surface localization of the enzyme. Ultrastructural studies confirmed this point too, and also revealed γ -glutamyl transferase activity in the Golgi and endoplasmic reticulum. The intracellular localization was attributed to stages in the processing of newly synthesized enzyme (Marathe, Tate and Haschemeyer, 1978).

Curthoys and Hughey (1979) supported the view that the physiological function of γ -glutamyl transferase is the hydrolysis of extracellular glutathione.

Mercapturic acid biosynthesis

In mammals, many foreign compounds, including various drugs and carcinogens, undergo chemical modifications in the body, such as oxidation, reduction, hydrolysis and synthesis (conjugations). In most cases transformations of foreign compounds lead to their partial or complete inactivation. Many of the enzymes which catalyze such transformation reactions occur in the liver, and the resulting metabolites are usually excreted through the kidney or bile duct. Biosynthesis of mercapturic acids is a transformation route, in which the initial step is the formation of a conjugate between the foreign compound and glutathione.

Mercapturic acids are compounds containing N-acetyl-L-cysteine residues. Conjugation of foreign compounds with the SH group of glutathione is catalyzed by glutathione S-transferases for many substrates (Boyland and Chasseaud, 1969). Several glutathione transferases with different substrate specificities have been identified and purified from rat liver (Fjellstedt et al., 1973; Habig, Pabst and Jakoby, 1974; Pabst, Habig and Jakoby, 1974).

The role of γ -glutamyl transferase in the mercapturic acid pathway is in hydrolyzing S-derivatives of glutathione. After γ -glutamyl group has been removed by γ -glutamyl transferase, glutathione conjugates are thought to be metabolized further by a reaction in which the glycine residue is cleaved by an aminopeptidase; this is followed by acetylation of the resultant free amino group of the cysteinyl residue to give the final product, a mercapturic acid. The latter reaction is catalyzed by an N-acetyltransferase (Curthoys and Hughey, 1979). Mercapturic acids are then excreted either in the urine or in the feces.

Since virtually no endogenous compounds have been shown to be excreted in the form of mercapturic acids, it has been suggested that the enzymes in the mercapturic acid pathway may probably have some other physiological functions as well (Curthoys and Hughey, 1979). Glutathione S-transferases are present in the liver in much larger quantities than in the kidney (Boyland and Williams, 1965; Johnson, 1966), and γ -glutamyl transferase activity in the kidney is much higher than in the liver. N-acetyltransferase and peptidases hydrolyzing cysteinylglycine are also found in the kidney (Robinson, Birnbaum and Greenstein, 1953; Semenza, 1957; Green and Elce, 1975; Hughey et al., 1978). Such distribution of the enzymes of the mercapturic acid pathway may indicate that the S-derivatives of glutathione are synthesized in the liver and then are converted to mercapturic acids in the kidney, which implies that probably both the liver and the kidney are involved in the biosynthesis of mercapturic acids. The hydrolysis of glutathione and its S-derivatives in the kidney involve the same enzymes.

Wendel et al. (1978) have suggested that the role of renal

γ -glutamyl transferase is in the extracellular, intraluminal hydrolysis of glutathione and in catalyzing a final step of mercapturic acid synthesis.

1.4 Role of γ -glutamyl transferase activity measurements in diagnosis

Raised serum γ -glutamyl transferase activity is found in diseases of the liver and is associated with cirrhosis, alcoholism, hepatic secondaries and cholestasis. Increased levels of serum activity of the enzyme are also observed in conditions associated with microsomal enzyme induction in the liver. Raised levels have also been reported in diseases without obvious hepatobiliary involvement, e.g., pancreatitis, diabetes, myocardial disorders, chronic renal failure, cerebrovascular accidents, cerebral tumours and chronic obstructive pulmonary disease (Goldberg and Martin, 1975; Rosalki, 1975).

Hepatobiliary disease. γ -Glutamyl transferase is regarded as one of the most sensitive tests for liver disease. Early studies of Szewczuk and Orłowski (1960), Szczeklik, Orłowski and Szewczuk (1961) have suggested that examination of γ -glutamyl transferase activity in serum of patients with liver diseases is useful in clinical diagnosis. The highest values of activity are observed in biliary obstruction, and space-occupying lesions such as hepatic metastases, in cirrhosis and chronic alcoholism.

Obstructive jaundice. Serum γ -glutamyl transferase activity in extra-hepatic jaundice is usually very high, the levels of activity being on average 12 times the upper limit of normal (Szczeklik, Orłowski and Szewczuk, 1961; Rutenburg, Goldberg and Pineda, 1963; Rosalki, 1975).

In patients with obstruction due to malignant disease, γ -glutamyl transferase activity tends to be higher than in patients with non-malignant obstruction. In obstructive jaundice γ -glutamyl transferase is elevated more than alkaline phosphatase (Orlowski, 1963; Aronsen, Hanson and Nosslin, 1965), and in cases of intra- and extra-hepatic biliary obstruction it is more active in relation to transaminases (Lum and Gambino, 1972). After relief of obstruction, serum γ -glutamyl transferase activity levels decline to normal. Serum γ -glutamyl transferase values in hepatitis are generally lower than in obstructive jaundice, but a considerable overlap of these levels does not permit accurate discrimination between these conditions on the basis of serum γ -glutamyl transferase measurements alone (Betro, Oon and Edwards, 1973; Keane et al., 1973). In hepatitis, serum transaminases are much more increased compared with γ -glutamyl transferase, whereas in obstructive jaundice, γ -glutamyl transferase activity is disproportionately increased as compared to transaminase activity (Lum and Gambino, 1972). To discriminate between hepatocellular and obstructive jaundice, the use of an ALT/ γ GT ratio has been suggested (Aronsen, Hanson and Nosslin, 1965), in which γ GT is used as an indicator of obstructive jaundice and ALT as an indicator of hepatocellular damage. Another study also suggested the use of this ratio to differentiate between the two conditions (Lum and Gambino, 1972).

Hepatic metastases. γ -Glutamyl transferase activity is raised in serum of patients with liver secondaries as well as in primary liver tumours (Szczeklik, Orlowski and Szewczuk, 1961; Goldberg et al., 1963; Orlowski, 1963; Aronsen, Hanson and Nosslin, 1965; Aronsen, Nosslin and

Pihl, 1967 and 1970; Zein and Discombe, 1970; Jacobs, 1972; Goldberg and Martin, 1975; Rosalki, 1975). In liver tumours, increased γ -glutamyl transferase activity may result from cholestasis in hepatic tissue due to tumour growth; it may also originate from the tumour tissue, since tumours growing in the liver have higher γ -glutamyl transferase activity than those growing in other organs (Gibinski, Grzybek and Zmudzinski, 1963; Kokot et al., 1965). Normal liver cells in the vicinity of tumour growth were shown to have increased γ -glutamyl transferase activity (Aronsen et al., 1969), so they may also contribute to the raised serum enzyme levels (Kokot et al., 1965). The increase in the activity of γ -glutamyl transferase in serum was shown to be parallel with an increase in the activity of this enzyme in hepatic tissue (Aronsen et al., 1969).

Rutenburg, Goldbarg and Pineda (1963), Kokot et al. (1965), Aronsen, Nosslin and Pihl (1970) observed that patients with primary or secondary liver neoplasms had increased serum γ -glutamyl transferase activity, irrespective of whether the patients were jaundiced or not, but jaundiced patients had much higher serum enzyme activities than the non-jaundiced ones. Szczeklik, Orłowski and Szewczuk (1961) have also shown elevated serum γ -glutamyl transferase levels in patients with primary and secondary liver cancer irrespective of the presence or absence of concomitant obstructive jaundice; sera of two patients with cancer of the stomach and peritonitis carcinomatosa, but no intrahepatic metastases, had normal activities of this enzyme.

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Rutenburg (1963) observed increased serum γ -glutamyl transferase activity in all jaundiced patients with liver metastases from cancer of the pancreas or extrahepatic biliary tract and in 91% of non-jaundiced

patients with liver metastases from other primary sites; in the same study, all patients with hepatoma had elevated serum γ -glutamyl transferase levels. Kokot et al. (1965) reported that in patients with primary or metastatic liver tumours γ -glutamyl transferase activity in serum was raised above normal levels in all cases with jaundice, whereas eight cases in the non-jaundiced group gave results within normal limits; in patients with tumours in organs other than the liver (either without metastases or with extrahepatic metastases) blood γ -glutamyl transferase activity was usually normal, or slightly raised in about one-quarter of the observed cases.

Since laboratory tests can sometimes give elevated values of serum γ -glutamyl transferase in patients with cancer in the absence of hepatic secondaries, it may be important to use reference values that allow discrimination between the presence and the absence of liver secondaries. Aronsen, Nosslin and Pihl (1970) used a group of patients with cancer in whom liver metastases were excluded (histologically, at operation or post-mortem examination) as control to another group of cancer patients, in whom the presence of liver metastases was established in a similar way. In 153 cases (out of which 77 were controls and 76 had liver secondaries), determination of serum γ -glutamyl transferase activity gave correct diagnosis in 90% of the cases, false positive results in 9% and false negative results in 1% for both jaundiced and non-jaundiced patients. However, since all patients with evidence of extrahepatic biliary obstruction were excluded, the actual percentage of false positive results could be expected to be higher than in this report. Results of other investigations (Rutenburg et al., 1964; Zein and Discombe, 1970) also

confirmed that measurement of serum levels of the enzyme may be helpful in the diagnosis of primary or secondary liver neoplasms; jaundice of any type and congestive heart failure were found to affect the correctness of the diagnosis since both caused increases in the activity of serum γ -glutamyl transferase (Zein and Discombe, 1970).

However, authors who examined 262 patients with carcinoma of the colon or rectum at operation and found evidence of the presence of liver metastases in 45 of them, reported that approximately one-quarter of the patients without and only about one-half of those with metastases had raised γ -glutamyl transferase activity (Baden et al., 1971). The use of the usual upper level of normal led to 18%, or 47 out of 262 patients, being misclassified, from which the authors concluded that the measurement of serum γ -glutamyl transferase activity as a test for detection of liver metastases was unsatisfactory and that the test was no better than the simple assumption that all the patients were without liver secondaries, the same conclusion was reached for the determination of serum alkaline phosphatase activity.

The non-specificity of measuring serum γ -glutamyl transferase activity has been confirmed in another study (Delarue et al., 1973), with altered levels of activity being observed in diseases other than liver secondaries. However, these authors found raised levels of serum γ -glutamyl transferase in all cases where the presence of liver metastases was confirmed by either laparoscopy or hepatic scintigraphy, with no false negative reactions. According to Rosalki (1975), γ -glutamyl transferase activity in serum may almost always be raised when liver metastases are obvious on clinical examination or by scintigraphy, but in the absence of

such evidence, the incidence of the increased levels of the enzyme has to be established at autopsy or laparotomy.

In summary, most studies support the view that γ -glutamyl transferase is probably a more sensitive test than other enzymes in the important clinical problem of the detection of hepatic secondaries, such as alkaline phosphatase, leucine aminopeptidase, 5'-nucleotidase, glutamate-pyruvate transaminase (Szczeklik, Orłowski and Szewczuk, 1961; Rutenburg et al., 1964; Aronsen, Hanson and Nosslin, 1965; Lukasik and Richterich, 1965; Aronsen, Nosslin and Pihl, 1970; Lum and Gambino, 1972; Boone and Routh, 1973; Cooper et al., 1975). Suggestions have been made to combine serum γ -glutamyl transferase assays with determination of carcino-embryonic antigen, CEA (Steele et al., 1974; Cooper et al., 1975). CEA values alone can differentiate between hepatic metastases and non-malignant hepatic disease with a high degree of certainty. While CEA may be elevated with metastases, irrespective of their site, γ -glutamyl transferase is usually elevated when hepatic metastases are present. Steele et al. (1974) suggested that by combination of CEA and γ -glutamyl transferase activity determinations, hepatic metastases may be identified and differentiated from local recurrences or metastases to other organs. Cooper et al. (1975) have found that when serum γ -glutamyl transferase in the same patient is measured repeatedly, a progressive rise, if it is accompanied by a rise in CEA, was indicative of metastases in the liver.

The absence of γ -glutamyl transferase in bone makes its estimation useful in confirming the hepatic origin of an elevated alkaline phosphatase activity (Lum and Gambino, 1972; Betro, Oon and Edwards, 1973a; Boone and Routh, 1973; Schwartz, 1973; Batsakis, 1974; Price and Sammons, 1974; Rosalki, 1975). Serum γ -glutamyl transferase activity is normal in patients

without liver metastases from cancer originating in sites other than the hepatobiliary tract or pancreas (Rutenburg, Goldbarg and Pineda, 1963; Zein and Discombe, 1970; Schwartz, 1973). Normal values of γ -glutamyl transferase activity were observed in patients with bone metastases (Lum and Gambino, 1972). According to Rosalki (1973 and 1975) the presence of bone metastases is not accompanied by elevation in the enzyme levels, unless liver disease is also present or the bone metastases originate from the prostate, in which case serum acid phosphatase activity is usually also raised.

Alcoholism. Serum γ -glutamyl transferase activity determination is regarded as a sensitive and highly specific test for the detection of the effect of alcohol on the liver. The serum enzyme activity may reach values that are 2 - 5 times the upper limit of normal and sometimes the elevation of activity is even higher (Rosalki, 1975).

Elevated levels of serum γ -glutamyl transferase activity in alcoholics have been reported in many studies (Konttinen, Hartel and Louhija, 1970; Zein and Discombe, 1970; Lum and Gambino, 1972; Rollason, Pincherle and Robinson, 1972; Rosalki and Rau, 1972; Batsakis, 1974; Goldberg and Martin, 1975; Rosalki, 1975; Collins and Johnson, 1978, Goldberg, 1979).

Zein and Discombe (1970) reported increased values of serum γ -glutamyl transferase in chronic alcoholics, the values returned to normal in a few patients after complete abstinence for a month or more.

In one study approximately 75% of the 76 patients (either non-jaundiced alcoholics or heavy drinkers) had elevated serum γ -glutamyl transferase, when more than half of these patients had no clinical evidence

of liver disease, and γ -glutamyl transferase was shown to be more sensitive than transaminases and alkaline phosphatase (Rosalki and Rau, 1972). These authors also reported that γ -glutamyl transferase was the only enzyme with elevated activity in a group of heavy drinkers who were not considered to be alcoholics.

Rollason, Pincherle and Robinson (1972) determined γ -glutamyl transferase in 5 groups of patients with different amounts of alcohol intake and found a correlation between alcohol consumption and γ -glutamyl transferase activity. Among social drinkers only 12% had elevated activity of the enzyme, whereas among heavy drinkers, who regularly had more than six drinks a day, about 50% had elevated serum γ -glutamyl transferase activity. Normal social drinking by normal healthy people does not lead to significant elevation of the enzyme level (Rosalki, 1975).

When drinking is stopped, raised values return to normal in 2 - 3 weeks (Rosalki, 1975) but in heavy drinkers a fall in γ -glutamyl transferase activity is slower and may take several months (Rollason, Pincherle and Robinson, 1972). Monitoring of γ -glutamyl transferase levels therefore may be useful in following the progress and controlling abstinence from alcohol in heavy drinkers. Serum γ -glutamyl transferase elevation could be the result of hepatic enzyme induction by alcohol; it could also result from the liver cell injury, since alcohol has been shown to cause pronounced ultrastructural changes in the liver (Lieber and Rubin, 1968; Rosalki and Rau, 1972).

Serum γ -glutamyl transferase activity is raised in patients with chronic hepatitis caused by alcoholism and in alcoholic cirrhosis (Szczeklik, Orłowski and Szewczuk, 1961; Orłowski, 1963; Rosalki, 1975).

Cirrhosis. Serum γ -glutamyl transferase activity levels in cirrhosis vary quite a lot. In about 90% of patients with cirrhosis they are elevated (Szewczuk, 1961; Orłowski, 1963; Lum and Gambino, 1972; Rutenburg^{et al.}, 1964; Konttinen, Hupli and Salmenkivi, 1971; Goldberg and Martin, 1975; Rosalki, 1975). There are some reports on normal values of γ -glutamyl transferase in some patients with cirrhosis (Betro, Oon and Edwards, 1973); the incidence of increased serum γ -glutamyl transferase is as high in jaundiced as in non-jaundiced patients and particularly high (up to 25^{times} the upper limit of normal) serum γ -glutamyl transferase activities were observed in biliary cirrhosis resulting from obstruction (Rutenburg, Goldbarg and Pineda, 1963).

Viral hepatitis. Serum γ -glutamyl transferase is elevated in nearly all patients with acute viral hepatitis; the increase in activity is usually gradual, with maximum values obtained in the second or third week of illness. The raised values may be 5 - 6 times the upper limit of normal, and the return of the activity of the enzyme to normal values can take 3 - 6 weeks. Persistent elevation of serum γ -glutamyl transferase levels (up to 7 times the upper limit of normal) indicate the onset of chronic hepatitis (Szczeklik, Orłowski and Szewczuk, 1961; Rutenburg, Goldbarg and Pineda, 1963; Rosalki, 1975).

Pancreatic disease. Raised serum γ -glutamyl transferase levels (on average up to 10 times the upper limit of normal) occur in acute and chronic pancreatitis (Goldbarg et al., 1963; Szczeklik et al., 1965; Lum and Gambino, 1972; Boone, Routh and Schrantz, 1974) and in carcinoma of the head of the pancreas with and without biliary obstruction (Boone, Routh and Schrantz, 1974) or hepatic metastases (Rutenburg, Goldbarg and Pineda, 1963; Boone, Routh and Schrantz, 1974; Rosalki, 1975).

In chronic pancreatitis the enzyme levels could be either elevated or normal (Orlowski, 1963).

Myocardial infarction. Although the content of the enzyme in cardiac tissue is low, elevations of serum γ -glutamyl transferase activity following myocardial infarction have been demonstrated by many investigators. Some reports indicate that the enzyme levels remain normal in the first three to four days after the infarction with an increase in activity starting thereafter. The values reach a peak at 7 - 11 days, remaining raised for up to 6 weeks after the onset of infarction (Orlowski, 1963; Agostini, Ideo and Stabilini, 1965; Hedworth-Whitty, Whitfield and Richardson, 1967; Ravens et al., 1969; Coodley, 1972; Jacobs, 1972; Szczeklik et al., 1972). However, other workers report no late increase in serum γ -glutamyl transferase in patients with myocardial infarction, finding that only 11 out of 33 patients developed increases at all (Betro, Oon and Edwards, 1973b). The increased activity in myocardial infarction is comparatively lower than in hepatobiliary disease.

The origin of the increased activity of serum γ -glutamyl transferase is not very clear, and both liver (Orlowski, 1963) and heart (Agostini, Ideo and Stabilini, 1965) were suggested as possible sources. Myocardial infarction resulting in disturbances in hepatic circulation, or changes occurring in the heart muscle may give rise to the activity of serum γ -glutamyl transferase (Szczeklik et al., 1972). Agostini, Ideo and Stabilini (1965) proposed that elevation of the serum enzyme could be caused by a higher rate of γ -glutamyl transferase synthesis as an expression of the process of repair of the myocardium.

Other disorders. Serum γ -glutamyl transferase activity is also elevated in epilepsy, in patients following neurosurgery with brain tumours (Ewen and Griffiths, 1973), multiple sclerosis (Rieder, Zurfluh and Jegge, 1977), diabetes mellitus (Jacobs, 1972; Goldbarg et al., 1963), chronic obstructive pulmonary disease (Barton, Powers and Lourenco, 1974) and during rejection of renal transplants (Lehmann, Prentice and Rosalki, 1970).

Enzyme induction. Enzyme induction is a term which refers to an increase in the activity of an enzyme, usually as a result of increased synthesis of the enzyme protein, and in response to some specific stimulus. Hepatic microsomal enzyme systems responsible for the detoxification of compounds foreign to the body, are known to be inducible by exposure of human beings and animals to a variety of chemicals. The enzymes involved in this system are located in the smooth endoplasmic reticulum of the hepatocyte, and the system comprises an electron transport chain and cytochrome P-450 (terminal oxidase). Many foreign substances are hydroxylated by these enzymes, the process of hydroxylation reducing their toxicity as well as making them more water soluble to allow excretion in the bile or urine (Marshall, 1978). Inducers such as phenobarbitone, insecticide DDT are well known.

Rosalki, Tarlow and Rau (1971) were first to discover increased levels of γ -glutamyl transferase in patients receiving enzyme-inducing drugs. In 16 patients receiving drug treatment for epilepsy or depression, 12 had elevated activities of the plasma enzyme: in 4 out of 5 receiving phenytoin, in 1 of 3 receiving phenobarbitone and in 7 of 8 patients

receiving both phenytoin and phenobarbitone. These authors suggested that the raised levels of plasma γ -glutamyl transferase may indicate microsomal injury or result from microsomal enzyme induction without microsomal damage.

Ideo et al. (1971) demonstrated that γ -glutamyl transferase in rat liver is inducible by oral phenobarbitone and ethanol administration. In another study (Whitfield et al., 1973) a significant rise in plasma γ -glutamyl transferase activity was observed in 13 out of 14 cases when patients on long-term oral treatment with anticoagulant warfarin were given amylbarbitone, quinalbarbitone or phenazone (antipyrine) for 30 days. Increase in plasma γ -glutamyl transferase activity was accompanied by a significant fall in plasma warfarin levels (which was used as a marker of hepatic enzyme induction). One patient showed no increase in the plasma enzyme activity but a significant increase in the rate of warfarin metabolism; and in another patient increase in plasma γ -glutamyl transferase activity was not accompanied by changes in warfarin metabolism. The authors suggested that the induction of hepatic drug-metabolizing enzymes is accompanied by induction of hepatic γ -glutamyl transferase, which is then released into the circulation.

Raised levels of serum γ -glutamyl transferase, 5'-nucleotidase and leucine aminopeptidase were observed in a patient who had been treated for several years with glutethimide, a potent inducer of hepatic microsomal enzymes, which structurally resembles phenobarbitone. Elevation of serum γ -glutamyl transferase was greater compared to those of alkaline phosphatase, 5'-nucleotidase and leucine aminopeptidase (Greenwood, Prunty and Silver, 1973).

Bartels et al. (1975) measured the activities of serum enzymes frequently used to diagnose liver disease in 103 epileptic children on long-term anticonvulsant drug therapy. γ -Glutamyl transferase accounted for the largest percentage of increased values, probably being more sensitive to hepatic microsomal enzyme induction. Bartels, Hauck and Vogel (1975) studied serum activities of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and γ -glutamyl transferase of children receiving the anti-inflammatory drug aminopyrine. Whereas other enzymes were more or less normal, serum γ -glutamyl transferase activity was above the upper limit of normal in every patient. The raised γ -glutamyl transferase activity returned to normal on withdrawal of aminopyrine.

Although enzyme induction by drugs seems to be the most likely explanation of such changes of activity, Rosalki (1975) has pointed out that a possibility that enzyme-inducing drugs may cause the increase in plasma γ -glutamyl transferase activity by enhancing the hepatic catabolism of endogenous inhibitors or repressors of this enzyme cannot be ruled out.

The monitoring of increases in serum γ -glutamyl transferase activity for screening purposes in populations exposed to potential inducers has been suggested as a useful index of induction (Marshall, 1978).

In conclusion, it can be said that the γ -glutamyl transferase activity level in serum is a sensitive index of the presence of hepatobiliary disease. Although it is not specific for any type of liver disease, it is useful in the assessment of alcoholism and, in the absence of jaundice, for the confirmation of both primary and secondary hepatic malignancy. It can also be useful in monitoring the effect on the liver

of the administration of enzyme-inducing drugs. Since the estimation of γ -glutamyl transferase activity is simple (see Chapter 2) and the correlation with alkaline phosphatase is high, it is useful in confirming the hepatic origin of raised serum alkaline phosphatase.

1.5 Origin and multiple forms of γ -glutamyl transferase in serum

Although γ -glutamyl transferase activity measurements provide the most sensitive enzymic test for hepatobiliary disease, attempts to demonstrate directly that the liver is the source of the increased activity in serum by experiments in animals have so far been unsuccessful or inconclusive, and the association of increased activity of this enzyme with diseases of liver and biliary tract is based entirely on evidence drawn from clinical observations.

Kryszewski et al. (1973) in experiments with bile-duct ligated rats showed that for γ -glutamyl transferase and 5'-nucleotidase the increase of activity in the liver was slow but the elevation of their activities in serum was more rapid. On the other hand, the increase of alkaline phosphatase activity in the liver as well as in the serum was very rapid. The authors concluded that bile duct ligation caused an early increase in the synthesis of liver alkaline phosphatase, but the increase in γ -glutamyl transferase and 5'-nucleotidase was delayed until the bile duct proliferation phase. When inhibitors of protein synthesis were given to the rats immediately before ligation of the common bile ducts, serum activities of γ -glutamyl transferase and 5'-nucleotidase still increased. The early increase in serum activities of these two enzymes were therefore not attributed to their rapid increased synthesis by the liver.

Several possible explanations of the mechanism of increase in serum γ -glutamyl transferase activity were proposed by the same authors:

(i) accumulation of the enzyme released from non-hepatic sources by, e.g., secondary effect of bile stasis on these tissues, or because the normal excretion in the bile was interrupted; (ii) leakage of the enzyme into the blood from cell membranes of the enzyme-containing cells as a consequence of damage by the obstruction of the bile flow; (iii) solubilization of the enzyme from the cell surface by accumulating bile salts; (iv) activation of the enzyme molecules in serum by an accumulation of some substance which is normally excreted in the bile.

A study of Wootton, Neale and Moss (1977) with different types of cells isolated from livers of normal and cholestatic rats also showed no rapid change in the specific activities of γ -glutamyl transferase and 5'-nucleotidase in any type of liver cells. It was suggested that the rapid increase of these two enzymes in plasma after bile duct ligation could be due to release of enzyme from cell membranes, especially from non-parenchymal cells.

Experiments of Huseby and Vik (1978) on guinea pig after bile duct ligation showed a very rapid increase in the activity of serum γ -glutamyl transferase (up to 10 - 20 times the normal value) 3 hours after operation and the mean activity over the first 3 days was about 8 times the normal value. A small decline in activity of the enzyme in the liver was observed, and high values in serum were not influenced by administration of the inhibitor of protein synthesis. Serum bile acids levels were also increased and their rate of increase initially paralleled the serum enzyme activity. The authors suggested that increase in the activity of serum γ -glutamyl transferase is the result of the solubilization by bile acids of liver membrane-bound enzyme.

In the case of several other enzymes, a resemblance in properties between the enzyme entering the circulation in certain diseases and its counterpart in particular tissues has been demonstrated. This both confirms the supposed tissue of origin of the enzyme in disease and improves the sensitivity and specificity of measurements carried out for diagnostic purposes. It is not surprising that the success of this approach in instances such as that of alkaline phosphatase should have directed attention to the comparative properties of γ -glutamyl transferase in serum and tissues, and to the occurrence in health and disease of multiple forms of the enzyme in serum.

Many reports have appeared on the electrophoretic heterogeneity of γ -glutamyl transferase in human serum. Such studies include separation of the serum enzyme by electrophoresis on paper (Kokot and Kuska, 1965; Kokot et al., 1965; Rutenburg, Smith and Fischbein, 1967), starch gel (Orlowski and Szczeklik, 1967; Kokot and Kuska, 1968), agar gel (Szewczuk, 1966; Miyazaki and Okumura, 1972; Wenham, Price and Sammons, 1979), agarose gel (Jacyszyn and Laursen, 1968; Hetland, Andersson and Gerner, 1975, and this work), cellulose acetate (Lehman, Prentice and Rosalki, 1970; Rosalki et al., 1970; Igartua, Domecq and Findor, 1973; Burlina, 1978; Kok et al., 1978), polyacrylamide gel (Azzopardi and Jayle, 1973). Up to seven different zones of activity could be detected following electrophoresis. Some reports indicated that different patterns of enzyme zones may be associated with various diseases (Kokot and Kuska, 1965; Orlowski and Szczeklik, 1967; Rutenburg, Smith and Fischbein, 1967; Miyazaki and Okumura, 1972; Igartua, Domecq and Findor, 1973; Hetland, Andersson and Gerner, 1975; Rosalki, 1975; Burlina, 1978).

However, no consistent change in patterns of serum γ -glutamyl transferase activity zones on electrophoresis has been reported which is specific to certain diseases. Attempts to separate γ -glutamyl transferase activity in serum by gel-filtration have so far been rather inconclusive (see introduction to Chapter 6).

The molecular basis of the observed heterogeneity remains unclear, and some investigators have attributed the various enzyme zones to such factors as aggregation of enzyme molecules with other constituents of serum, such as lipids, lipoproteins and other proteins, rather than to the existence of distinct enzyme forms or isoenzymes (Orlowski and Szczeklik, 1967; Jacyszyn and Laursen, 1968; Miyazaki and Okumura, 1972; Von Freise, Magerstedt and Schmidt, 1976; Huseby, 1978). Since the enzyme is a glycoprotein with a carbohydrate content as high as 35% (Goldberg, 1979) its ability to form complexes is not surprising.

Attempts to elucidate the nature of the heterogeneity of γ -glutamyl transferase in serum, and to compare the enzymes from serum and liver tissue, represent the major objectives of the work described in this thesis.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

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A. MATERIALS

2.1 Sources of γ -glutamyl transferase

Human liver tissue was obtained at autopsy as soon as possible after death, the causes of which were considered not to affect the enzyme activity in the liver. The liver tissue was frozen at -25°C immediately and was stored at this temperature until required, then it was left at room temperature to thaw.

Bile samples were obtained from patients with obstruction of the common bile duct due to malignant growth either in the pancreas or in the bile duct. Bile was collected in the course of percutaneous intra-hepatic decompression. The samples were stored at $0-4^{\circ}\text{C}$; before use, each bile sample was filtered through glass wool.

Sera from patients with either normal or elevated γ -glutamyl transferase activities were randomly chosen from samples submitted to the laboratory for diagnostic purposes.

2.2 Chemicals and column chromatography materials and suppliers

Albumin	from bovine serum, crystallized and lyophilized (M.W. approx. 67,000);
Blue Dextran	(average M.W. 2,000,000);
Catalase	from bovine liver, purified powder, thymol free (M.W. approx. 210,000);

Cytochrome C	from horse heart, type VI (M.W. 12,384);
Diazo Blue B	(O-Dianizidine, tetrazotized, Fast Blue B Salt; Fast Blue B Salt BN), practical grade, ZnCl_2 complex. (M.W. 475.5);
γ -Globulins	bovine, Cohn Fraction II (M.W. approx. 160,000)
L- γ -Glutamyl-p-nitroanilide	
Glutathione	oxidized form, disodium salt, Grade IV (M.W. 656.0);
Glutathione	reduced form (M.W. 307.3);
Glycylglycine	free base (M.W. 132.1);
Lubrol WX	(Ethylene oxide condensates of fatty alcohols);
α -Methyl-D-glucoside	(1-O-Methyl- α -D-glucoside, M.W. 194.2);
Neuraminidase	from <i>Clostridium perfringens</i> , purified, type V;
Ovalbumin	grade III (M.W. 43,000);
Phospholipase A ₂	(Lecithinase A ₂ , phosphatide 2-acyl-hydrolase, EC 3.1.1.4), from bovine pancreas, suspension in 3.2 mmol/l $(\text{NH}_4)_2\text{SO}_4$ solution, pH~5.5;
Phospholipase C	(Lecithinase C, EC 3.1.4.3), Type I, from <i>Cl. welchii</i> , 7.4 units/mg protein;
Sodium deoxycholate	($\text{C}_{24}\text{H}_{39}\text{NaO}_4$, M.W. 414.56);
Trypsin	from bovine pancreas, type XI (EC 3.4.4.4, M.W. 23,800)
Thyroglobulin	bovine, type I (M.W. approx. 670,000).

The above were all purchased from Sigma London Chemical Company Limited, Fancy Road, Poole, Dorset BH17 7NH, England.

The following chemicals were obtained from the British Drug Houses Limited, (B.D.H. Chemicals Limited), Poole, England:

Acetic acid glacial, AR;

Acetone AR;

Amidoschwartz "10B"

Barbitone (diethylbarbituric acid: $(C_2H_5)_2C.CO.NH.CO.NH.CO$,
M.W. 184.2);

Barbitone, sodium $(C_2H_5)_2C.CO.NH.C.(ONa):N.CO$, M.W. 206.18;

n-Butanol AR;

Diethanolamine $[CH_2(OH).CH_2]_2NH$ (M.W. 105.14);

Di-iso-propyl ether AR;

Magnesium chloride $(MgCl_2.6H_2O)$, M.W. 203.31);

Para-nitrophenylphosphate (4-Nitrophenyl disodium orthophosphate:
 $NO_2C_6H_4Na_2PO_4.6H_2O$, M.W. 371.15)

Peroxidase from horse radish (M.W. 40,000);

Sodium azide (NaN_3) , M.W. 65.01);

Trichloroacetic acid $(CCl_3.COOH)$, M.W. 163.39),

and

γ -Glutamyl- α -naphthylamide AR;

Tris (Tris-(hydroxymethyl)-methyllamine, 2-amino-2(hydroxy-
methyl)-1,3-propanediol Tris-buffer, $C_4H_{11}NO_3$,
M.W. 121.14) and

Triton X-100 (purified)

were obtained from Koch-Light Laboratories Limited, Colnbrook, Bucks.,
England.

Hydrochloric acid,

Sodium chloride and

Sodium hydroxide (all AR grade) were purchased from Hopkin and Williams,
Chadwell Heath, Essex, England.

- Agarose [®] (Indubiose A37); Produits Biologiques, l'Industrie Biologiques Française S.A., 35 Quai du Moulin de Cage, 92 Gennevilliers.
- 'Carbowax' 20m (Polyethylene glycol); Raymond A Lamb, Waxes and General Laboratory Supplies, 6 Sunbeam Road, North Acton, London NW10 6JL, England.
- Human Fibrinogen (M.W. approx. 330,000); Blood Products Laboratory, Lister Institute, Elstree, Herts., England.
- Methanol AR; James Burrough Limited, Fine Alcohols Division, 60 Montford Place, London SE11, England.

Column Chromatography Materials:

- Sephadex [®] G-200 (dry powder, particle size 40-120 μ);
- Sepharose [®] 6B (a suspension of swollen beads in distilled water, containing 0.02% sodium azide as a bacteriostatic agent) and
- Concanavalin A - Sepharose 4B (Concanavalin A covalently bound to Sepharose 4B, supplied as a suspension of 100 ml sedimented gel in acetate buffer solution (0.1 mol/l, pH 6) containing 1 mol/l NaCl, 1 mmol/l CaCl_2 , 1 mmol/l MgCl_2 , 1 mmol/l MnCl_2 with 0.02% merthiolate added as a preservative),

were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

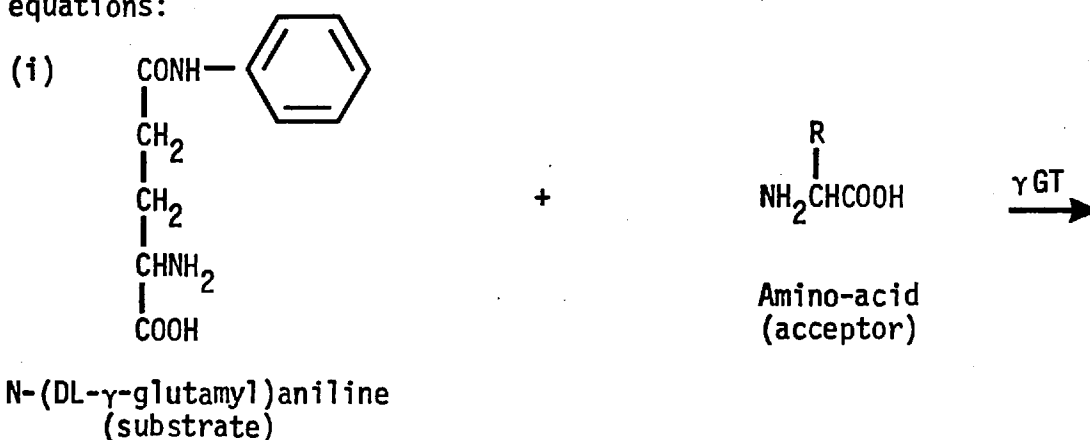
DE 52 Diethylaminoethyl cellulose (DEAE-cellulose, pre-swollen, microgranular anion exchanger for column chromatography) was obtained from Whatman Limited, Springfield Mill, Maidstone, Kent, England.

B. METHODS

2.3 Enzyme assays

a) Determination of γ -glutamyl transferase activity

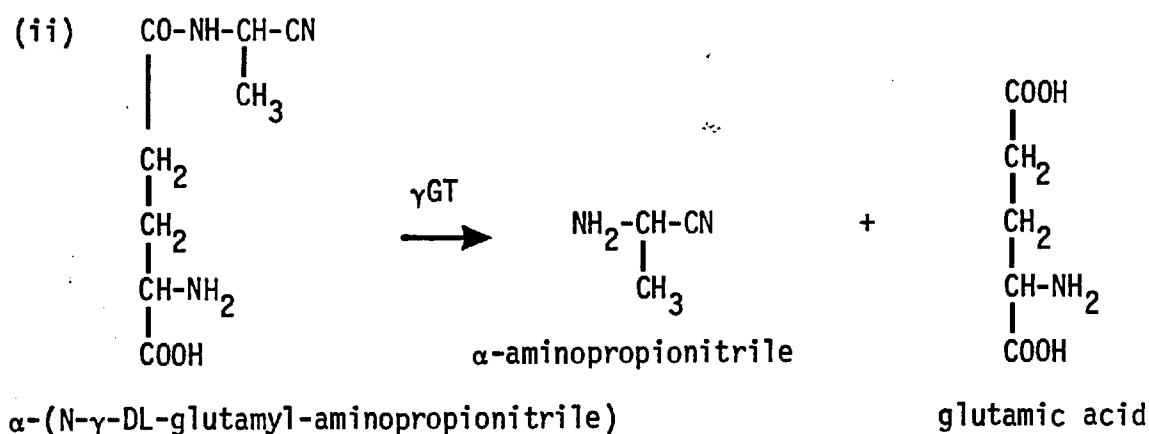
Glutathione was the first substrate used for the measurement of γ -glutamyl transferase activity. With glutathione, the rate of its disappearance or of appearance of its products of transpeptidation or hydrolysis was measured by, for example, chromatographic methods (Hanes, Hird and Isherwood, 1950, and 1952); by reacting glutathione with alloxan and measuring the rate of its disappearance in the ultraviolet light (Ball, Revel and Cooper, 1956); by measuring the formation of cysteinylglycine (Ball, Revel and Cooper, 1956); by determining γ -glutamylarginine when arginine was used as acceptor (Kinoshita and Ball, 1953) or by estimating the residual glutathione using glyoxalase (Hird and Springell, 1954). Later, synthetic γ -glutamyl substrates which also serve as substrates for γ -glutamyl transferase were introduced, the first being N-(DL- γ -glutamyl)aniline (Goldbarg et al., 1960; Goldbarg et al., 1963) and α -(N- γ -DL-glutamyl)-aminopropionitrile (Szewczuk and Orlowski, 1960; Orlowski and Szewczuk, 1961). Enzymic reactions with each of these substrates proceed according to the following equations:





γ -glutamyl peptide

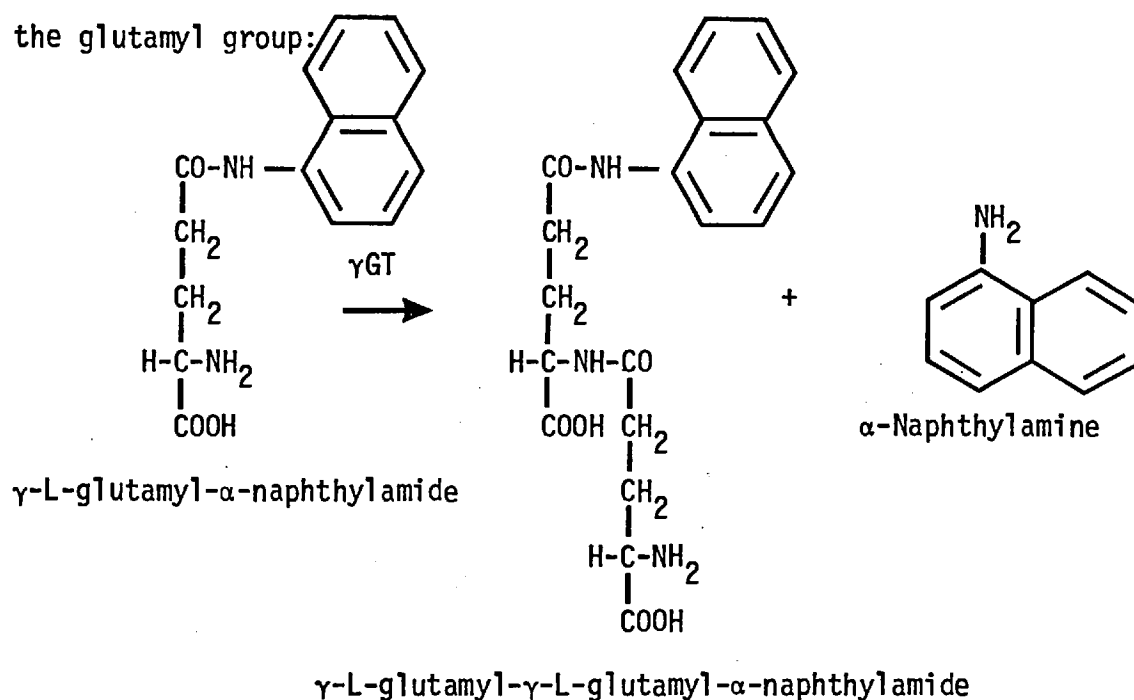
In the hydrolytic reaction where the acceptor is water, aniline and glutamic acid are formed. Aniline can be measured colorimetrically, e.g., by its conversion to an azo dye by a modified method of Bratton and Marshall (1939).



α -Aminopropionitrile, on reaction with bromine, gives a substance which, in presence of pyridine and benzidine hydrochloride, produces an intense red colour that is determined colorimetrically.

Since the synthesis of γ -glutamyl- α -aminonitriles and their use as substrates had presented difficulties, γ -glutamyl-naphthylamides were recommended as substrates for determination of γ -glutamyl transferase activity (Albert, Orłowski and Szewczuk, 1961; Orłowski and Szewczuk, 1962).

As substrate, γ -glutamyl- α -naphthylamide has usually been preferred to γ -glutamyl- β -naphthylamide because the solubility of the α - form is about 10 times greater than that of the β - form (Orlowski and Szewczuk, 1962). With both forms of γ -glutamyl-naphthylamides, γ -glutamyl transferase catalyzes a transpeptidation reaction in which the substrate acts, if no amino acids or peptides have been added, both as donor and acceptor of the glutamyl group:

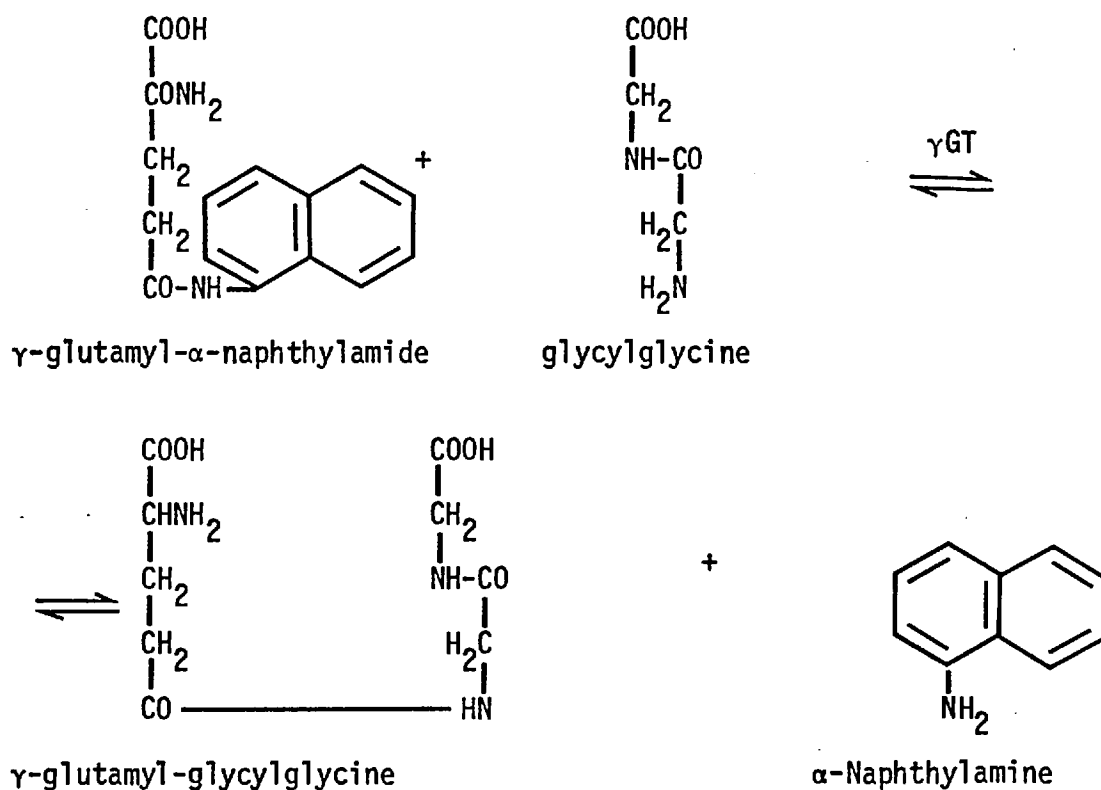


α -Naphthylamine liberated in this reaction is converted to an azo dye (by a modification of the Bratton and Marshall procedure) (Bratton and Marshall, 1939; Goldberg and Rutenburg, 1958) and is measured colorimetrically.

In the absence of added acceptors for the glutamyl moiety, all synthetic substrates for γ -glutamyl transferase are either hydrolyzed with the liberation of glutamic acid, or the reaction of transpeptidation takes place, in which the substrate itself acts as an acceptor. When suitable

acceptor peptides or L-amino acids are added, activation takes place, and hydrolysis is reduced in favour of transpeptidation to the acceptor. Glycylglycine was shown to be the most potent acceptor (activator) (Binkley, 1961; Orlowski and Szewczuk, 1961; Goldbarg et al., 1963). The transpeptidation reaction in the presence of glycylglycine is pH-dependent (Ball, Revel and Cooper, 1956; Naftalin et al., 1969a).

When glycylglycine is used as the acceptor with γ -glutamyl- α -naphthylamide as the substrate, the reaction with γ -glutamyl transferase proceeds as follows:



Kulhanek and Dimov (1966) suggested determination of γ -glutamyl transferase activity by this reaction, which is followed by a direct coupling of the liberated α -naphthylamine with a stable diazonium compound (Fast Blue B salt). Their method is quicker, simpler and more

sensitive than older methods which did not use glycylglycine and in which α -naphthylamine was determined by a modified Bratton-Marshall reaction.

Determination of γ -glutamyl transferase activity was further simplified by the introduction of the directly chromogenic substrate L- γ -glutamyl-p-nitroanilide (Orlowski and Meister, 1963). This is a colourless, non-carcinogenic substrate which has a maximum absorbance at 315 nm and does not absorb at 405 nm. p-Nitroaniline liberated by the action of γ -glutamyl transferase exhibits an absorbance maximum at 405 nm. γ -Glutamyl transferase activity therefore can be determined either by measuring liberated p-nitroaniline colorimetrically after stopping the reaction with acid addition, or by continuous monitoring at 405 nm of the increase in absorbance of the incubation mixture due to the formation of p-nitroaniline (Orlowski, 1963).

γ -Glutamyl-p-nitroanilide has been gradually replacing γ -glutamyl-naphthylamides as substrate, γ -glutamyl-p-nitroanilide/glycylglycine being a quicker, simpler and cheaper method. However, for histochemical localization of γ -glutamyl transferase and for the staining of the zones of activity of the enzyme following electrophoresis, γ -glutamyl naphthylamides are still preferred to γ -glutamyl-p-nitroanilide as substrates because their solutions are more stable, especially at higher incubation temperatures, and because the products of the chromogenic reaction are less diffusible and more easily visualized (Albert, Orlowski and Szewczuk, 1961; Szewczuk, 1966; Kokot and Kuska, 1968; Igartua, Domecq and Findor, 1973; Hetland, Andersson and Gerner, 1975).

Some methods with L- γ -glutamyl-p-nitroanilide as a substrate use no

special acceptor for γ -glutamyl moiety and the substrate is converted into γ -glutamyl- γ -glutamyl-p-nitroanilide (Orlowski and Meister, 1963), other methods involve the use of glycylglycine as acceptor (Naftalin et al., 1969a; Szasz, 1969; Rosalki et al., 1970; Jacobs, 1971; Haesen, Berends and Zondag, 1972; Rosalki and Tarlow, 1974). Optimal conditions for this method which take account of both the dependence of the rate of reaction on such factors as substrate concentration and the solubility and stability of the substrate have been recommended (Fuke et al., 1976; Strømme and Theodorsen, 1976).

In recent years, γ -glutamyl-3-carboxy-4-nitroanilide (London et al., 1976; Shaw et al., 1977), γ -glutamyl-3-sulfo-4-nitroanilide (Bernt et al., 1975), γ -glutamyl-sulfanilide (Bucolo, Jaffe and Madappally, 1976), γ -L-glutamyl-5-amino-isophthalic acid dimethyl ester (MacQueen, Driscoll and Gargiulo, 1976) and γ -L-glutamyl-p-aminobenzoic acid (Szewczuk and Wellman-Bednawska, 1978) have been used for the assay of serum γ -glutamyl transferase activity. These newly synthesized substrates are more soluble than γ -glutamyl-p-nitroanilide, so that higher substrate concentrations can be attained.

In this work, γ -glutamyl transferase activity was determined by two methods: (i) at 37°C by the method of Rosalki and Tarlow (1974) with the LKB Produkter AB 8600 Reaction Rate Analyzer (LKB Instruments Ltd., Surrey, England), equipped with 410 nm interference filters; (ii) at 37°C with the slightly different conditions recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1976), using the same Reaction Rate Analyzer with 410 nm filters.

The latter method is more economical of reagents, although results are some 4% lower than by the Rosalki and Tarlow (1974) method (Hopkins and Moss, 1979). The method of Rosalki and Tarlow was used in earlier work, i.e., for γ -glutamyl transferase activity measurements during purification procedure of human liver γ -glutamyl transferase and in the experiments on electrophoretic patterns of γ -glutamyl transferase activity eluted from liver tissue. Later results were obtained by the Scandinavian method. However, since comparisons of activity within each experiment or group of experiments were made by a single method, the differences between the two methods are not significant.

(i) Reagents and procedure for the Rosalki and Tarlow method

- Buffer: - Tris (hydroxymethyl)aminomethane (115 mmol/l) -
glycylglycine (138 mmol/l) buffer, pH 8.5 at 37°C.
- Substrate: - L- γ -glutamyl-p-nitroanilide (104 mmol/l, in 0.5 mol/l
hydrochloric acid).

50 μ l of sample and 1.0 ml of buffer were pre-warmed to 37°C. The reaction was started by adding 0.10 ml of substrate and the rate of increase of absorbance of the reaction mixture was continuously monitored at 410 nm at 37°C. Instrument background absorbance compensation was set to a value of 0.5 and absorbance increases were usually monitored for 2 minutes (1 minute for samples with high γ -glutamyl transferase activities).

Samples with activities higher than 1,500 I.U./l were further diluted with the buffer in which they were dissolved (or with 0.9% NaCl in the case of serum samples) and their activities were re-determined to

get a more accurate estimation of their rate of absorbance increase. The volume of the assay mixture was 1.15 ml and the final reaction conditions were: Tris (hydroxymethyl)aminomethane - 100 mmol/l; glycylglycine - 120 mmol/l; γ -glutamyl-p-nitroanilide - 9.0 mmol/l; pH 8.0 at 37°C.

Calculation

One International Unit (I.U.) of γ -glutamyl transferase is the enzyme activity which liberates 1 μ mole of p-nitroaniline per minute under the test conditions. At 410 nm, the absorbance of a 1 mol/litre p-nitroaniline solution is 8.5×10^3 (for 1 cm lightpath cells), i.e., 1 μ mole of p-nitroaniline in 1 ml has an absorbance of 8.5.

The final test volume is 1.15 ml, so 1 μ mole of p-nitroaniline would increase the absorbance of the test mixture by $\frac{8.5}{1.15} = 7.39$.

The number of μ moles of p-nitroaniline liberated per minute is therefore equal to: $\frac{\Delta A/\text{min observed}}{7.39}$

This number of μ moles of p-nitroaniline are produced by 0.050 ml of sample containing the enzyme, therefore the number of μ moles of p-nitroaniline liberated per minute from 1 litre of sample, or I.U./l, is equal to:

$$\frac{\Delta A/\text{min}}{7.39} \times 20,000 \quad \text{or} \quad A/\text{min} \times 2706$$

(ii) Reagents and procedure for the Scandinavian method of determination of γ -glutamyl transferase activity

Buffer: - Tris (hydroxymethyl)aminomethane (120 mmol/l) -
Glycylglycine (90 mmol/l, containing $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
(12 mmol/l), pH 7.8 at 37°C (pH 8.2 at 25°C).

Substrate: - L- γ -glutamyl-p-nitroanilide (48 mmol/l, in 0.15 mol/l hydrochloric acid).

100 μ l of sample and 1.0 ml of buffer were pre-warmed to 37°C and the reaction was started by the addition of 100 μ l of substrate solution. The rate of increase of absorbance in the reaction mixture at 410 nm was continuously monitored for 2 minutes (1 minute for samples with very high γ -glutamyl transferase activity). The LKB 8600 Reaction Rate Analyzer background absorbance compensation was set to a value of 0.1.

Samples with activities above 1,500 I.U./l were diluted as in the Rosalki and Tarlow method.

The final volume of the reaction mixture was 1.20 ml and the assay conditions were: Tris (hydroxymethyl)aminomethane - 100 mmol/l; glycylglycine - 75 mmol/l; γ -glutamyl-p-nitroanilide - 4 mmol/l, pH 7.6 at 37°C.

Calculation

The final volume of the assay mixture is 1.20 ml, the increase in absorbance by 1 μ mole p-nitroaniline would be: $\frac{8.5}{1.20} = 7.08$.

The number of μ moles of p-nitroaniline liberated per minute is equal to:

$$\frac{\Delta A/\text{min observed}}{7.08}$$

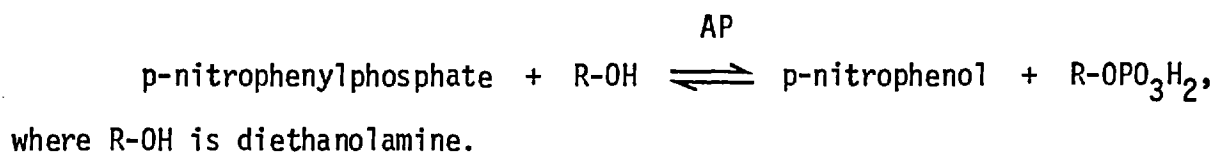
$$\text{Then } \frac{\Delta A/\text{min}}{7.08} \times 10,000, \quad \text{or} \quad A/\text{min} \times 1411$$

is the number of μ moles of p-nitroaniline liberated per minute from 1 litre sample (I.U./l).

b) Determination of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) activity

Alkaline phosphatase activity was measured by the method recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974). p-Nitrophenylphosphate ($\text{NO}_2\text{C}_6\text{H}_4\text{Na}_2\text{PO}_4 \cdot 6\text{H}_2\text{O}$) was used as the substrate, with diethanolamine as the phosphate acceptor and buffer.

The reaction proceeds according to the following equation:



The initial reaction rate was determined by continuous measurement (for 1 or 2 minutes) of the p-nitrophenol formation at 410 nm, using the LKB 8600 Reaction Rate Analyzer. The background absorbance compensation of the analyzer was set at 0.

Reagents and procedure

- Buffer: - Diethanolamine, $(\text{CH}_2\text{OHCH}_2)_2\text{NH}$ (1.01 mol/l), containing MgCl_2 (0.505 mmol/l) pH 9.8 at 37°C (pH 10.0 at 25°C).
- Substrate: - Para-nitrophenylphosphate (0.111 mol/l), in diethanolamine- MgCl_2 buffer.

20 μl of sample and 1.0 ml of buffer were pre-warmed to 37°C, and the reaction was started by the addition of 0.10 ml of p-nitrophenylphosphate. The rate of increase of absorbance in the reaction mixture was measured continuously at 410 nm.

The final volume of the reaction mixture was 1020 μl and the assay conditions were: diethanolamine - 1 mol/l; MgCl_2 - 0.5 mmol/l; paranitro-

phenylphosphate - 10 mmol/l; pH 9.8 at 37°C.

Calculations of the catalytic activity of alkaline phosphatase

$$\frac{\Delta A_{410} \text{ nm.min}^{-1}}{17200} \times \frac{1120}{20} \times 10^6, \text{ or}$$

$$\Delta A/\text{min} \times 3255.814,$$

where 17200 litre mol⁻¹ cm⁻¹ is the molar^{absorption} coefficient of p-nitrophenol at 410 nm in diethanolamine buffer (1 mol/l), pH 10 (25°C).

Precision of enzyme assays

A sample of pooled serum was introduced into each batch of γ -glutamyl transferase activity determinations. An estimate of long-term between-batch reproducibility of $\pm 4.7\%$ c.v. at the 75 I.U./l level was obtained for the assay of activity in 45 successive batches.

The estimation of long-term between-batch reproducibility for alkaline phosphatase activity measurements gave a value of $\pm 2.7\%$ c.v. at the 400 I.U./l level for the assay of activity (Whitaker, Whitby and Moss, 1977).

2.4 Zone electrophoresis

This was carried out in agarose gel (0.8% agarose (w/v) in barbitone buffer of ionic strength 0.05, pH 8.6) on glass plates (7.5 x 7.5 cm) in an MBI Immunoelectrophoresis tank (Medical and Biological Instrumentation Ltd., Ashford, Kent) in the cold room (4°C) for 2-2½ hours, with a voltage of 6 volts/cm.

After electrophoresis, zones of γ -glutamyl transferase activity

were located by overlaying the gel with a second layer of agarose gel containing 2-5 mg/ml γ -glutamyl- α -naphthylamide dissolved in a solution of 200 mmol/l Tris and 100 mmol/l glycylglycine, pH 7.4 at 37°C. γ -Glutamyl- α -naphthylamide was dissolved by boiling on a water-bath; preparation of solutions of the higher concentrations of this substrate needed rather prolonged heating (up to 30 minutes). α -Naphthylamine liberated by the action of γ -glutamyl transferase was rendered visible by immersing the gels on their supporting plates in a solution of Fast Blue B salt (1 mg/ml distilled water, containing 0.1% Triton X-100) to give a reddish-coloured precipitate. The zones of activity were scanned at 540 nm in a DCD-16 Digital Computing Densitometer, Model 39431 (Gelman Instrument Company, 600 S. Wagner Road, Ann Arbor, Michigan 48106, U.S.A.).

After scanning, the same plates were stained for protein with Amidoschwartz 10B (2.5 g/l solution in methanol, containing 3% trichloroacetic acid), then washed several times with methanol:glacial acetic acid solution (9:1 v/v) to get rid of the excess stain. Protein-stained gels were scanned again at 590 nm and the mobility of albumin in the marker serum on each plate was determined. The mobilities of γ -glutamyl transferase activity zones were expressed as fractions of the mobility of albumin.

2.5 Gel-filtration

(a) Through Sephadex G-200

Sephadex is a bead-formed gel prepared by cross-linking dextran (an anhydrous polymer of glucose units) with epichlorohydrin. It contains a large number of hydroxyl groups in the polysaccharide chains, which makes the gel very hydrophilic. Sephadex swells in water and electrolyte solutions. It is available in different particle-size grades.

Sephadex G-200, used in this work, has a protein- and peptide-molecular weight fractionation range of 5,000 - 800,000. The gel, fully swollen and pre-equilibrated with 0.1 mol/l Tris-HCl buffer pH 8.0, was packed into a column (2.6 x 100 cm; Pharmacia, Uppsala, Sweden) at a flow rate of 12 ml/hr. This column was used in the purification of γ -glutamyl transferase from human liver.

A smaller column (0.9 x 30 cm; Pharmacia, Uppsala, Sweden) was used for determination of the molecular weights of various γ -glutamyl transferase preparations. The flow rate for this column was 6 ml/hr.

(b) Through Sepharose 6B

Sepharose is the trade name for spherical-formed gel beads prepared from agarose. Agarose is a linear polysaccharide which consists of alternating residues of D-galactose and 3,6-anhydro-L-galactose units. Sepharose contains only a very small number of residual charged groups, so the interference of non-specific ionic binding with gel-filtration is minimal. There are three types of Sepharose available; they differ in agarose concentrations and have different fractionation ranges.

Sepharose 6B has agarose concentration of approximately 6% of swollen beads suspended in distilled water and has a molecular weight fractionation range of $1 \cdot 10^4 - 4 \times 10^6$ for proteins and $1 \cdot 10^4 - 1 \times 10^6$ for polysaccharides (Pharmacia Fine Chemicals Booklets: "Gel Filtration - Theory and Practice" and "Beaded Sepharose 2B-4B-6B").

Sepharose 6B pre-equilibrated in 0.1 mol/l Tris-HCl buffer pH 8.0 containing 0.1 mol/l NaCl was packed into a "Quickfit" glass column (size 1.5 x 95 cm; Jobling Corning Ltd., Durham, England) at a flow rate of

12 ml/hr. This column was used for the preparation of γ -glutamyl transferase fractions from human bile and serum and for the determination of molecular weights of γ -glutamyl transferase preparations. A smaller Sepharose 6B column (size 0.6 x 45 cm) was used for the gel-filtration analysis of serum samples from individual patients. This column was operated at a flow rate of 6 ml/hr.

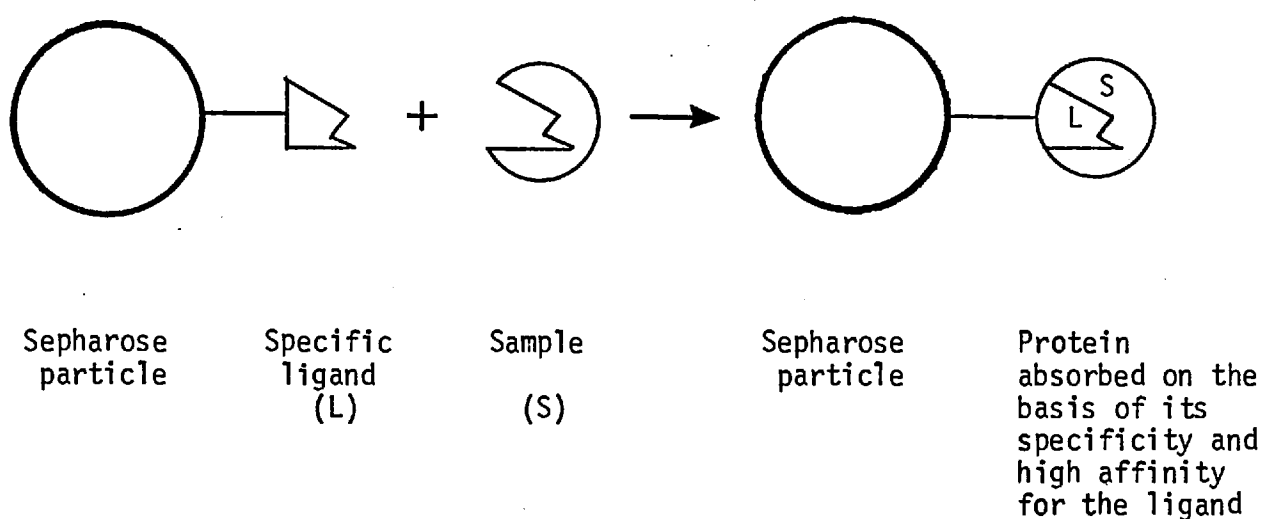
2.6 Ion-exchange chromatography on DE-52 Diethylaminoethyl (DEAE) cellulose

In this work, DE-52 DEAE-cellulose was used in the purification of human liver γ -glutamyl transferase. The exchanger preparation before packing the column included precycling, degassing, removal of fines and equilibration as described in the Whatman Company Ltd. laboratory manual and information leaflet IL-2 on "Advanced ion-exchange celluloses". The DEAE-cellulose thus prepared was packed into a column (2.2 x 30 cm; Wright Scientific Ltd., England) at a flow rate of 45 ml/hr. Elution conditions are described under "Results".

2.7 Affinity chromatography on Concanavalin A-Sepharose 4B

Affinity chromatography is a type of adsorption chromatography in which the bed material has a specific capacity to bind the substance to be isolated. The molecule to be purified is non-covalently and reversibly absorbed by a binding group (a ligand), which is immobilized on an insoluble supporting material (the matrix).

The principle of affinity chromatography can be illustrated as follows:



Specific absorptive properties of the bed material are obtained by finding an appropriate ligand (such as a substrate or an inhibitor of an enzyme) and coupling it covalently to an insoluble matrix. The ligand can then selectively absorb the substance of interest (e.g., the enzyme) from solution and, after unbound materials have been washed away, the bound substance can be desorbed by changing the experimental conditions. Affinity-chromatography separations can be highly specific and rapid, since usually only small bed volumes are required.

Sephacrose, which is a bead-formed agarose gel, is usually used as a matrix since it has good flow properties and does not interfere with the separation due to any non-specific effects. Sepharose 4B has an open pore structure, its exclusion limit in gel-filtration of proteins is about 20×10^6 molecular weight, and the hydroxyl groups on its sugar residues can be easily derivatized for covalent attachment of a ligand.

Concanavalin A was chosen as the ligand for γ -glutamyl transferase preparations from human liver, bile and serum since it binds glycoproteins such as γ -glutamyl transferase. Concanavalin A is a protein from the jack bean belonging to a group of plant proteins known as lectins and it is a specific absorbent for terminal α -D-glucopyranosyl, α -D-mannopyranosyl or sterically similar residues. The Concanavalin A-Sepharose 4B (Concanavalin A covalently bound to Sepharose 4B) used in this work is supplied as a suspension of sedimented gel in acetate buffer solution (see "Column chromatography materials"): 1 ml of swollen gel contains about 8 mg of Concanavalin A.

Preparation of column for affinity chromatography

Concanavalin A-Sepharose 4B was washed in 0.1 $\frac{\text{mol}}{\text{l}}$ Tris-HCl buffer pH 8.0, containing 0.1 $\frac{\text{mol}}{\text{l}}$ NaCl, 1 $\frac{\text{mmol}}{\text{l}}$ MgCl_2 and 1 $\frac{\text{mmol}}{\text{l}}$ CaCl_2 (buffer 1) several times, then loaded on to the column (0.9 x 30 cm; Pharmacia, Uppsala, Sweden) at a flow rate of 30 ml/hr. The bed material was washed with 30 ml of buffer 1, then with 60 ml of the same buffer containing 50 mg/ml α -methyl-D-glucoside (1-O-methyl- α -D-glucopyranoside) and then again with about 400 ml of buffer 1.

The loading of samples and elution conditions are described under "Results".

CHAPTER 3

OBSERVATIONS ON γ -GLUTAMYL TRANSFERASE IN HUMAN LIVER, BILE AND SERUM

CHAPTER 3

OBSERVATIONS ON γ -GLUTAMYL TRANSFERASE IN HUMAN LIVER, BILE AND SERUM

3.1 Introduction

The aims of the investigations reported in this chapter were to identify and characterize naturally-occurring fractions of γ -glutamyl transferase in human liver, bile and serum. The experiments included purification of the enzyme from human liver, preparation of γ -glutamyl transferase fractions from human bile and serum, and characterization of their physical and catalytic properties. Studies on the activity and different molecular weight forms of γ -glutamyl transferase in individual bile samples are also included.

3.2 Purification of human liver γ -glutamyl transferase

Human liver tissue was obtained at autopsy as soon as possible after death from patients in whom the liver was not considered to be affected by the cause of death. The tissue was stored in the deep-freezer at -25°C until required. The purification procedure was mainly according to Huseby (1977) with some modifications. The main modification in the purification method was the absence of the following steps: (a) treatment of γ -glutamyl transferase with a proteolytic enzyme, and (b) affinity chromatography on Concanavalin A-Sepharose 4B. Proteolysis and binding to Concanavalin A were used later as means of characterizing the partially-purified liver enzyme.

At each step of the purification, the volume of the solution, γ -glutamyl transferase activity and protein concentration were measured. γ -Glutamyl transferase activity was determined by the method of Rosalki and Tarlow (1974) with the LKB 8600 Reaction-Rate Analyzer. Protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Homogenization

763.5 g of liver tissue thawed at room temperature was homogenized thoroughly in four parts (3,050 ml) of ice-cold 80 mmol/l MgCl_2 containing 0.75 mmol/l NaOH in a Waring blender for 2 mins, then with an Ultra-Turrax homogenizer for 2.5 mins. The homogenate was filtered through gauze.

Solubilization

The filtrate was incubated for 2 h at 37°C and kept in the cold room overnight. After centrifugation at 25,000 x g for 30 mins, the supernatant containing about 3% of the total γ -glutamyl transferase activity was discarded. The pellet was homogenized with the Ultra-Turrax homogenizer in ice-cold 0.1 mol/l Tris-HCl buffer, pH 8.5, for 2 - 3 mins. Lubrol WX and Na-deoxycholate were added to final concentrations of 0.5% (w/v) each, and this mixture was stirred overnight in the cold-room (4°C). After centrifugation at 25,000 x g for 30 mins, 95% of initial γ -glutamyl transferase activity was recovered in the supernatant, and the pellet was discarded.

Acetone precipitation

Cold acetone (-15°C) was added dropwise, slowly and with continuous

stirring, to the Na-deoxycholate-Lubrol WX extract to give a final concentration of 25%. After one hour, the suspension was centrifuged at $2,200 \times g$ for 30 mins and cold acetone (-15°C) was added to the supernatant to a 57% final concentration. This mixture was further stirred on ice for 1 h, then centrifuged for 30 mins at $2,200 \times g$. The pellet was resuspended in 0.1 mol/l Tris-HCl buffer, pH 8.5, containing 0.5% Na-deoxycholate. The suspension was stirred overnight in the cold room, then centrifuged at $25,000 \times g$ for 30 mins. The pellet was re-extracted with the same buffer containing 0.5% Na-deoxycholate for 2 h and centrifuged again at $27,000 \times g$ for 30 mins. The two supernatants were pooled and dialyzed against the solution of Na-deoxycholate in buffer to remove any traces of acetone.

Butanol treatment

Cold n-butanol (-15°C) was added dropwise, over a period of 30 mins, to the combined supernatants, stirred on ice, to a 25% concentration. The suspension was further stirred for 30 mins and centrifuged at $40,000 \times g$ for half an hour.

The butanol phase on top was removed by aspiration and the precipitate discarded. The water phase was filtered through several layers of gauze to get rid of a lipid-like substance. Na-deoxycholate was added to a final concentration of 0.2% (w/v). This solution was extensively dialyzed versus 0.1 mol/l Tris-HCl buffer, pH 8.5, containing 0.2% Na-deoxycholate.

Ammonium sulphate precipitation

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the dialyzed solution to 20%

saturation. After half an hour, the precipitate was removed by centrifugation at 12,000 x g for 10 mins. Further $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to give 70% saturation. After one hour, the suspension was centrifuged at 20,000 x g for 30 mins. The pellet was redissolved in 0.1 mol/l Tris-HCl buffer, pH 8.5, containing 0.1% Na-deoxycholate, and dialyzed against 10 litres of this buffer overnight. The dialyzed solution was concentrated with "Carbowax" 20m to a volume of 62 ml. The solution was very clear initially, but as the volume went down below about 90 ml some precipitate appeared, which did not redissolve on dialysis versus the same buffer.

DEAE-cellulose ion-exchange chromatography

The concentrated solution was centrifuged at 30,000 x g for 30 mins and the supernatant applied to the column (2.2 x 30 cm) pre-equilibrated with 0.1 mol/l Tris-HCl Buffer, pH 8.5, containing 0.1% Na-deoxycholate. The column was washed with two sample volumes (about 120 ml) of dialyzing buffer and the enzyme was eluted with a linear gradient of increasing chloride concentration generated by gradual addition of 0.5 mol/l Tris-HCl buffer, pH 9.0, containing 0.5 mol/l NaCl and 0.1% Na-deoxycholate to 500 ml of the dialyzing buffer. A flow rate of 45 ml/hr was used and 9 ml fractions were collected. The elution profile of human liver γ -glutamyl transferase at this step is shown in Fig. 3.1.

The active fractions (Nos. 66 - 76) were pooled, concentrated by dialysis against "Carbowax" 20m to a volume of 7 ml, and dialyzed against several changes of 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1% Na-deoxycholate. The recovery of the enzyme activity after DEAE-cellulose ion-exchange chromatography was 76.7%.

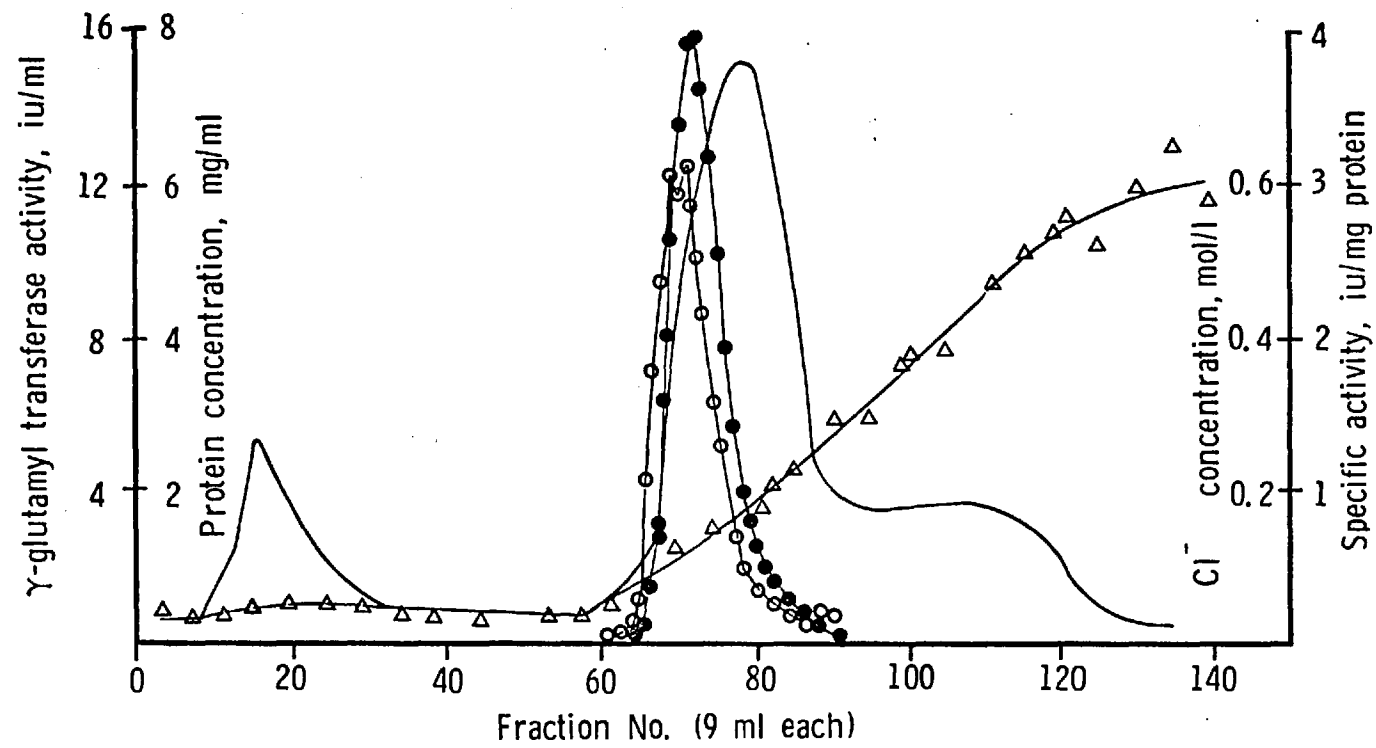


Fig. 3.1

DEAE-cellulose ion exchange chromatography of human liver γ -glutamyl transferase.

γ -glutamyl transferase activity ●—●
 protein concentration —
 specific activity ○—○
 Cl⁻ concentration △—△

Sephadex G-200 gel-filtration

The concentrated enzyme solution was applied to a Sephadex G-200 column (2.6 x 100 cm) pre-equilibrated with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1% Na-deoxycholate. The column was eluted with the same buffer, at a flow rate of 12 ml/hr, and 5 ml fractions were collected (see Fig. 3.2).

Fractions Nos. 40 - 52, which contained most of the γ -glutamyl-transferase activity, were pooled. The peak of the enzyme activity was eluted at 230 ml, i.e., at 1.3 x void volume of 176 ml.

After gel-filtration on Sephadex G-200 column, 67.5% of the applied γ -glutamyl transferase activity was recovered.

The Sephadex G-200 (2.6 x 100 cm) column was calibrated with the following marker proteins: catalase, γ -globulins, bovine serum albumin, ovalbumin, peroxidase and cytochrome C (for source and molecular weight values see Chapter 2). A sample of serum with alkaline phosphatase activity of 1,900 I.U./l (represented mainly by the liver isoenzyme) was also used for calibration. Blue Dextran with average molecular weight of 2,000,000 was used for determination of the void volume.

The elution volume of γ -glutamyl transferase which was probably complexed with Na-deoxycholate corresponded to a molecular weight value of approximately 160,000.

Removal of Na-deoxycholate

The pH of the solution of the combined fractions was adjusted to 7.5 with 1 mol/l HCl and MgCl_2 was added to a concentration of 80 mmol/l.

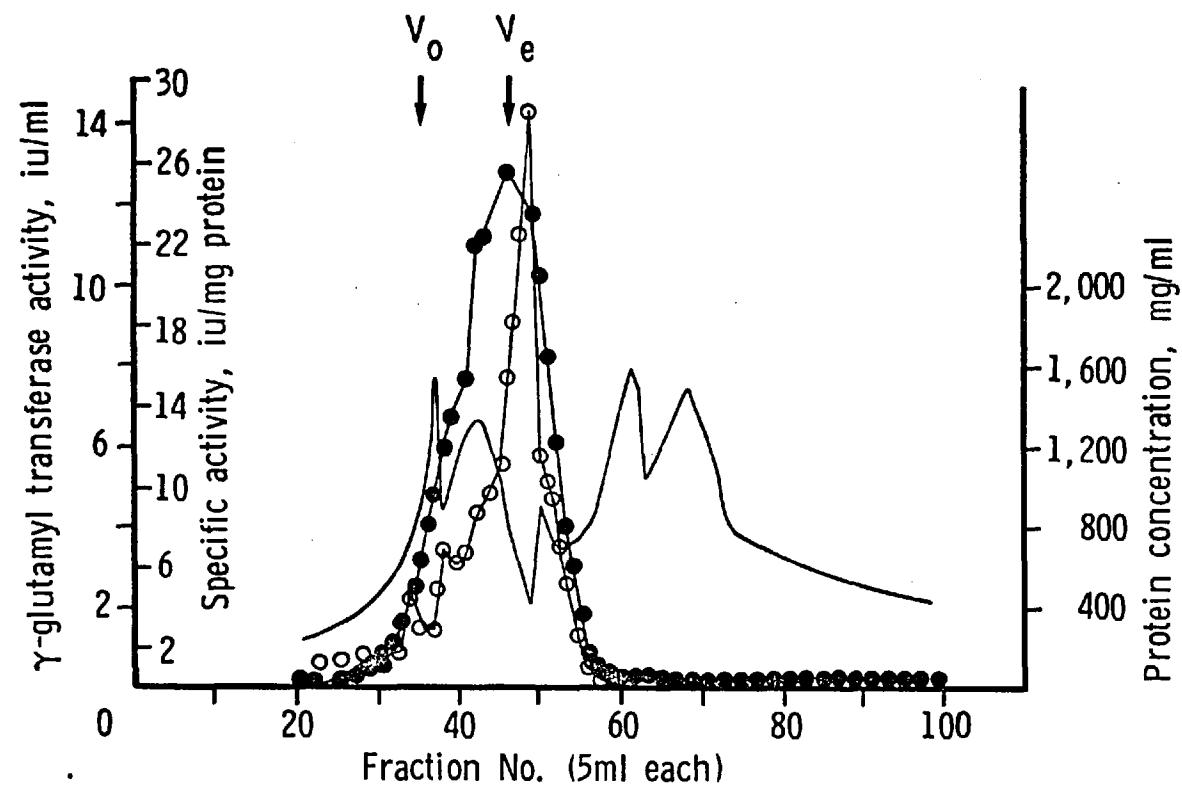


Fig. 3.2

Sephadex G-200 gel-filtration of human liver
γ-glutamyl transferase activity.

γ-glutamyl transferase activity ●—●
protein concentration —
specific activity ○—○
 V_0 = void volume, V_e = elution volume of
γ-glutamyl transferase

This mixture was stirred in the cold room for 6 hours and then centrifuged at 20,000 x g for 15 mins. The supernatant containing all the γ -glutamyl transferase activity was concentrated with "Carbowax" 20m to a 12 ml volume, then dialyzed versus 0.1 mol/l Tris-HCl buffer, pH 8.0.

Upon the removal of the detergent, the enzyme tended to precipitate out of solution rather easily. This was probably due to the formation of aggregates of the enzyme molecules and led to an increase in its apparent molecular weight. The average molecular weight of γ -glutamyl transferase purified from human liver was, in the absence of detergents, about 550,000. This preparation of the enzyme was used in all experiments described later in the thesis.

The summary of the purification procedure of human liver γ -glutamyl transferase is given in Table 3.1.

3.3 Human bile γ -glutamyl transferase

γ -Glutamyl transferase activity in the bile is usually higher than that in serum (Orlowski, 1963; Kuska, 1965).

Human bile γ -glutamyl transferase has been partially purified and some of its properties studied by Indirani and Hill (1977), who suggested that bile may serve as a useful source of this enzyme for further studies on the physiological role and mechanism of action of γ -glutamyl transferase.

Measurement of γ -glutamyl transferase activity in individual bile samples

Bile samples were obtained from several patients with either cancer of the pancreas or cancer of the bile duct by means of percutaneous

Table 3.1 - Summary of purification of γ -glutamyl transferase from human liver

Steps	Total volume, ml	Protein		γ -Glutamyl transferase activity			Recovery of activity %	Purification fold
		concentration mg/ml	total mg	I.U./l	Total I.U.	Specific I.U./mg protein		
I Homogenization	3,770	25.18	94,923	970	3,659	0.038	100	1.0
II Detergent extraction (0.5% Na-deoxycholate and 0.5% Lubrol WX)	1,807	13.22	23,882	1,928	3,484	0.146	95	3.8
III Acetone precipitation	1,805	9.20	16,600	1,455	2,626	0.158	72	4.2
IV Butanol treatment	1,760	1.92	3,372	919	1,617	0.480	44	12.6
V Ammonium sulphate precipitation	57	18.88	1,076	22,947	1,308	1.216	36	32.0
VI DEAE-cellulose ion-exchange chromatography	99	4.69	464	10,131	1,003	2.162	27	56.9
VII Sephadex G-200 column chromatography	65	0.94	61	10,410	677	11.10	18.5	292.0

intrahepatic decompression. All samples were stored in the refrigerator ($0 - 4^{\circ}\text{C}$) until required. Some sediment appeared in the bile under storage; therefore each sample was filtered through glass wool before use.

γ -Glutamyl transferase activity was measured by the Scandinavian method for this enzyme, as described in "Materials and Methods". Because of their intense yellow to greenish colouration, whole bile samples had to be diluted at least 1:4 for the enzyme activity to be measured. Alkaline phosphatase activity was measured by the appropriate Scandinavian method (see "Materials and Methods" section).

Bilirubin concentration in bile samples was measured by the Technicon SMA 12/60 method, in which bilirubin is reacted with diazotized sulphanilic acid in the presence of caffeine-sodium benzoate reagent to form coloured azobilirubin. The absorbance of the alkaline azobilirubin in the sample analytical stream was measured at 600 nm in a 15 mm length by 1.5 mm internal diameter flowcell. The blank was determined by mixing the sample stream with caffeine, sulphanilic acid (instead of the diazo-reagent) and tartrate. The absorbance of the blank analytical stream was measured at 600 nm in a 15 mm length by 1.5 mm internal diameter flowcell, and the blank value was automatically subtracted by differential colorimetry.

Bile acids concentration was measured according to Talalay (1960).

Effect of dilution on γ -glutamyl transferase activity in human bile

Eighteen samples were diluted with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl. The following serial dilutions of bile

in the assay mixture were made: 1:48, 1:96, 1:192, 1:384, 1:768 and 1:1,536. γ -Glutamyl transferase activity was measured in all samples. The activity measured at the lowest possible dilution of bile in the assay mixture here will be referred to as "initial activity".

As can be seen from Table 3.2, γ -glutamyl transferase activity increased with dilution in all bile samples with the maximum increase (78%) in sample No. 10, which had an initial activity of 127 I.U./l, measured at the 1:48 dilution of bile. The smallest increase in γ -glutamyl transferase activity was observed in bile sample No. 12 (initial activity 1,271 I.U./l). For each dilution factor in Table 3.2 the mean γ -glutamyl transferase activity was calculated and the increase in activity was compared to that of the mean initial activity in the 18 bile samples. Fig. 3.3 shows the plot of the average increase in γ -glutamyl transferase activity versus dilution factor and the change of the enzyme activity with dilution for bile samples No. 10 (highest degree of activation) and No. 12 (minimum activation effect).

To compare the effect of dilution on γ -glutamyl transferase with another biliary enzyme, alkaline phosphatase activity was measured in the diluted bile samples, the dilution factors for the bile in the reaction mixture being: 1:56, 1:112, 1:224, 1:448, 1:896, 1:1,792 and 1:3,584. The results obtained are presented in Table 3.3. As with γ -glutamyl transferase, an increase in alkaline phosphatase activity with increase in dilution factor was observed for all bile samples, except sample No. 15, which had the highest initial activity of 10,842 I.U./l. The highest activation of alkaline phosphatase activity with dilution was observed in

Table 3.2 - Effect of dilution on γ -glutamyl transferase activity in human bile samples. Figures in parentheses are percentage increases in activity compared with initial activity

Sample No.	Final dilution of bile in reaction mixture					
	1:48	1:96	1:192	1:384	1:768	1:1,536
1	271	294 (8.5)	294 (8.5)	294 (8.5)	317 (17.0)	362 (33.6)
2	345	362 (4.9)	367 (6.4)	407 (18.0)	407 (18.0)	497 (44.1)
3	356	373 (4.8)	390 (9.6)	396 (11.2)	429 (20.5)	542 (52.2)
4	548	588 (7.3)	616 (12.4)	644 (17.5)	768 (40.2)	814 (48.6)
5	249	263 (5.6)	294 (18.1)	294 (18.1)	316 (26.9)	316 (26.9)
6	881	994 (12.8)	1,107 (25.7)	1,107 (25.7)	1,152 (30.8)	1,175 (33.4)
7	689	746 (8.3)	791 (14.8)	814 (18.2)	859 (24.7)	859 (24.7)
8	808	915 (13.3)	994 (23.0)	1,028 (27.2)	994 (23.0)	1,130 (39.9)
9	1,090	1,209 (10.9)	1,288 (18.2)	1,299 (19.2)	1,356 (24.4)	1,536 (40.9)
10	127	130 (2.4)	136 (7.1)	147 (15.7)	226 (78.0)	226 (78.0)
11	486	520 (7.0)	565 (16.3)	576 (18.5)	655 (34.8)	723 (48.8)
12	1,271	1,367 (7.6)	1,446 (13.8)	1,491 (17.3)	1,491 (17.3)	1,581 (24.4)
13	271	288 (6.3)	283 (4.4)	305 (12.5)	316 (16.6)	452 (66.8)
14	970	1,243 (28.1)	1,310 (35.1)	1,333 (37.4)	1,378 (42.1)	1,400 (44.3)
15	2,132	2,259 (6.0)	2,372 (11.3)	2,666 (25.0)	2,711 (27.2)	2,802 (31.4)
16	263	302 (14.8)	305 (16.0)	328 (24.7)	328 (24.7)	362 (37.6)
17	780	859 (10.1)	859 (10.1)	915 (17.3)	904 (15.9)	994 (27.4)
18	396	430 (8.6)	441 (11.4)	430 (8.6)	543 (37.1)	543 (37.1)
AVERAGE	663	730 (10.1)	770 (16.1)	804 (21.3)	842 (27.0)	906 (36.7)

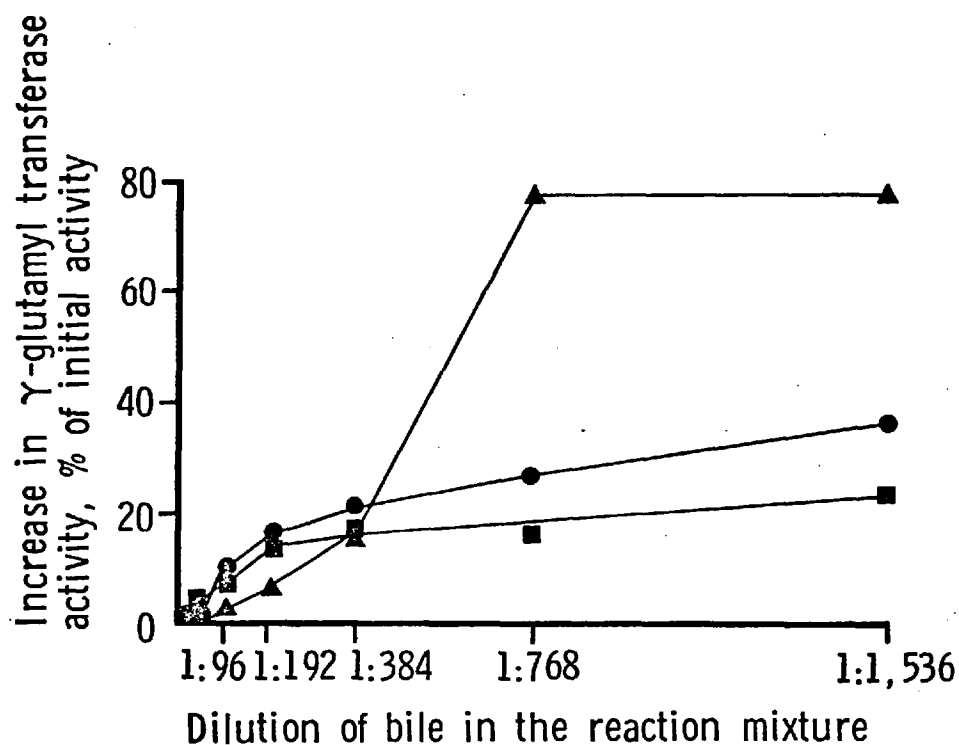


Fig. 3.3

Effect of dilution on γ -glutamyl transferase activity in human bile samples: bile sample no. 10, with highest increase in activity (▲—▲); bile sample no. 12 with lowest increase in activity (■—■) and the mean increase in activity for 18 bile samples (●—●).

Table 3.3 - Effect of dilution on alkaline phosphatase activity in human bile samples. Figures in parentheses are percentage increases in activity compared with initial activity.

Sample No.	Final dilution of bile in reaction mixture						
	1:56	1:112	1:224	1:448	1:896	1:1,792	1:3,584
1	151	268 (77.5)	439 (190.7)	586 (288.1)	599 (296.7)	610 (304.0)	632 (318.5)
2	75	248 (230.7)	413 (450.7)	502 (569.3)	526 (601.3)	---	---
3	110	298 (170.9)	350 (218.2)	454 (312.7)	470 (327.3)	---	---
4	105	190 (81.0)	325 (209.5)	555 (428.6)	785 (647.6)	1,005 (857.1)	1,166 (1,010.4)
5	73	145 (98.6)	253 (246.6)	366 (401.1)	442 (505.5)	623 (753.4)	---
6	391	611 (56.3)	1,059 (170.8)	1,264 (223.3)	1,360 (247.8)	1,724 (340.9)	2,004 (412.5)
7	1,095	1,374 (25.5)	1,695 (54.8)	2,043 (86.6)	2,487 (127.1)	3,051 (178.6)	3,127 (185.6)
8	1,303	1,413 (8.4)	1,622 (24.5)	1,634 (25.4)	1,842 (41.4)	1,988 (52.6)	2,093 (60.6)
9	1,251	1,528 (22.1)	1,748 (39.7)	2,311 (84.7)	2,742 (119.2)	3,139 (150.9)	3,022 (141.6)
10	171	239 (39.8)	343 (100.6)	390 (128.1)	433 (153.2)	418 (144.4)	490 (186.5)
11	487	1,223 (151.1)	1,518 (211.7)	1,654 (239.6)	1,784 (266.3)	1,726 (254.4)	1,858 (281.5)
12	2,015	2,146 (6.5)	3,087 (53.2)	2,910 (44.4)	3,433 (70.4)	3,592 (78.3)	3,654 (81.3)
13	877	900 (2.6)	1,040 (18.6)	1,070 (22.0)	1,213 (38.3)	1,367 (55.9)	1,353 (54.3)
14	3,820	4,490 (17.5)	5,233 (37.0)	5,059 (32.4)	5,442 (42.5)	5,735 (50.1)	5,944 (55.6)
15	10,842	8,896 (0)	9,796 (0)	8,791 (0)	8,948 (0)	8,790 (0)	8,624 (0)
16	791	858 (8.5)	942 (19.1)	895 (13.1)	895 (13.1)	945 (19.5)	832 (5.2)
17	2,540	2,721 (7.1)	2,878 (13.3)	2,981 (17.4)	3,056 (20.3)	3,537 (39.2)	3,608 (42.0)
18	1,277	1,361 (6.6)	1,392 (9.0)	1,499 (17.4)	1,517 (18.8)	1,538 (20.4)	1,758 (37.7)
AVERAGE	1,521	1,606 (5.6)	1,896 (24.7)	1,943 (27.7)	2,110 (38.7)	2,487 (63.5)	2,678 (76.1)

sample No. 4, where the activity of this enzyme increased more than ten times at a dilution of 1:3,584. Sample No. 16 on the other hand, exhibited the lowest degree of activation of alkaline phosphatase by dilution. The apparent decrease in activity (about 20%) in sample No. 15 could be caused by inaccurate dilutions.

A plot for the average increase in alkaline phosphatase activity for 18 bile samples and also plots representing maximum (sample No. 4) and minimum (sample No. 16) degrees of activation of this enzyme by dilution are shown in Fig. 3.4.

γ -Glutamyl transferase activity in individual bile samples

γ -Glutamyl transferase activity changes in several specimens of bile of each of the 4 patients undergoing percutaneous intrahepatic decompression for obstructive jaundice were studied. Alkaline phosphatase activity and bilirubin levels were also measured. Because of the effect of dilution on the activity of γ -glutamyl transferase and alkaline phosphatase in the bile, all specimens were diluted about one hundred times (with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl) before the activities of these two enzymes were estimated.

It cannot be said that at a dilution factor of the whole bile of about 1:100, which corresponds to final dilutions in the assay mixtures of 1:1,200 for γ -glutamyl transferase and 1:1,560 for alkaline phosphatase, maximum enzyme activities have been obtained. However, the samples were not diluted further, because of the decreasing reliability of the measurements of the resulting low enzyme activities.

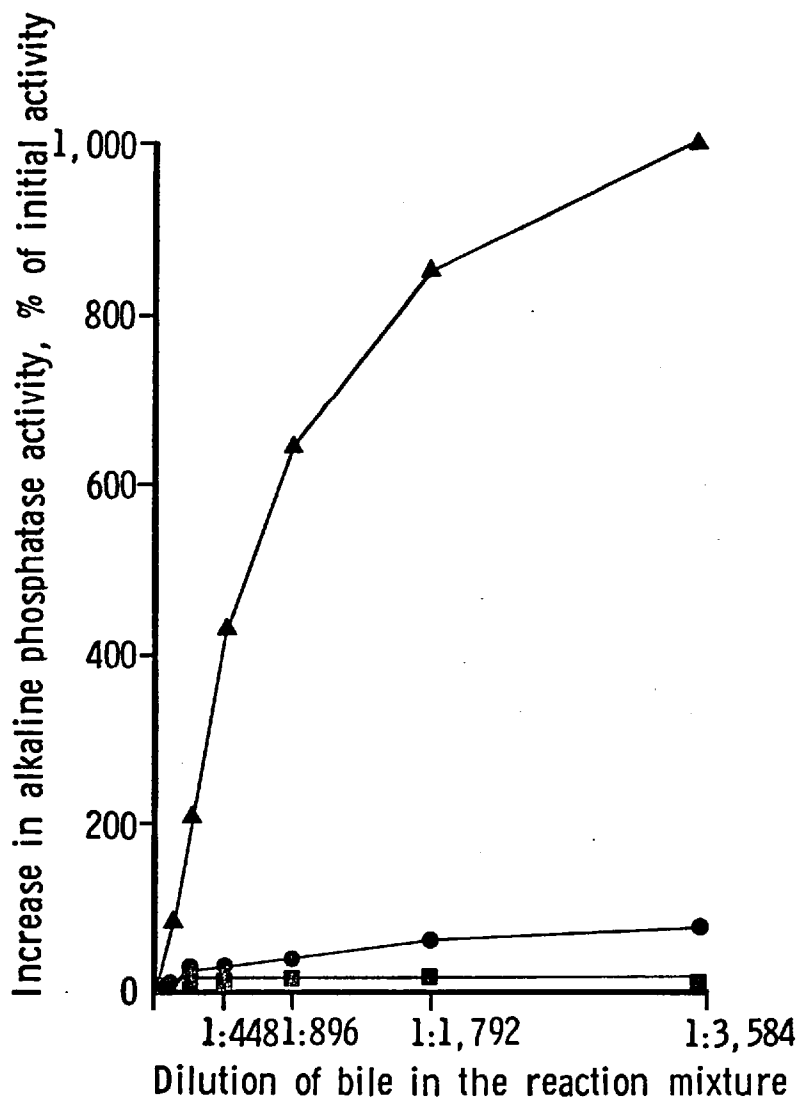


Fig. 3.4

Effect of dilution on alkaline phosphatase activity in human bile samples: bile sample no. 4, with highest increase in activity (▲—▲); bile sample no. 16, with lowest increase in activity (■—■), and the mean increase in activity for 18 bile samples (●—●).

The following patients were studied:

Patient I (M, D.O.B. 1916) - cancer of the pancreas

Patient II (M, D.O.B. 1921) - cancer of the pancreas

Patient III (M, D.O.B. 1932) - cancer of the bile duct

Patient IV (M, D.O.B. 1945) - suspected cancer of the bile duct

Results obtained for bile samples of each of these patients were plotted against the date of collection of bile specimen, and these plots are shown in Figs. 3.5, 3.6, 3.7 and 3.8a and b.

The pattern of changes of γ -glutamyl transferase and alkaline phosphatase activities were rather similar for all patients, except that of patient III. In all the patients studied, the activity of alkaline phosphatase in the bile was always higher than that of γ -glutamyl transferase.

In patients I and II, an overall tendency towards gradual increase in the activities of both enzymes was observed until about 13 - 15 days after the start of the decompression. After that, both activities dropped considerably. The levels of bilirubin in the bile of patients I and II gradually increased until the maximum level was reached between the 6th and 8th day after the beginning of decompression (Figs. 3.5 and 3.6). In patient II, average γ -glutamyl transferase activity in the bile was about 1.5 times lower and average alkaline phosphatase activity was about the same as in patient I. Average bilirubin concentration in the bile of patient II was approximately twice that of patient I.

In patient III (Fig. 3.7), γ -glutamyl transferase activity in the bile did not change as much as that of alkaline phosphatase; the latter

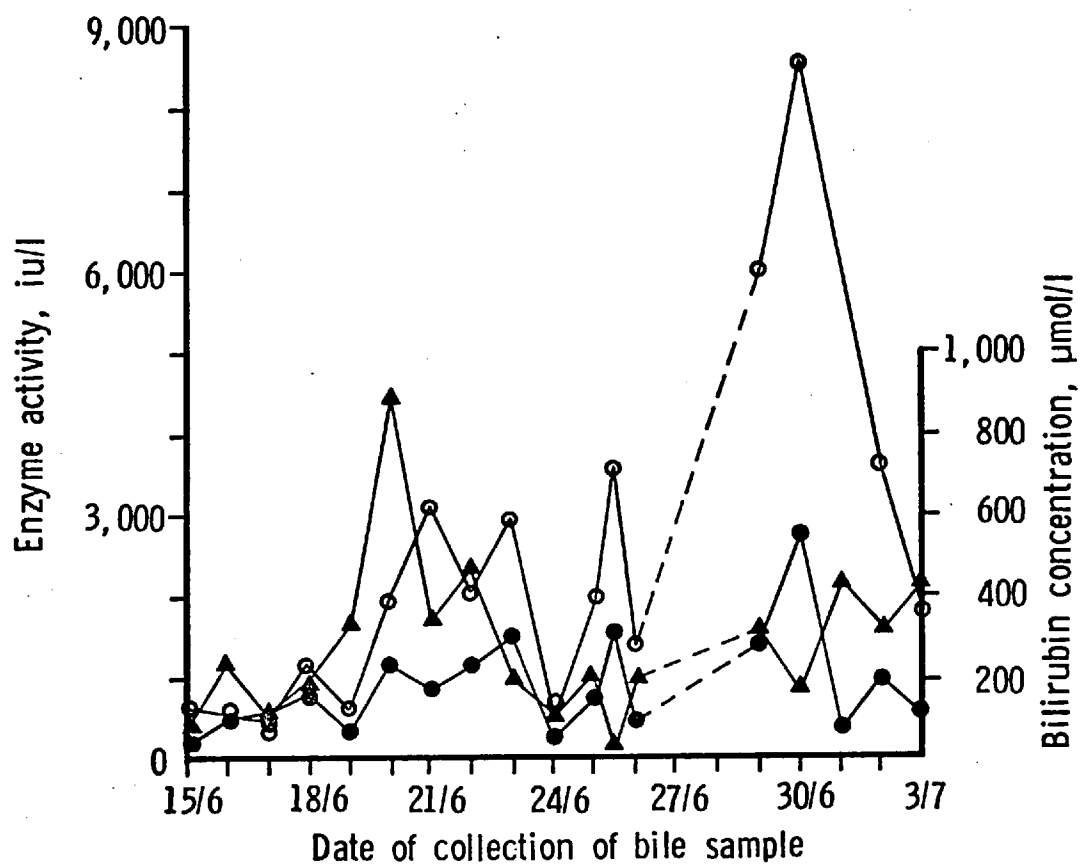


Fig. 3.5 γ -Glutamyl transferase activity (●—●), alkaline phosphatase activity (○—○) and bilirubin concentration (▲—▲) in bile samples from patient I.

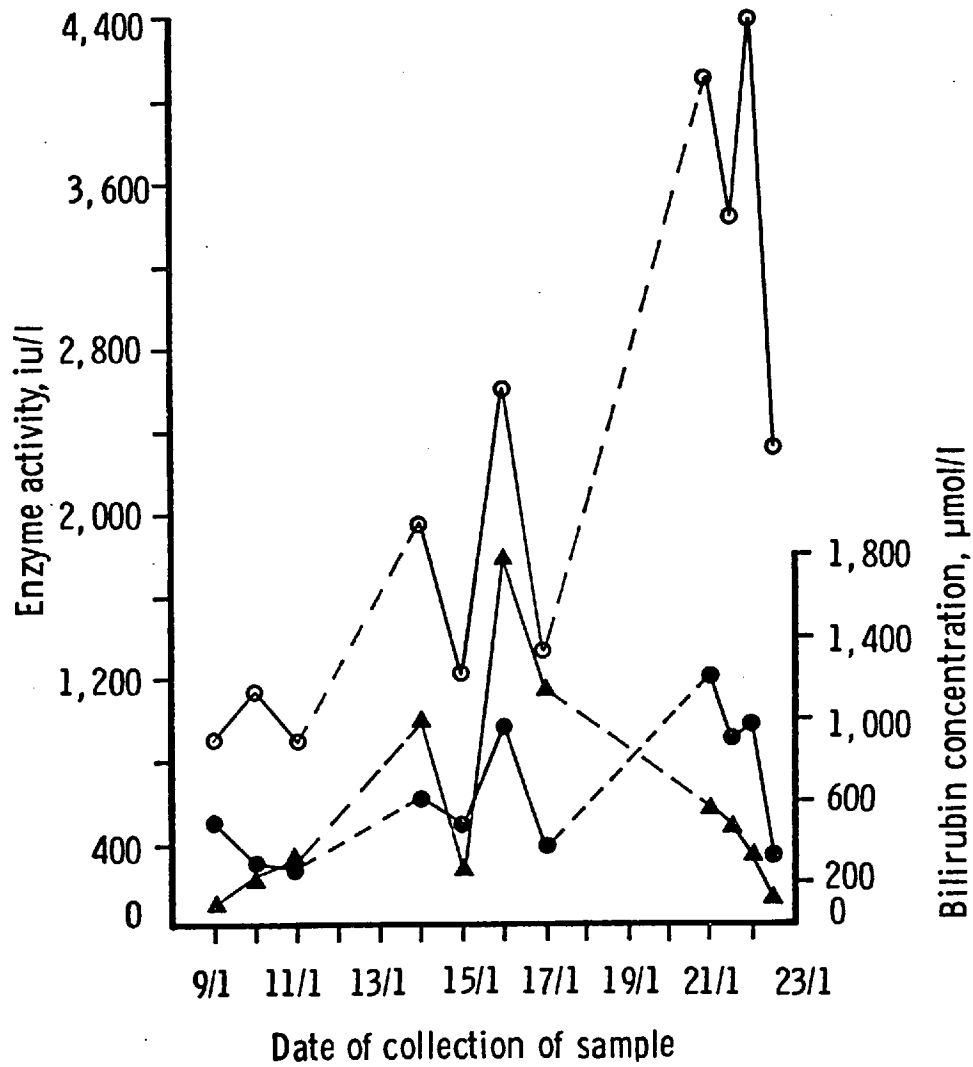


Fig. 3.6

γ -Glutamyl transferase activity (●—●), alkaline phosphatase activity (○—○) and bilirubin concentration (▲—▲) in bile samples from patient II.

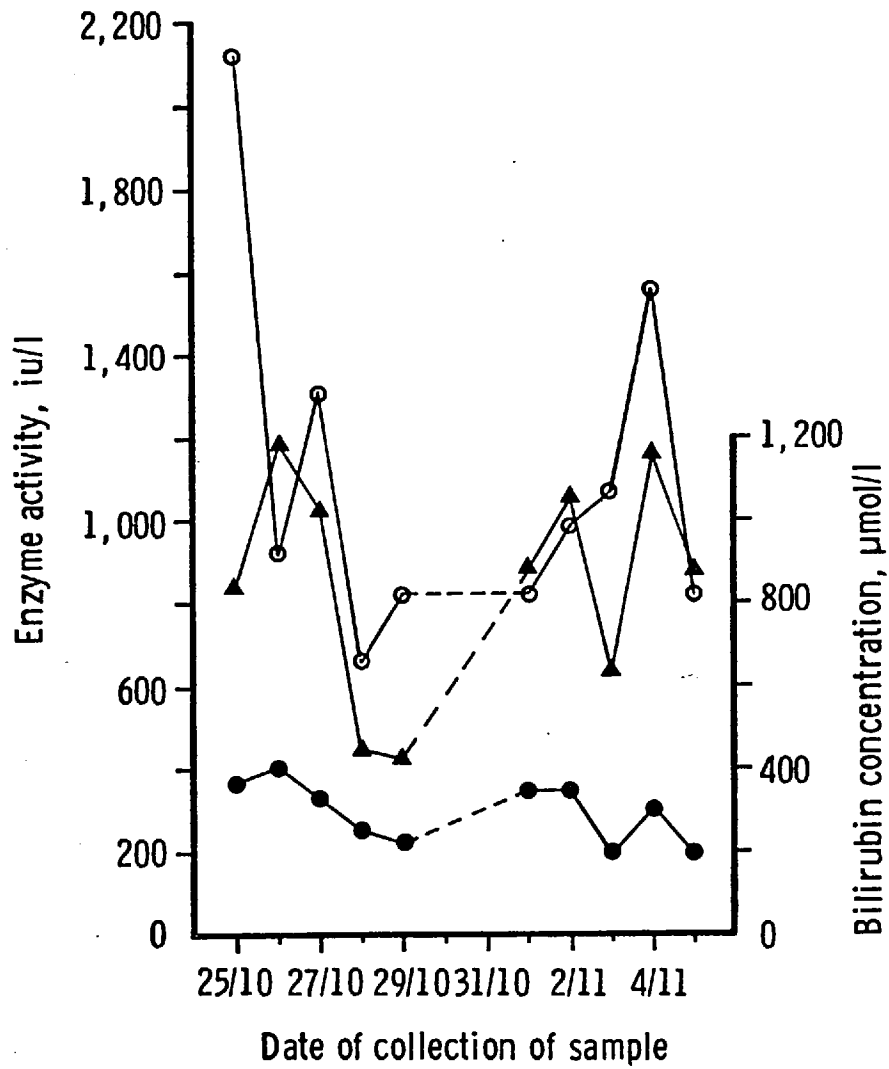


Fig. 3.7

γ -Glutamyl transferase activity (●—●), alkaline phosphatase activity (○—○) and bilirubin concentration (▲—▲) in bile samples from patient III.

one was highest in the first-day sample and lowest in the fourth-day sample. The minimal values for bilirubin concentration in the bile of this patient were observed in the 4th and 5th day samples.

In the bile of patient IV (Fig. 3.8), the highest values for γ -glutamyl transferase and alkaline phosphatase activities were in the 8th day sample. Bilirubin concentration reached a maximum in the 15th day specimen. For this patient, the concentration of bile salts in bile was also measured. The pattern of changes in the bile salts concentration did not quite resemble those of γ -glutamyl transferase and alkaline phosphatase activities.

For the bile of patients I, II, III and IV, respectively, the average values for γ -glutamyl transferase activity were: 906, 636, 286 and 697 I.U./l; those for alkaline phosphatase were 2,321, 2,212, 1,056 and 1,521 I.U./l; the average bilirubin concentration values were 278, 578, 805 and 542 μ mol/l.

Gel-filtration patterns of γ -glutamyl transferase in individual bile samples

A column (1.5 x 95 cm) of Sepharose 6B pre-equilibrated with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl was used for gel-filtration of bile. Each bile sample was filtered through glass-wool, its volume and γ -glutamyl transferase activity were measured and it was then applied to the column at a flow rate of 12 ml/hr. About 60 fractions of 3 ml were collected with an LKB 7000 Ultrorac Fraction Collector.

In most bile samples, total γ -glutamyl transferase activity after gel-filtration separated into two main peaks of activity, one between

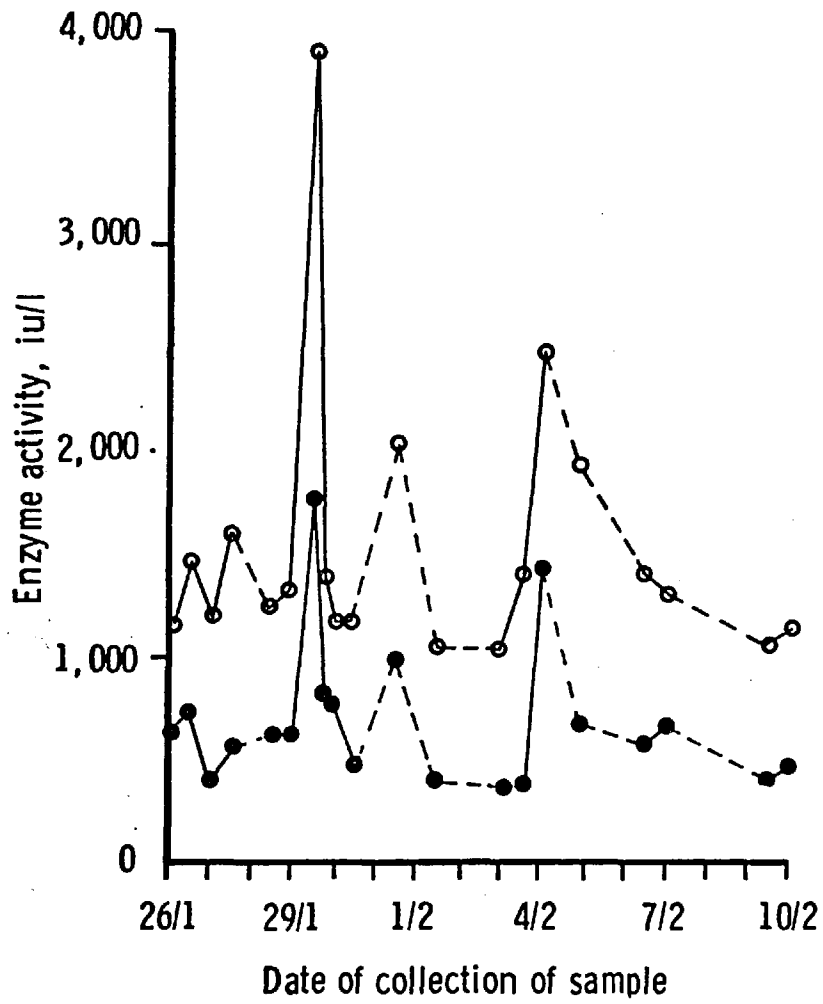


Fig. 3.8a

γ -Glutamyl transferase activity (●—●), and alkaline phosphatase activity (○—○) in bile samples from patient IV.

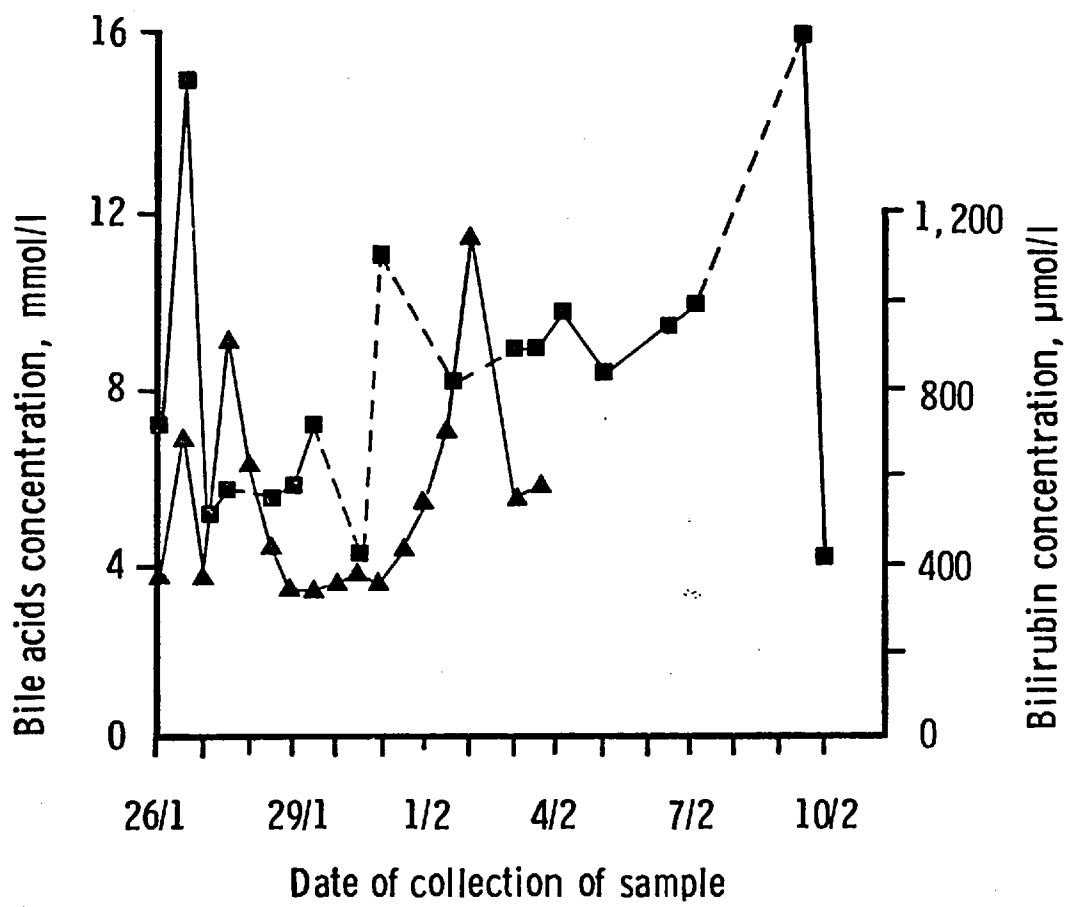


Fig. 3.8b. Bilirubin concentration (▲—▲) and bile salts concentration (■—■) in bile samples from patient IV.

fractions No. 20 - 28 (60 - 84 ml of effluent) and another between fractions No. 38 - 50 (114 - 150 ml of effluent). In some samples, however, nearly all the γ -glutamyl transferase activity was found to be present in either one or other of these two peaks. Examples of separations where most of γ -glutamyl transferase activity eluted in the higher molecular weight peak, was more or less equally distributed between the two peaks, or was present mostly in the lower molecular weight form, are given in Figs. 3.9, 3.10 and 3.11.

In a few cases, another zone of γ -glutamyl transferase activity between fractions No. 29 - 38 (87 - 114 ml effluent volume) was observed after gel-filtration (Fig. 3.12).

It is interesting to note, however, that when the bile samples were stored at 0 - 4°C for a long time (about 2 - 4 months) and then were subjected to gel-filtration on the same Sepharose 6B column again, only the small molecular weight peak of γ -glutamyl transferase activity was observed, which presumably was the result of conversion of larger molecular weight forms of the enzyme into a smaller molecular weight form (Fig. 3.13).

γ -Glutamyl transferase activity in bile fractions No. 38 - 50 was designated B₁, in fractions No. 29 - 37, B₂, and in fractions No. 20 - 28, B₃. Fractions from various separations representing these types of γ -glutamyl transferase activity were combined and concentrated with "Carbowax" 20m to provide pools of each of the three types for further experiments. Since only a few bile specimens contained B₂- γ -glutamyl transferase, the volume of this pool was small, limiting the range of experiments carried out on it.

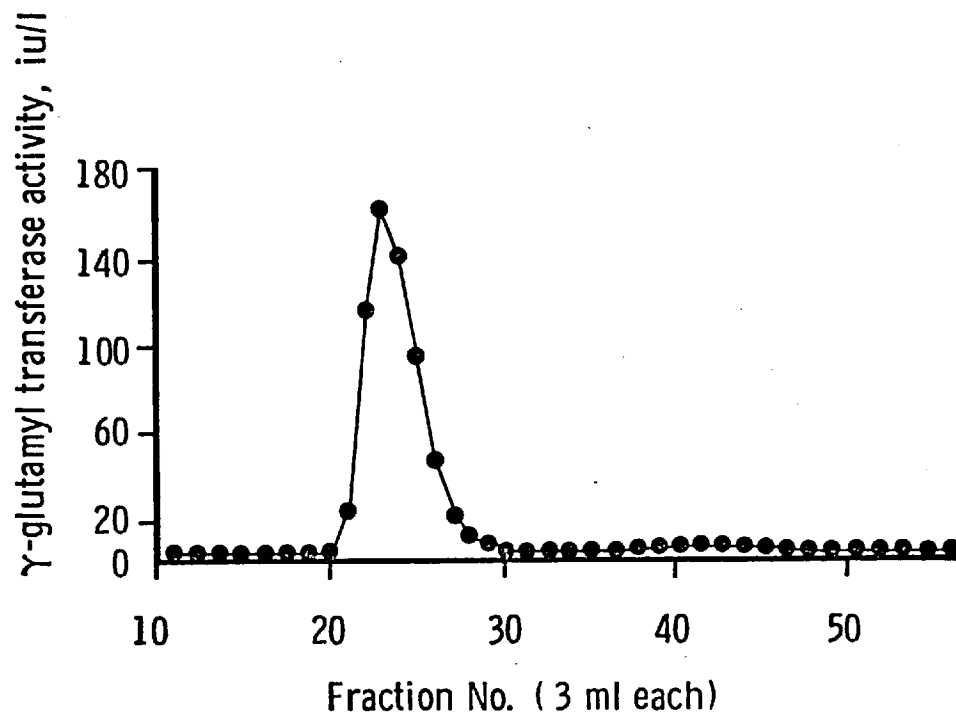


Fig. 3.9

Sephrose 6B gel-filtration of human bile samples: an example where nearly all γ -glutamyl transferase activity is present in the high molecular weight form (8 ml of sample with the total activity of 252 I.U./l was applied; for column details and elution conditions see the text).

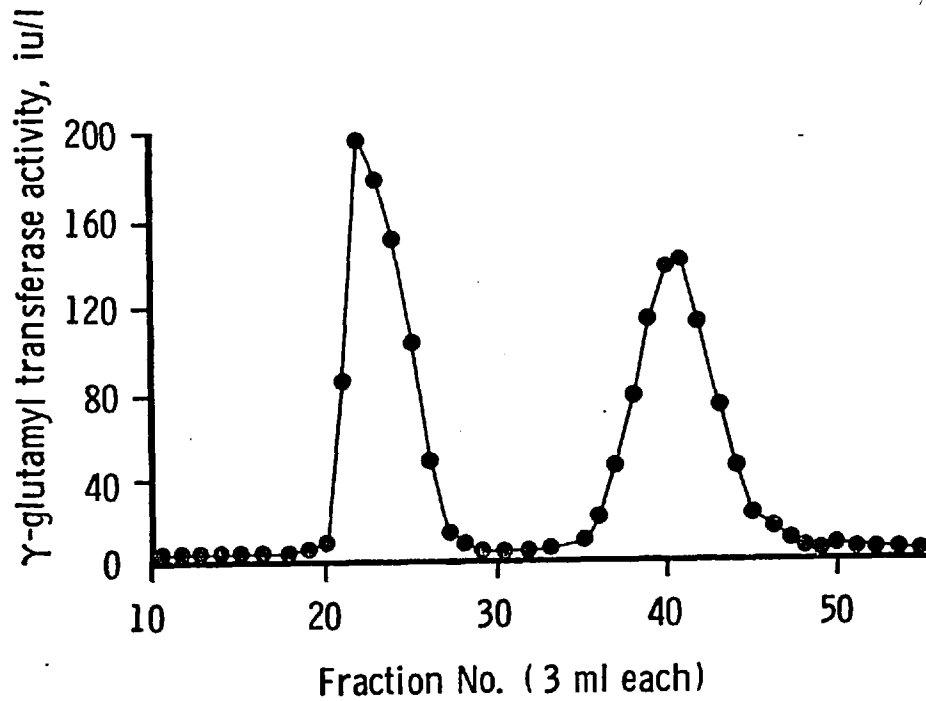


Fig. 3.10

Sepharose 6B gel-filtration of human bile samples: an example where γ -glutamyl transferase activity is more or less equally distributed between the high and the low molecular weight forms (8.4 ml of sample with the total activity of 881 I.U./l was applied; for column details and elution conditions see the text).

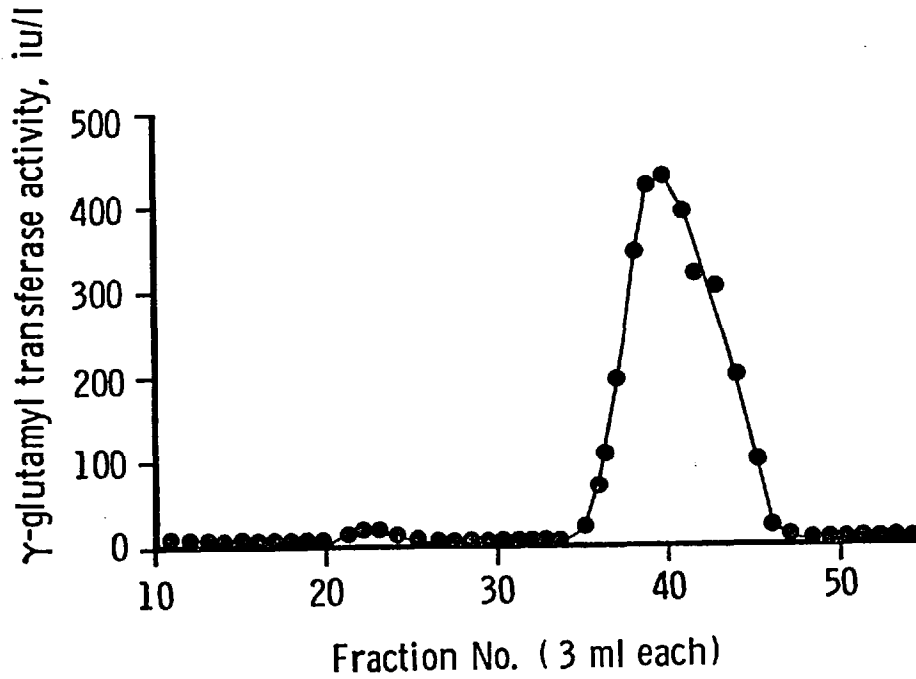


Fig. 3.11

Sepharose 6B gel-filtration of human bile samples: an example where γ -glutamyl transferase activity is present mostly in the low molecular weight form (8.6 ml of sample with total activity of 808 I.U./l was applied; for column details and elution conditions see the text).

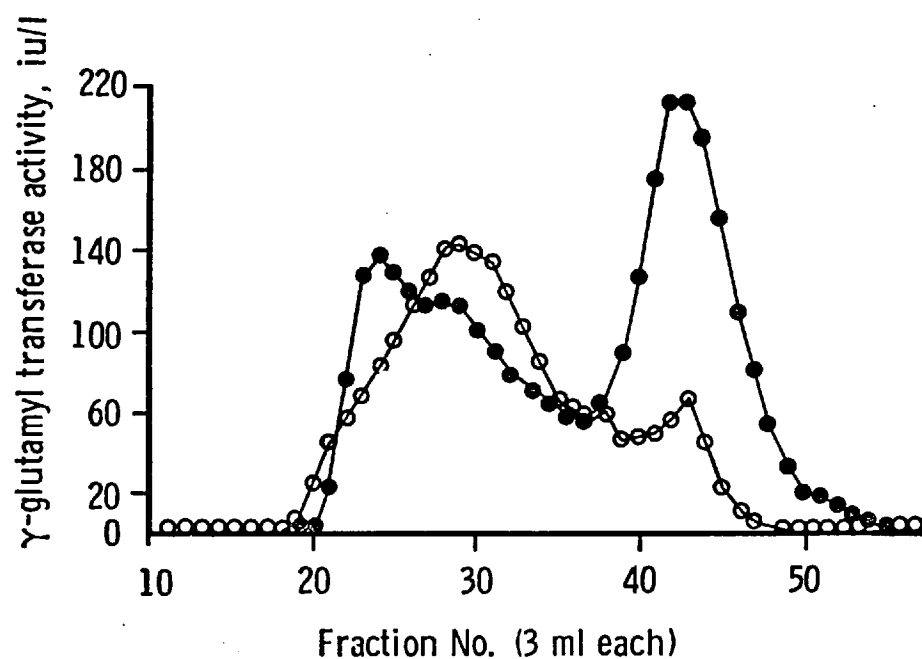


Fig. 3.12

Sephacrose 6B gel-filtration of human bile samples: examples of the presence of activity of the intermediary molecular weight form of γ -glutamyl transferase—8.0 ml of sample 1 with the total activity of 1,202 I.U./l (●—●); 8.0 ml of sample 2 with the total activity of 970 I.U./l (○—○); for column details and elution conditions see the text.

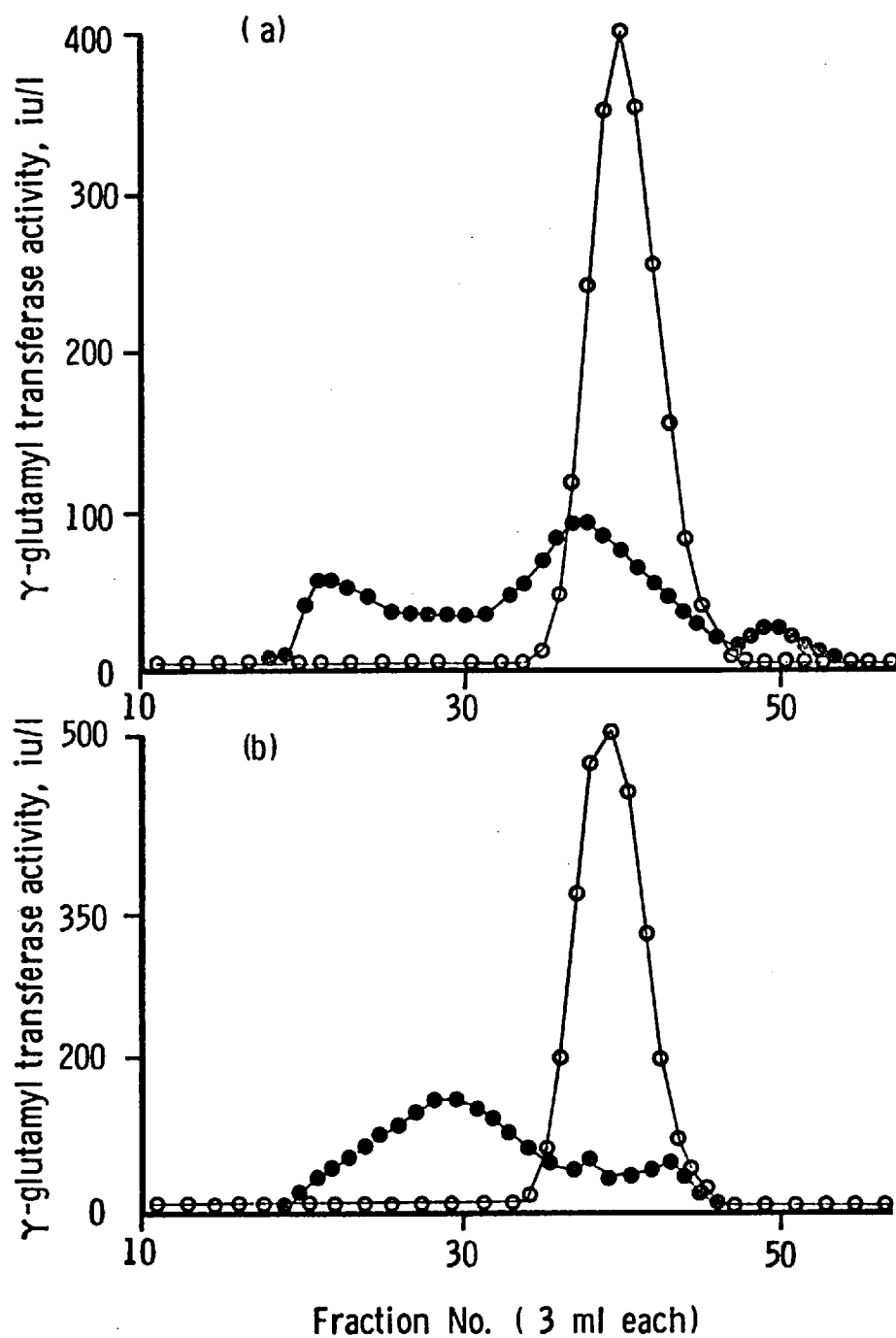


Fig. 3.13

Sephacrose 6B gel-filtration of human bile samples: examples of conversion of the larger molecular weight forms of γ -glutamyl transferase into small molecular weight forms after 2-4 months storage at 0-4°C. Initial distribution of activity (●—●) and distribution of activity in the same samples after 2-4 months (○—○).

Stability of biliary γ -glutamyl transferase to heat

100 μ l of a pool of unfractionated human bile, prepared by mixing equal volumes of all the bile specimens available, was heated in glass Dreyer tubes for $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, $3\frac{1}{2}$, 4, $4\frac{1}{2}$, 5, $5\frac{1}{2}$, 6, $6\frac{1}{2}$, 7, $7\frac{1}{2}$, 8, $8\frac{1}{2}$, 9, $9\frac{1}{2}$, 10, 15, 20, 25 and 30 mins at 60°C in a thermostatted water-bath. At the end of the heating time for each tube, the specimens were placed in ice to stop the effect of heating as quickly as possible. γ -Glutamyl transferase activity in each unheated (0 mins at 60°C) and heated sample of bile was measured, and logarithms of percentage of residual activity were plotted versus time of heating at 60°C.

The half-life of γ -glutamyl transferase in the bile pool (i.e. time at 60°C in mins, at which 50% of the initial enzyme activity was lost) was estimated to be 1.2 mins (Fig. 3.14 a). However, when the bile pool was extensively dialyzed against 0.9% NaCl and then heated, the half-life of the enzyme increased to 5.2 mins (Fig. 3.14b).

To confirm and extend the implication that some component (or components) of bile reduce the heat-stability of γ -glutamyl transferase, the enzyme in the bile pool and in the "high γ -glutamyl transferase serum pool" (see section 3.3 on human serum γ -glutamyl transferase) was inactivated by heating at 60°C for 2 hours (after this no enzyme activity was detected in either pool), and the following experiments were carried out.

(1) Purified human liver γ -glutamyl transferase was added to the inactivated bile pool; the enzyme activity in this mixture was 294 I.U./l. 100 μ l of this mixture was heated at 60°C as described above. The half-life of the purified human liver γ -glutamyl transferase which was mixed with the

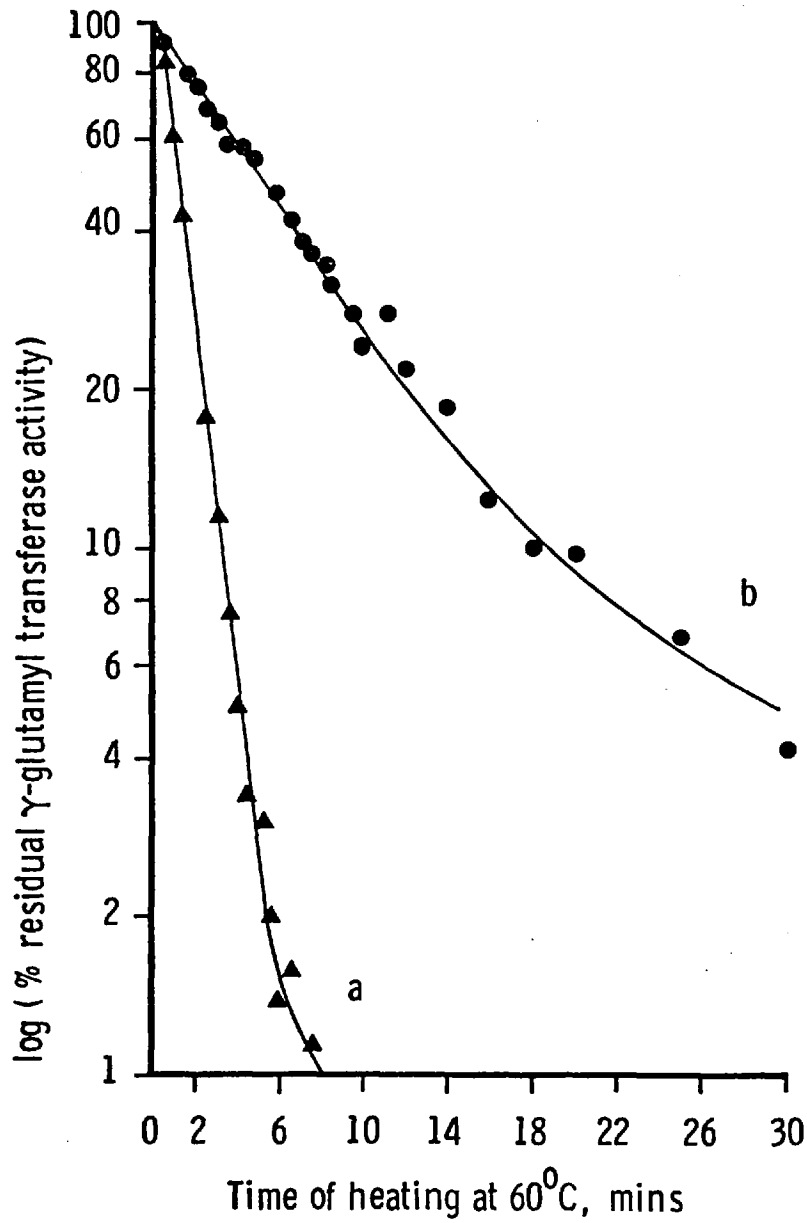


Fig. 3.14

Stability of biliary γ -glutamyl transferase to inactivation by heat: (a) in the undialyzed bile pool ($\blacktriangle$ — $\blacktriangle$) and (b) in the dialyzed bile pool ($\bullet$ — $\bullet$).

inactivated bile pool, was found to be much lower than that of the purified liver enzyme in 0.1 mol/l Tris-HCl, pH 8.0, buffer; the presence of bile decreased it from about 10 mins to 1 min.

(2) Purified liver γ -glutamyl transferase was also mixed with the inactivated "high γ -glutamyl transferase serum pool". The enzyme activity in the mixture was 271 I.U./l. After heating this mixture at 60°C, the half-life of γ -glutamyl transferase purified from human liver was practically unaltered, i.e., the composition of human serum did not affect the heat stability of the enzyme.

(3) Heat-inactivated "high γ -glutamyl transferase serum pool" was mixed with the active bile pool in the proportion of v:v = 4:1. This mixture was also heated at 60°C. The half-life of the biliary γ -glutamyl transferase in this experiment had increased from 1.2 mins to about 2.8 mins.

In summary, these experiments show that a certain factor(s) present in the human bile affects the resistance of γ -glutamyl transferase to inactivation by heat. This factor(s) can be dialyzed out, which is demonstrated by the increase of half-life of γ -glutamyl transferase in the dialyzed bile. Whole bile causes the enzyme purified from human liver to become more heat labile. The heat stability of the biliary enzyme increases when the bile is mixed with the serum inactivated by heat: this could be either the result of some protective action of serum on the biliary γ -glutamyl transferase, or simply the effect of diluting out the labilizing factor in the bile. However, the results emphasize the need to standardize conditions, including the nature of the sample where this is possible, before making use of differences in heat stability to characterize γ -glutamyl transferase fractions.

3.4 γ -Glutamyl transferase in human serum

Preparation and designation of serum γ -glutamyl transferase fractions

When human sera with normal or elevated γ -glutamyl transferase activities were subjected to gel-filtration on a Sepharose 6B column, the total activity of the enzyme usually separated into two peaks of activity - one of a very high molecular weight, which came out of the column with the void volume, and another of lower molecular weight, which was eluted some time after the void volume. In order to characterize the two molecular forms of γ -glutamyl transferase in serum and study their properties, a sufficient quantity of each of these peaks had to be prepared.

For this purpose, serum samples with γ -glutamyl transferase activities greater than 300 I.U./l were selected from those that came to the laboratory for routine estimation of enzymes. These samples were pooled and filtered through glass-wool. Up to 8 ml of filtrate, called "high γ -glutamyl transferase serum pool" was applied to a Sepharose 6B column (1.5 x 95 cm), pre-equilibrated with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl. The column was eluted with the same buffer at a flow rate of 12 ml/hr, and 3 ml fractions were collected using LKB 7000 Ultrarac Fraction Collector. The enzyme activity in the "high γ -glutamyl transferase serum pool" separated into two main zones, the first one of the higher molecular weight eluted from the column usually between fractions No. 21 - 26 (63 - 78 ml of effluent), and the second one, of smaller molecular weight, between fractions No. 28 - 41 (84 - 123 ml effluent volume).

The peak of γ -glutamyl transferase activity that emerged from the column later was designated S_1 , and the bigger molecular weight peak of activity was named S_2 . In preparative experiments with "high γ -glutamyl transferase serum pool" there was usually more activity in the S_1 -peak

than in the S_2 . Column fractions containing either S_1 - or S_2 - γ -glutamyl transferase forms were pooled and concentrated with "Carbowax" 20m.

A typical separation of the "high γ -glutamyl transferase serum pool" by gel-filtration on the Sepharose 6B column is shown in Fig. 3.15.

3.5 Properties of γ -glutamyl transferase fractions prepared from human liver, bile and serum

(a) Physical properties

Molecular weight is an important characteristic of a given protein. Several different methods can be used to establish it, some of which require pure preparations of the protein under study. For the determination of molecular weights of various γ -glutamyl transferase preparations, the method of molecular-exclusion chromatography (gel-filtration) was used, which can be applied to mixtures of globular proteins. The presence of other proteins in such mixtures does not affect the estimation, since each protein passes through the column independently of the other proteins, at a rate which is determined by its molecular weight. Strictly speaking, gel-filtration columns provide an estimate of the Stokes radius, which is the radius of a perfect unhydrated sphere having the same rate of passage through the column as the unknown protein. Molecular weights are estimated directly if the unknown and known calibrating proteins have spherical shape. The elution volume is usually an approximately linear function of the logarithm of the molecular weight over a considerable range of molecular weights.

A Sepharose 6B column (1.5 x 95 cm) was used for determination of molecular weights of γ -glutamyl transferase preparations from human liver,

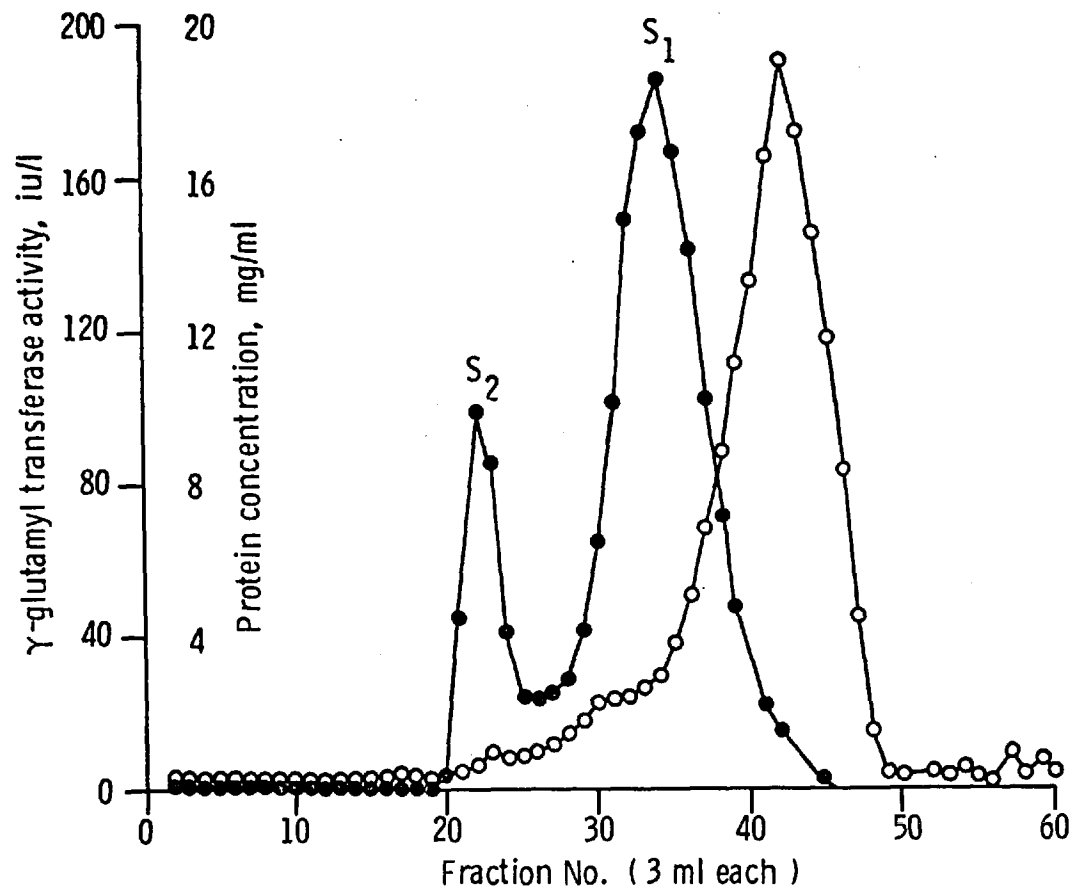


Fig. 3.15 Sepharose 6B gel-filtration of high γ -glutamyl transferase serum pool (7.0 ml sample with γ -glutamyl transferase activity of 780 I.U./l applied to the column)

γ -glutamyl transferase activity (●—●), protein concentration (○—○).

bile and serum. After Sepharose 6B column chromatography, smaller molecular weight fractions of the enzyme were subjected to gel-filtration on a Sephadex G-200 column (0.9 x 28 cm) for a more accurate estimation of their molecular weights.

Calibration of the Sepharose 6B column

The following proteins were used for calibration of the Sepharose 6B column: thyroglobulin (bovine), ferritin (from horse spleen), catalase (from bovine liver), γ -globulins (bovine), albumin (from bovine serum), ovalbumin, peroxidase (from horse radish), trypsin (from bovine pancreas), cytochrome C (from horse heart) and a sample of human serum with high alkaline phosphatase activity. For the determination of the exclusion volume of this column, Blue Dextran with average molecular weight of 2,000,000 was used.

The column was pre-equilibrated with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl, and 10 mg of a marker protein dissolved in 1.0 ml of the same buffer was carefully applied onto the column at a flow rate of 12 ml/hr. Collection of fractions (3 ml each) was started after one-half of the sample volume had gone into the gel. V_e (elution volume) was estimated for each of the calibrating proteins and, knowing V_0 (elution volume for the Blue Dextran, M.W. 2,000,000), the partition coefficient K_{av} ($K_{av} = \frac{V_e - V_0}{V_t - V_0}$, where V_t is the total volume of the gel bed) was calculated in each case and plotted against logarithm of molecular weight of that protein. The calibration curve obtained for the Sepharose 6B column is given in Fig. 3.16.

Partially-purified human liver γ -glutamyl transferase and each of the B_1 , B_2 , S_1 and S_2 preparations were chromatographed on the same column

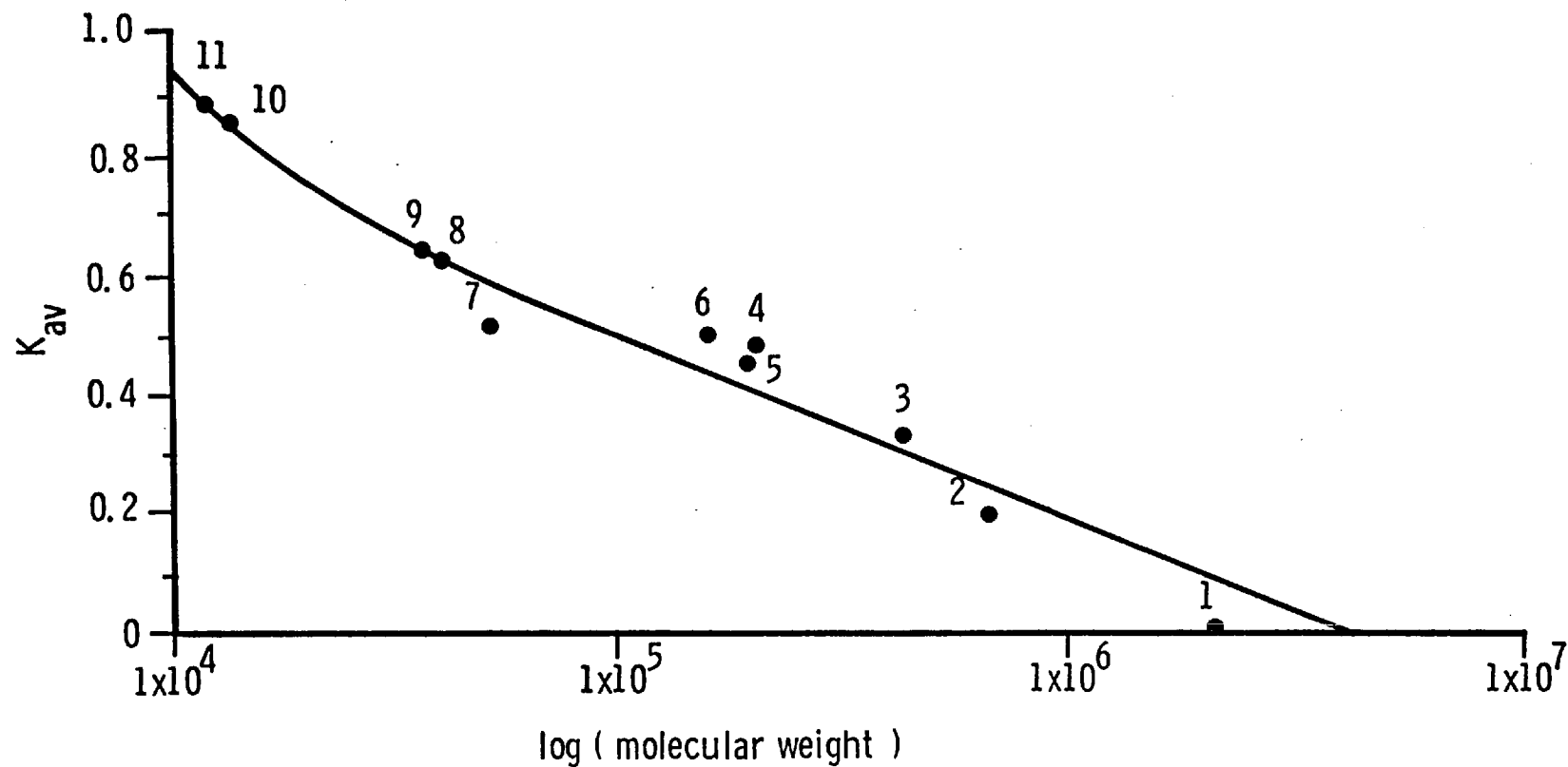


Fig. 3.16

Calibration of Sepharose 6B column (1.5 x 95 cm) with Blue Dextran and marker proteins: 1 - Blue Dextran, 2 - Thyroglobulin, 3 - Ferritin, 4 - Catalase, 5 - Alkaline phosphatase, 6 - γ -globulins, 7 - Bovine serum albumin, 8 - Ovalbumin, 9 - Peroxidase, 10 - Trypsin and 11 - Cytochrome C.

separately, and their elution volume was taken as the volume of the fraction (No. of the fraction x 3 ml) which contained the highest γ -glutamyl transferase activity. K_{av} values were calculated as above, and the corresponding molecular weights were obtained from the calibration curve.

The Sephadex G-200 column was calibrated in a similar way using the following proteins: γ -globulins, human serum alkaline phosphatase, bovine serum albumin, ovalbumin, peroxidase and cytochrome C. For this column, a flow rate of 6 ml/hr was used and 0.3 ml fractions were collected. The calibration curve obtained for the Sephadex G-200 column is shown in Fig. 3.17.

The molecular weight estimations of γ -glutamyl transferase in human liver, bile and serum are summarized in Table 3.4.

Table 3.4 - Estimated molecular weights of γ -glutamyl transferase fractions

No.	γ -Glutamyl transferase preparation	Molecular weight	Molecular weight determined by gel-filtration on:
1	Purified from human liver	550,000	Sepharose 6B (excluded from Sephadex G-200)
2	B ₁	82,000	Sepharose 6B and Sephadex G-200
3	B ₃	>1,000,000	Sepharose 6B (excluded)
4	S ₁	300,000	Sepharose 6B (excluded from Sephadex G-200)
5	S ₂	>1,000,000	Sepharose 6B (excluded)

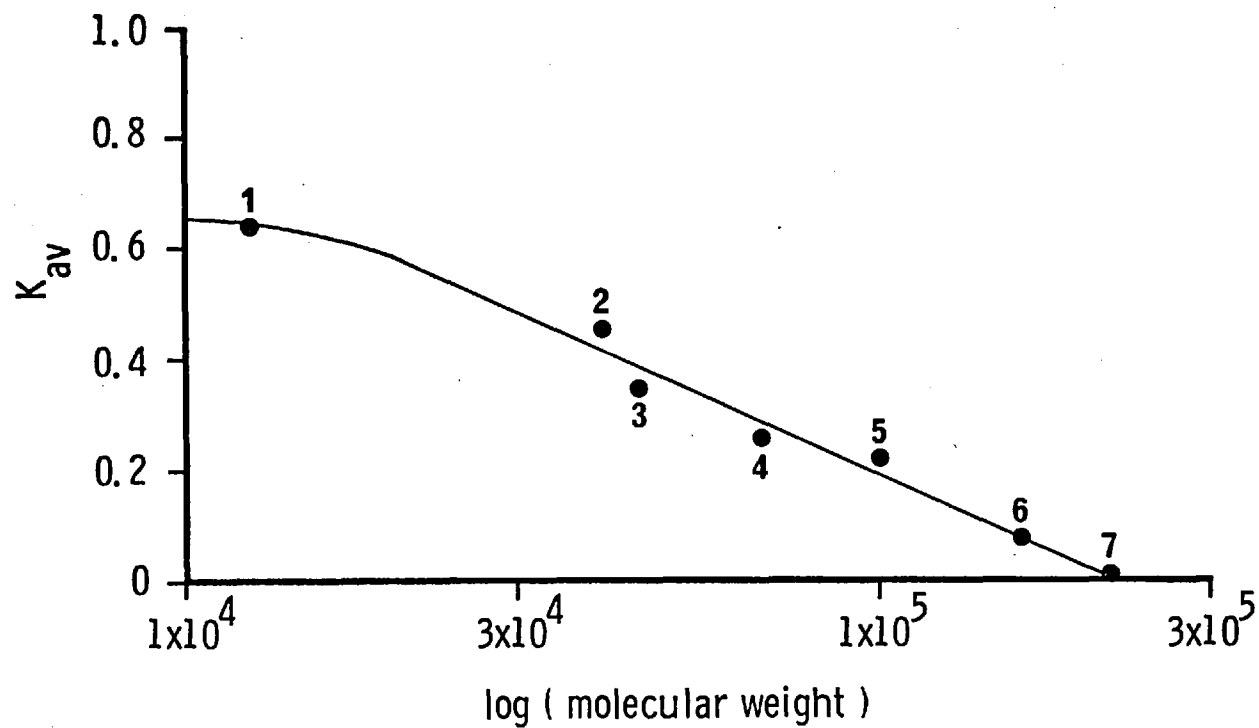


Fig. 3.17

Calibration of Sephadex G-200 column (0.9 x 28 cm) with Blue Dextran and marker proteins: 1 - Cytochrome C, 2 - Peroxidase, 3 - Ovalbumin, 4 - Bovine serum albumin, 5 - Alkaline phosphatase, 6 - γ -globulins, 7 - Blue Dextran.

Electrophoretic mobility

Electrophoretic separation and location of the separated zones of γ -glutamyl transferase activity were carried out as described in "Materials and Methods". A marker serum was applied to each plate in order to determine the electrophoretic mobility of albumin, and the mobilities of purified liver γ -glutamyl transferase and the fractions prepared from human liver, bile and serum were expressed as fractions of the mobility of albumin on that plate. The results obtained are given in Table 3.5

Table 3.5 - Electrophoretic mobilities relative to albumin of γ -glutamyl transferase fractions

No.	γ -glutamyl transferase preparation	Electrophoretic mobility (Albumin = 1.0)
1	Purified from human liver	0.8
2	B ₁	0.8
3	B ₂	0.8
4	B ₃	0 and 0.6
5	S ₁	0.5 and 0.95
6	S ₂	0 and 0.6 - 0.7

The γ -glutamyl transferase preparations were somewhat heterogeneous on electrophoresis, giving rise to rather broad bands of activity, as was the case with the S₁- γ -glutamyl transferase (Fig. 3.18).

In the case of high molecular weight fractions of the enzyme (B₃

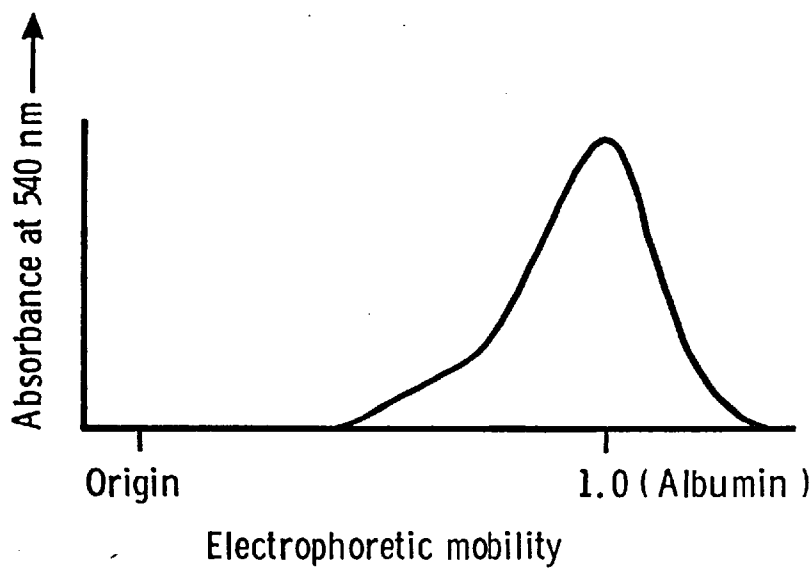


Fig. 3.18 Agarose-gel electrophoresis of S₁-pool.

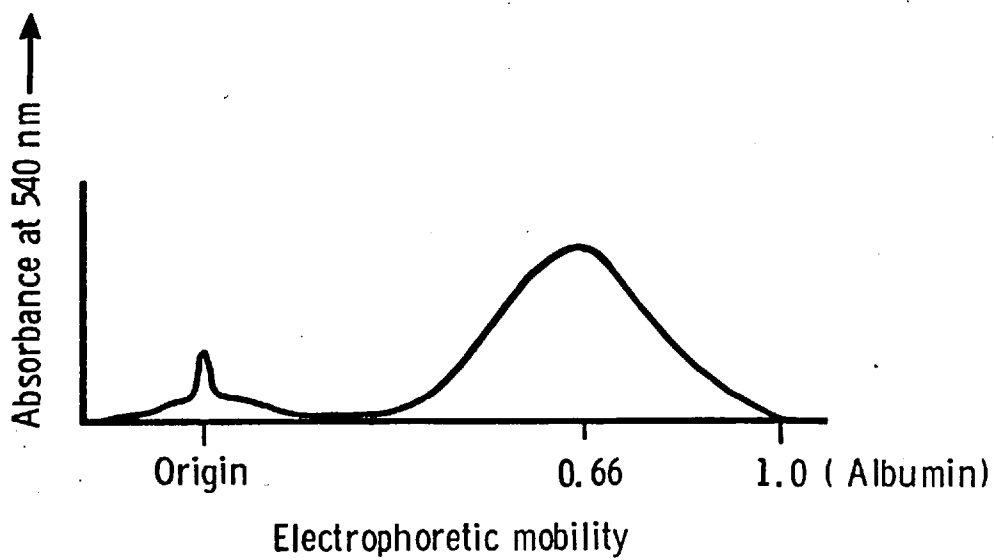


Fig. 3.19 Agarose-gel electrophoresis of S₂-pool.

and S_2), after electrophoresis part of the activity had mobility 0.6 - 0.7 (albumin = 1.0), while another, smaller part stayed at the origin (Fig. 3.19).

When electrophoresis was carried out on 89 serum samples with normal or elevated total γ -glutamyl transferase activities, up to three rather diffuse zones of activity were observed. One zone remained at the origin, while the other two had electrophoretic mobilities of 0.3 - 0.5 and 0.7 - 0.9, respectively, relative to serum albumin. All three bands were present in 66 sera. The two faster zones were observed in 13 sera and the origin zone together with the fastest zone were seen in 7 sera. In 3 sera all γ -glutamyl transferase activity was in one zone of mobility 0.7 - 0.9.

A typical densitometric scan of γ -glutamyl transferase activity in a serum which contained all the three zones is shown in Fig. 3.20.

Concanavalin A binding

Preparation of the column for affinity chromatography

Concanavalin A-Sepharose 4B was washed in 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl, 1 mmol/l $MgCl_2$ and 1 mmol/l $CaCl_2$ (buffer I) several times, then loaded into a column (0.9 x 30 cm) at a flow rate of 30 ml/hr. After the packing was completed the column was washed with further 30 ml of buffer I, then with 60 ml of the same buffer I containing 50 mg/ml α -methyl-D-glucoside (1-O-methyl- α -D-glucopyranoside), and then again with about 400 ml of buffer I.

Partially purified human liver γ -glutamyl transferase and the

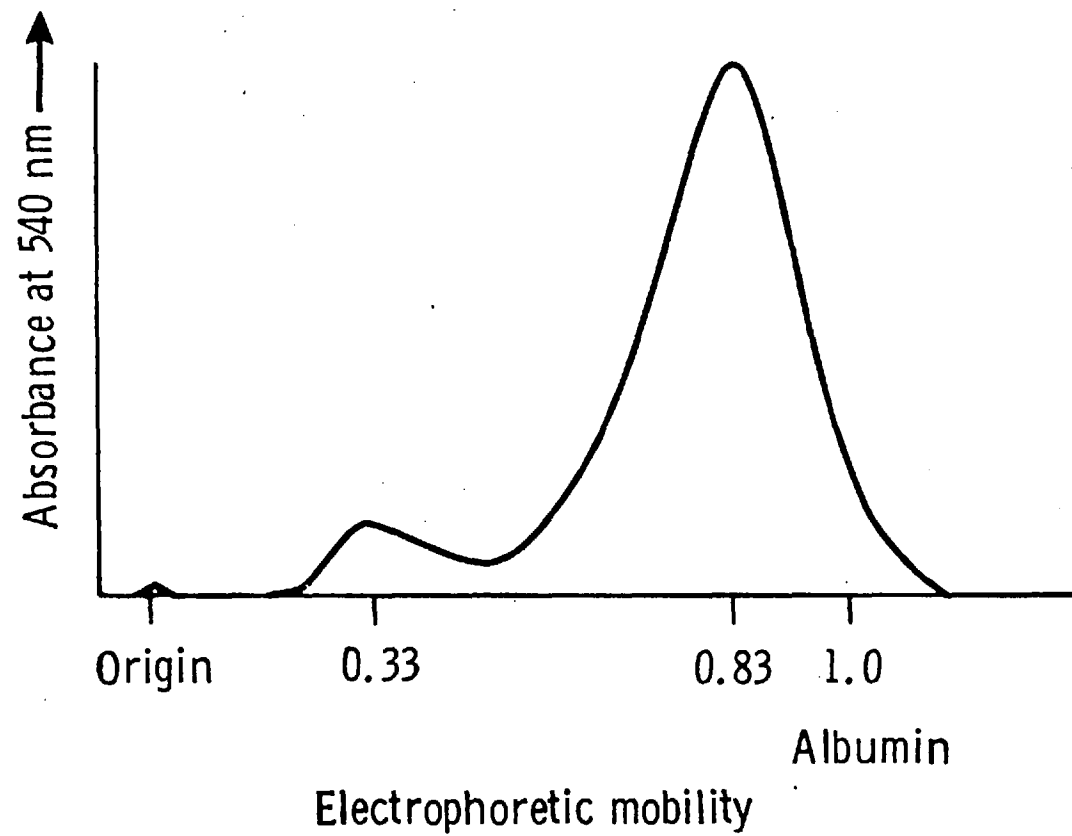


Fig. 3.20. Typical distribution of γ -glutamyl transferase activity after electrophoresis of serum in agarose gel at pH 8.6.

enzyme fractions prepared from the bile and serum were extensively dialyzed against buffer I and their γ -glutamyl transferase activities after dialysis were measured. A 6 - 8 ml sample of each of these fractions was applied onto the Concanavalin A-Sepharose 4B column, and the sample was left to stand in the column for 1 hour. The non-bound portion of γ -glutamyl transferase activity was eluted with 90 ml of buffer I. The enzyme bound to Concanavalin A was washed out with 90 ml of buffer I containing 50 - 100 mg/ml α -methyl-D-glucoside. A flow rate of 30 ml/hr was used and 3 ml fractions were collected in all Concanavalin A affinity chromatography experiments.

γ -Glutamyl transferase activity in fractions containing the non-bound enzyme and the enzyme which bound to Concanavalin A was measured. The total γ -glutamyl transferase activity eluted from the column was calculated, and the non-bound and bound portions were expressed as percentages of the total activity recovered from the column. The recovery of bound and unbound γ -glutamyl transferase averaged 80% of the activity originally applied to the column.

Examples of affinity chromatography on Concanavalin A-Sepharose 4B for two of the γ -glutamyl transferase preparations (S_1 - and B_1 - pools) are shown in Figs. 3.21 and 3.22.

The bound and non-bound portions of γ -glutamyl transferase activity of the samples were each concentrated with "Carbowax" 20m and their electrophoretic mobilities were determined as described in the previous section. The results of Concanavalin A-Sepharose 4B affinity chromatography for the enzyme purified from human liver and for the fractions from the bile and serum are summarized in Table 3.6.

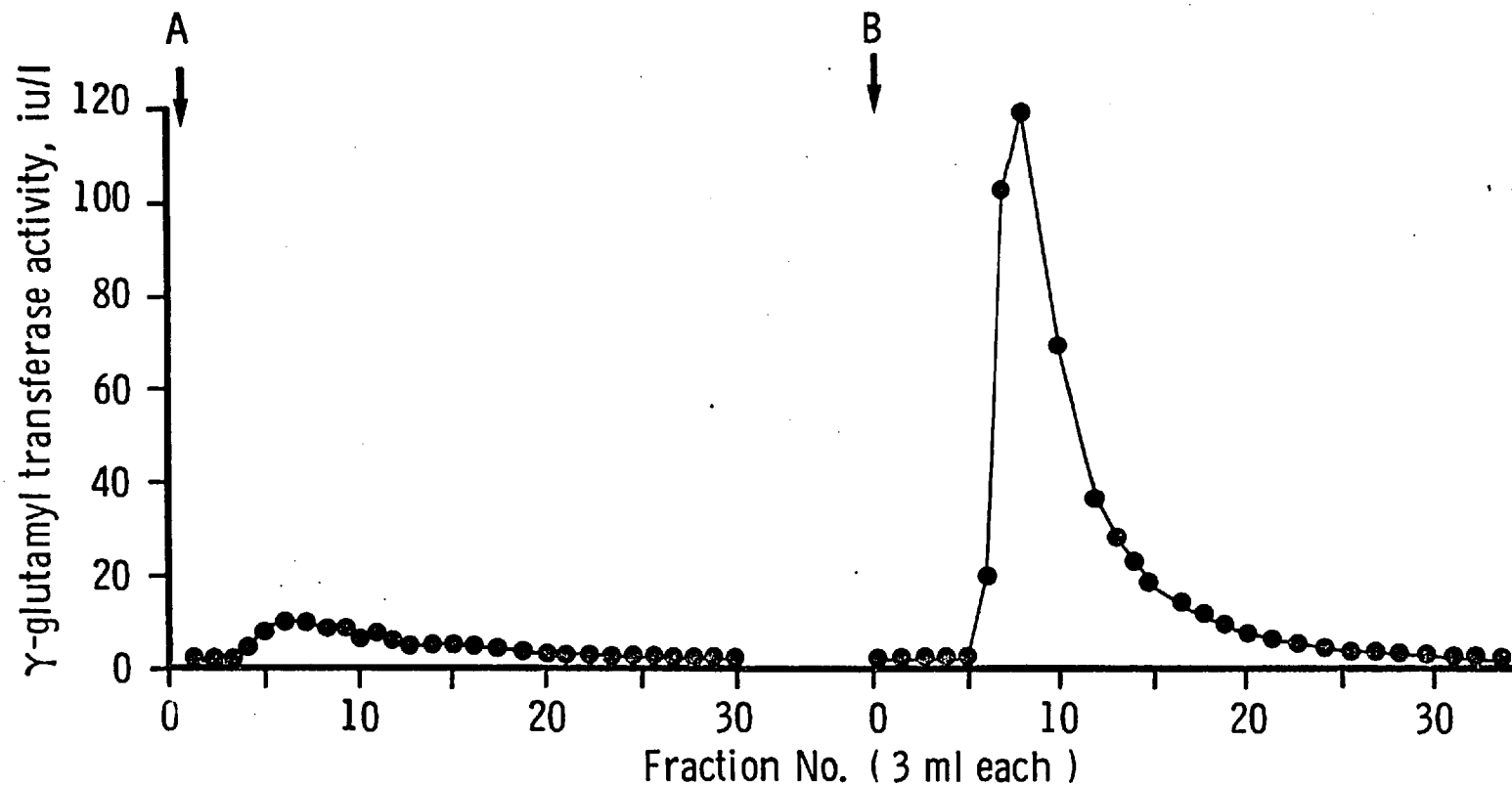


Fig. 3.21. Affinity chromatography on Concanavalin A-Sepharose 4B: 6 ml of S_1 -pool with total γ -glutamyl transferase activity of 416 iu/l applied to the column (A — elution of the non-bound enzyme, B — elution of the bound enzyme; for column and elution details see the text).

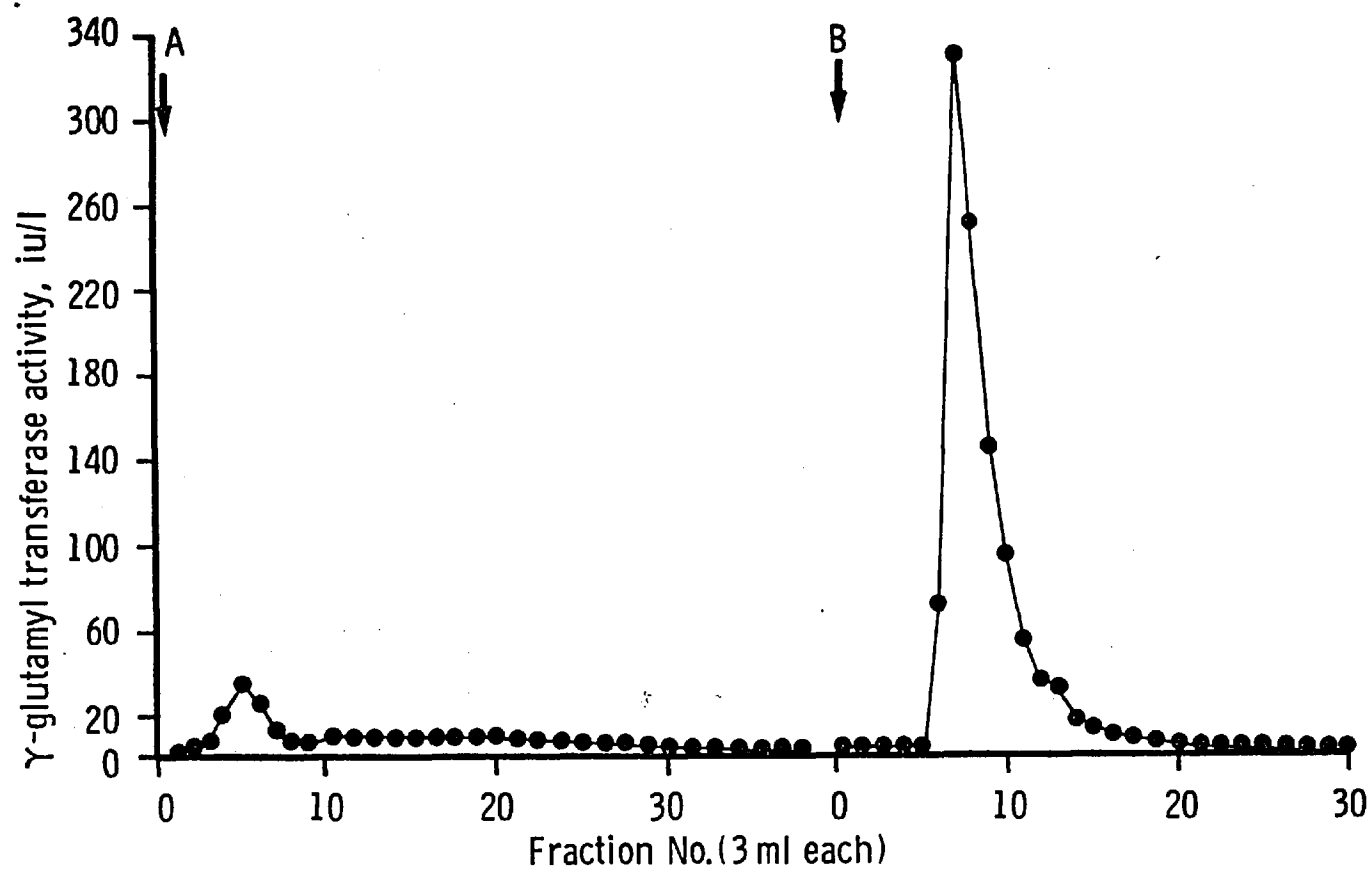


Fig. 3.22

Affinity chromatography on Concanavalin A-Sepharose 6B: 6 ml of B₁-pool with total γ -glutamyl transferase activity of 774 I.U./l applied to the column (A - elution of the non-bound enzyme, B - elution of the bound enzyme; for column and elution details see the text).

Table 3.6 - Affinity chromatography of γ -glutamyl transferase in human liver, bile and serum

No.	γ -glutamyl transferase preparation	γ -glutamyl transferase bound to Concanavalin A		γ -glutamyl transferase not bound to Concanavalin A	
		% of the total activity	Electrophoretic mobility	% of the total activity	Electrophoretic mobility
1	Purified from human liver	89.1	0 0.73	10.9	0 0.74
2	B ₁	79.3	0 0.75	20.7	0 0.75
3	B ₂	62.5	0 0.81	37.5	0 0.83
4	B ₃	52.7	0 0.80	47.3	0
5	S ₁	83.3	0.9 - 1.1	16.7	0 0.6 and 1.0
6	S ₂	81.0	0 0.4 and 0.8	19.0	0

Effect of treatment with neuraminidase

Neuraminidase from *Clostridium perfringens* was used in this type of experiment. 25 μ l of stock neuraminidase solution (10 mg/ml 0.1 mol/l Tris-HCl buffer, pH 7.7) was added to 0.5 ml of each of the γ -glutamyl transferase preparations; this gave a final concentration of neuraminidase of 0.48 mg/ml. Corresponding controls were prepared by adding 25 μ l of 0.1 mol/l Tris-HCl buffer, pH 7.7, instead of the neuraminidase stock solution. γ -Glutamyl transferase activity was measured in both controls and samples with neuraminidase, after which all the samples were incubated at 37°C for 48 h. At the end of the incubation the enzyme activity was measured again and the samples were subjected to agarose-gel electrophoresis.

Electrophoretic mobility of γ -glutamyl transferase in both neuraminidase-treated preparations and their corresponding controls was determined. This experiment (incubation at 37°C and electrophoretic mobility estimation) was repeated four times.

γ -Glutamyl transferase activity decreased after incubation at 37°C for 48 h in nearly all the samples. The incubation did not affect the electrophoretic mobility of γ -glutamyl transferase in control samples, whereas the mobilities in neuraminidase-treated fractions were lower than in controls. The results obtained (mean values of four experiments) are given in Table 3.7.

Heat stability of γ -glutamyl transferase preparations

To determine the stability of various γ -glutamyl transferase

Table 3.7 - Treatment of γ -glutamyl transferase from human liver, bile and serum with neuraminidase

No.	γ -Glutamyl transferase preparation	γ -Glutamyl transferase activity			Electrophoretic mobility	
		Before incubation I.U./l	In controls after 48 h incubation at 37°C I.U./l (% of initial activity left)	In neuraminidase- treated samples after 48 h incubation at 37°C I.U./l (% of initial activity left)	Before treatment with neuraminidase	After incubation with neuraminidase (48 h at 37°C) ± S.D.
1	Purified from human liver	456	418 (91.7)	415 (91.0)	0.8	0.21 ± 0.01
2	B ₁	670	670 (100)	657 (98.1)	0.74	0.13 ± 0.005
3	B ₂	201	201 (100)	190 (94.5)	0.74	0.12 ± 0.00
4	B ₃	329	329 (100)	326 (99.1)	0.7	0.12 ± 0.005
5	S ₁	509	449 (88.2)	302 (59.3)	0.5 and 0.95	1) 0 ± 0 2) 0.20±0.14 3) 0.54±0.0 4) 0.82±0.0
6	S ₂	233	230 (98.7)	179 (76.8)	0.6 - 0.7	1) 0 ± 0 2) 0.26±0.03

preparations to inactivation by heat, 100 μ l of each of these fractions was heated in glass Dreyer tubes in a thermostatted water-bath at 60°C. The time of heating at 60°C ranged from 0 to 60 minutes, and at the end of a heating period, each tube was placed in ice for quick cooling. γ -Glutamyl transferase activities in unheated and each of the heated samples were measured and percentage of residual activity, which is equal to:

$$\frac{\text{enzyme activity in the heated sample}}{\text{enzyme activity in the unheated sample}} \times 100\%,$$

was calculated for each case. Logarithms of percentage or residual activity at 60°C were plotted against time of heating (Figs. 3.23a and 3.23b) and half-life at 60°C (i.e. time at which half of the initial γ -glutamyl transferase activity was lost) was determined from these plots for each preparation of the enzyme. Heating at 60°C was repeated four times for each γ -glutamyl transferase fraction, and the results obtained were as in Table 3.8.

Table 3.8 - Stability of γ -glutamyl transferase from human liver bile and serum to inactivation by heat.

No.	γ -glutamyl transferase preparation	Range of half-life values obtained in 4 separate experiments at 60°C, mins (mean of 4 estimations)
1	Purified from human liver	range 8.3 - 10.9 (9.6)
2	B ₁	range 6.0 - 10.2 (8.1)
3	B ₃	range 5.0 - 11.0 (8.0)
4	S ₁	range 12.1 - 15.1 (13.6)
5	S ₂	range 12.1 - 13.3 (12.7)

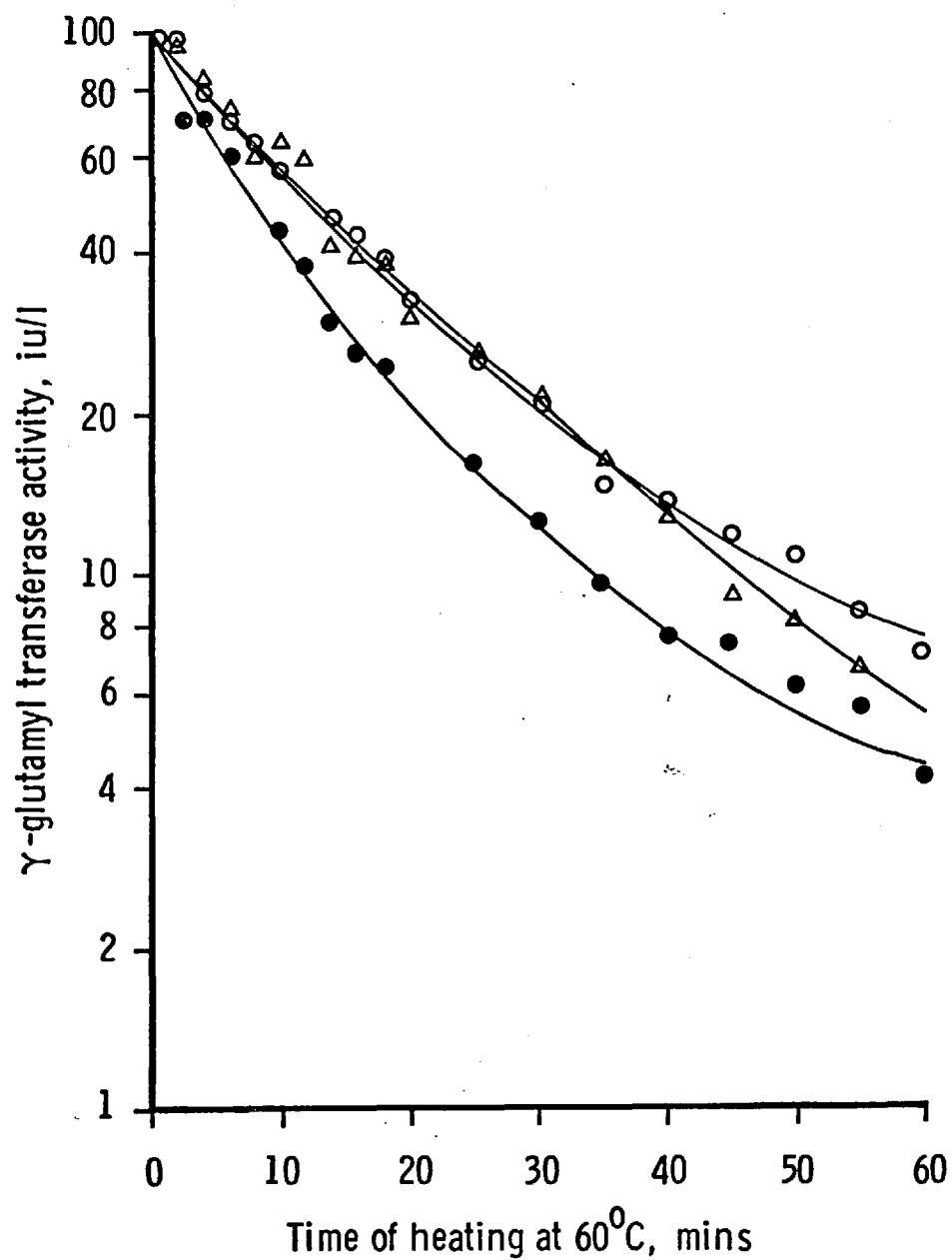


Fig. 3.23a Heat stability of γ -glutamyl transferase fractions: S₁-pool ($\circ$ — $\circ$), S₂-pool (Δ — Δ) and the enzyme purified from human liver ($\bullet$ — $\bullet$).

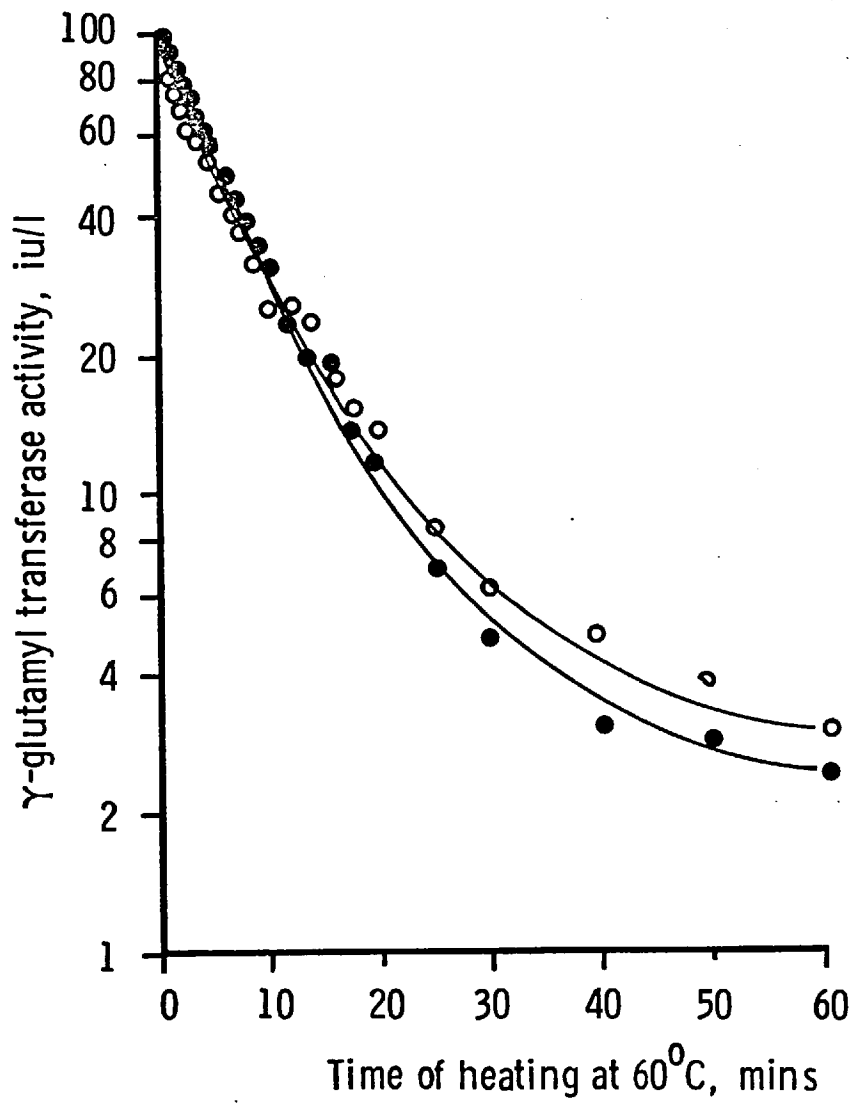


Fig. 3.23b Heat stability of γ -glutamyl transferase fractions: B₁ (●—●) and B₃ (○—○).

The stability of the enzyme purified from human liver and that of the bile fractions were rather similar. B_1 - and B_3 -preparations had to be dialyzed versus 0.1 mol/l Tris-HCl buffer, pH 8.0 in view of the earlier experiments on heat stability of γ -glutamyl transferase in human bile (Section 3.2). However, the rather low values in the range of half-life at 60°C for B_1 - and B_3 -pools were most probably contributed by the aliquots of these pools from which the labilizing factor(s) was not dialyzed out fully. The heat stabilities of the two serum fractions of γ -glutamyl transferase were rather similar to each other; but their half-life values seem to be somewhat higher than those of the purified liver enzyme and B_1 - and B_3 -fractions. This slightly higher heat stability of the two serum fractions of γ -glutamyl transferase could probably be caused by some protective action on the enzyme of the serum proteins contained in the S_1 - and S_2 -preparations and may not be very significant.

b) Catalytic properties

Determination of K_m values for γ -glutamyl-p-nitroanilide and glycylglycine

K_m , or the Michaelis constant, is an important characteristic of an enzyme. K_m is not a fixed value for any given enzyme, it may vary with different substrates of this enzyme, with pH and with temperature. Enzymes obtained from different sources, but catalyzing the same reaction, may differ in their K_m for the same substrate. For one-substrate enzymic reactions, K_m is equal to the substrate concentration at which the initial reaction velocity is half of its maximum value, as can be seen from the Michaelis-Menten equation:

(1)

$$V_o = \frac{V_{\max} \cdot S}{K_m + S} \quad (\text{if } K_m = S, V_o = \frac{V_{\max}}{2})$$

As has already been mentioned in Chapter 1, the reaction of transfer of γ -glutamyl group from a donor substrate to an acceptor substrate catalyzed by γ -glutamyl transferase was shown to proceed by "ping-pong bi-bi" mechanism. The rate equation for such a reaction is expressed as:

(2)

$$V_o = \frac{V_{\max} \cdot [S_1][S_2]}{[S_1] \cdot [S_2] + [S_2] \cdot K_m^{S_1} + [S_1] \cdot K_m^{S_2}}$$

where V_o is the initial velocity and $V_{\max}$ is the maximum velocity of the reaction, $[S_1]$ and $[S_2]$ are the concentrations of substrates S_1 and S_2 , respectively; $K_m^{S_1}$ is the Michaelis constant for substrate S_1 and $K_m^{S_2}$ is the Michaelis constant for substrate S_2 .

For a two-substrate enzymic reaction the Michaelis constant for a given substrate is the concentration of that substrate which leads to half the maximum rate when the enzyme is saturated with the other substrate.

In reciprocal form, the following expressions of equation (2) can be obtained:

$$(3) \quad \frac{1}{V_o} = \frac{K_m^{S_1}}{V_{\max}} \cdot \frac{1}{[S_1]} + \frac{1}{V_{\max}} \cdot \left(1 + \frac{K_m^{S_2}}{[S_2]} \right) \quad \text{and}$$

$$(4) \quad \frac{1}{V_o} = \frac{K_m^{S_2}}{V_{\max}} \cdot \frac{1}{[S_2]} + \frac{1}{V_{\max}} \cdot \left(1 + \frac{K_m^{S_1}}{[S_1]} \right)$$

When $1/V_0$ is plotted as a function of $1/[S_1]$ (see equation (3)), the intercept on the ordinate will change with the concentration of S_2 ; i.e., the value of $V_{\max}$ changes as $[S_2]$ is varied; the slope of the line will not be affected by varying $[S_2]$. If $1/V_0$ is plotted against $1/[S_2]$ (equation (4)), the slope of this plot will not be affected by changes in the concentration of S_1 , but $V_{\max}$ will change depending on $[S_1]$. As can be seen from equation (2), a particular initial rate of the reaction could be given by an infinite number of pairs of values of $[S_1]$ and $[S_2]$.

If the concentration of substrate S_2 is much greater than $K_m^{S_2}$, equation (3) can be reduced to:

$$\frac{1}{V_0} = \frac{K_m^{S_1}}{V_{\max}} \cdot \frac{1}{[S_1]} + \frac{1}{V_{\max}}$$

This is an equation corresponding to a one-substrate reaction, and $K_m^{S_1}$ can be determined, for example, by either of the following methods:

- (i) by plotting the reaction rate measured at various substrate concentrations against $[S]$;
- (ii) using the plot suggested by Lineweaver and Burk (1934) ($1/V$ against $1/[S]$);
- (iii) by the method of Hofstee (1959) (V plotted against $V/[S]$);
- (iv) by plotting $[S]/V$ against $[S]$ (Hanes, 1932).

The value of $K_m^{S_2}$ can be obtained by repeating the procedure with $S_1 \gg K_m^{S_1}$.

However, the choice of very high substrate concentrations in

practice can be difficult because of such factors as the limited solubility of the substrates, the light absorbance or the viscosity of their concentrated solutions. In such cases, the individual constants for enzymes are more accurately estimated by the so-called re-plotting procedure (Dalziel, 1957; Florini and Vestling, 1957). In this method, initial rates of the reaction are measured when $[S_1]$ is kept at a fixed value $[S_1]^1$ at a series of S_2 concentrations. The results of $1/V$ are plotted against $1/[S_2]$ (primary plot); the intercept of this plot (I^1) on the ordinate will be equal to:

$$I^1 = \frac{1}{V[S_1]^1} = \frac{1}{V_{\max}} \left(1 + \frac{K_m^{S_1}}{[S_1]^1} \right)$$

The measurements are repeated again at several fixed values of $[S_1]$ ($[S_1]^{II}$, $[S_1]^{III}$, $[S_1]^{IV}$, etc), so that a series of slopes and intercepts are obtained. These intercepts and slopes are re-plotted against the corresponding values of $1/[S_1]$. The plot of intercept against $1/[S_1]$ is a straight line that cuts the abscissa at $1/[S_1] = -1/K_m^{S_1}$. Re-plotting of slopes in the case of a reaction proceeding by "ping-pong bi-bi" mechanism should give a straight horizontal line which intersects the ordinate at $K_m^{S_2}/V_{\max}$.

The whole operation is repeated with various constant values of $[S_2]$ and $1/V$ is plotted against $1/[S_1]$ for each fixed concentration of S_2 . By re-plotting the intercepts on the ordinate and the slopes against the corresponding $1/[S_2]$, another pair of $K_m^{S_2}$ and $K_m^{S_1}$ can be obtained. The mean values of $K_m^{S_1}$ and $K_m^{S_2}$ are then calculated using the two re-plotting estimations.

In this work, K_m values for γ -glutamyl-p-nitroanilide (donor substrate) and glycylglycine (acceptor substrate) were determined in order to compare the kinetic properties of various preparations of γ -glutamyl transferase from human liver, bile and serum. However, it quickly became apparent that the fractions did not differ noticeably in this respect, and that kinetic measurements were unlikely to contribute to characterization of the fractions. It was therefore decided to simplify the procedure and to determine K_m values of each of the two substrates at only one fixed concentration of the other substrate. For the estimation of K_m values for γ -glutamyl-p-nitroanilide, the concentration of glycylglycine was kept constant at the level of 75 mmol/l, as is recommended by the Scandinavian method for measurement of γ -glutamyl transferase activity; the concentration of the donor substrate varied between 0 and 6 mmol/l. K_m values for glycylglycine were obtained when the concentration of γ -glutamyl-p-nitroanilide was fixed at the value of 4 mmol/l recommended by the Scandinavian method and the acceptor concentration varied between 0 and 120 mmol/l. All the concentrations mentioned here were the final concentrations of the substrates in the reaction mixture.

For each of the γ -glutamyl transferase preparations and at each substrate concentration, the enzyme activity measurements were repeated three to four times. K_m values for the donor substrate were determined graphically, by plotting γ -glutamyl-p-nitroanilide concentration versus $[\gamma\text{-glutamyl-p-nitroanilide}]/V$. Three such plots were made and three K_m values were obtained for each enzyme fraction; from these, the values of the mean $K_m \pm \text{S.D.}$ were calculated.

K_m values for the acceptor substrate were obtained similarly, by plotting glycylglycine concentration against $[\text{glycylglycine}]/V$. For each enzymic fraction the mean $K_m \pm \text{S.D.}$ was calculated based on the K_m values obtained in each of the four separate experiments.

Concentrations of γ -glutamyl-p-nitroanilide greater than 6 mmol/l and of glycylglycine greater than 120 mmol/l were found to inhibit all γ -glutamyl transferase preparations used.

Examples of plots obtained for estimation of K_m for γ -glutamyl-p-nitroanilide and glycylglycine are given in Figs. 3.24 and 3.25.

Determination of K_i values for reduced and oxidized forms of glutathione

Enzyme inhibition studies can help to elucidate such problems as the substrate specificity of enzymes, the mechanism of enzyme action, the nature of the functional groups at the active site of an enzyme molecule, the role of the functional groups in maintaining the active conformation of the enzyme molecule, the regulation of intermediary metabolism by certain metabolites acting as enzyme inhibitors.

Reversible enzyme inhibition is usually divided into three groups: competitive, non-competitive and uncompetitive inhibition.

In competitive inhibition, the inhibitor which resembles the substrate structurally, competes with the substrate for the active site on the enzyme molecule. A competitive inhibitor reversibly binds to the enzyme at the substrate-binding site to form an enzyme-inhibitor complex EI, just like the substrate forms an ES complex:



The dissociation constant of the EI complex, or the inhibitor constant, is expressed as $K_i = \frac{[E] \cdot [I]}{[EI]}$.

K_i reflects the affinity of the inhibitor for the enzyme. The velocity

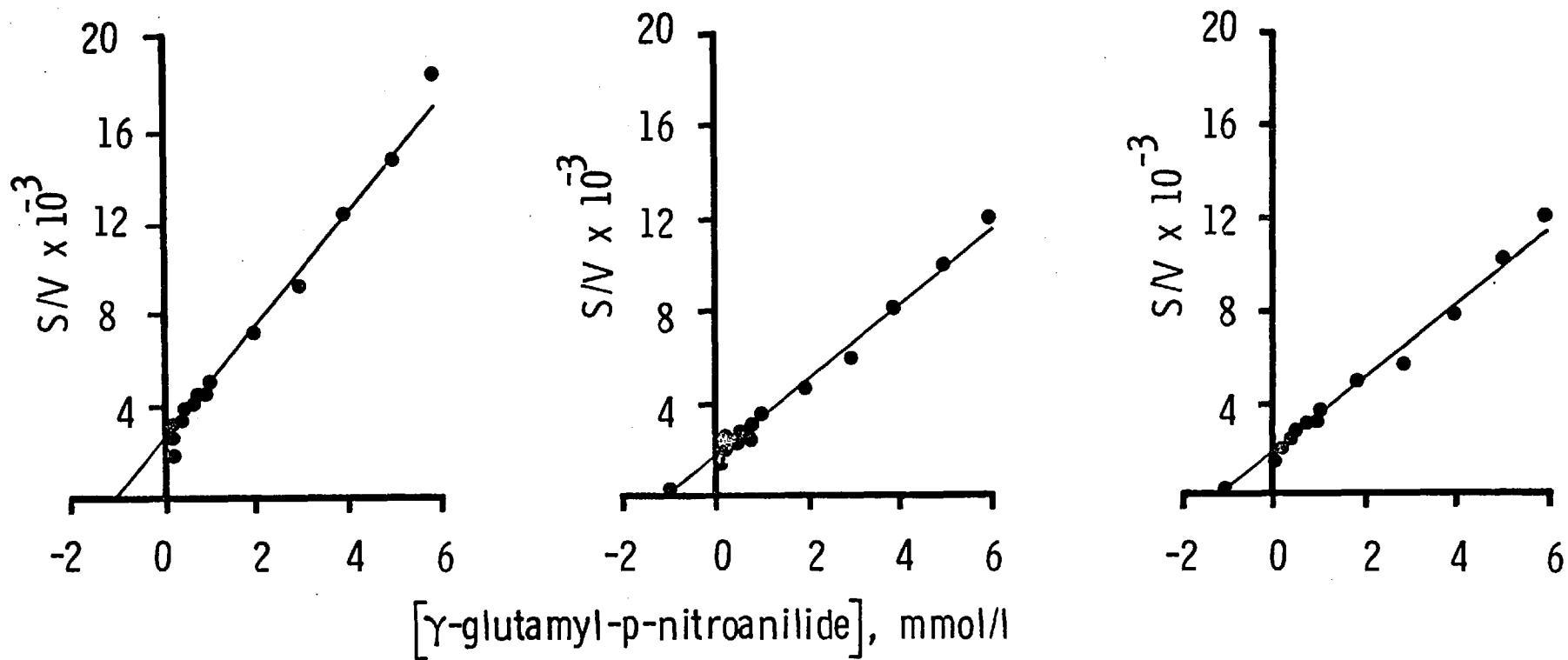


Fig. 3.24

Three replicate determinations of K_m value of γ -glutamyl transferase purified from human liver for γ -glutamyl-p-nitroanilide.

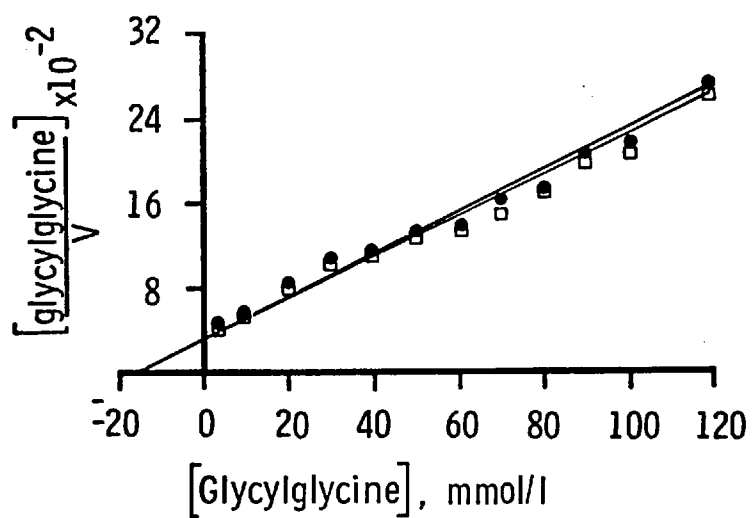
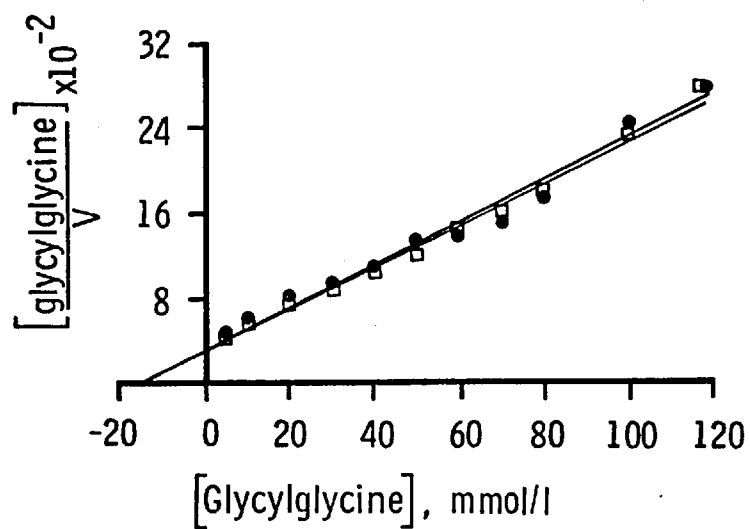


Fig. 3.25 Four replicate determinations of K_m value of S1-pool for glycylglycine.

of the reaction in the presence of the competitive inhibitor is a modified form of the Michaelis-Menten equation:

$$\frac{V_{\max} \cdot [S]}{[S] + K_m \cdot (1 + \frac{[I]}{K_i})}$$

where, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration and K_i is the inhibitor constant. $V_{\max}$ is the maximum velocity of the reaction.

The presence of a competitive inhibitor increases the K_m of the enzyme for the substrate, which means that the enzyme will require a higher concentration of the substrate to achieve its maximum velocity. This increased apparent K_m is greater than the true K_m by the factor of $(1 + \frac{[I]}{K_i})$; $V_{\max}$ remains unaltered.

Glutathione and γ -glutamyl-p-nitroanilide are known to compete for the binding site of γ -glutamyl transferase from rat and human kidney (Shaw, London and Petersen, 1978).

In our experiments, both oxidized (GSSG) and reduced (GSH) forms of glutathione were found to competitively inhibit γ -glutamyl transferase in human liver, bile and serum.

γ -Glutamyl transferase activity measurements were made over GSSG and GSH concentration range of 0 to 4.0 mmol/l, glycylglycine concentration was kept constant at 75 mmol/l; two γ -glutamyl-p-nitroanilide concentrations were used - 1.0 mmol/l and 4.0 mmol/l. For each γ -glutamyl transferase preparation and at each of the two γ -glutamyl-p-nitroanilide concentrations, the activity measurements were repeated three times over the inhibitor concentration range.

K_i values were determined graphically, using Dixon plots (Dixon, 1953). The values of $[I]$ versus $1/V_{\text{reaction}}$ were plotted three times for each enzyme fraction. Mean $K_i \pm \text{S.D.}$ were calculated using the three K_i values obtained. Examples of the Dixon plots for GSSG and GSH are shown in Figs. 3.26 and 3.27.

The values of K_m for γ -glutamyl-p-nitroanilide and glycylglycine and K_i for oxidized and reduced forms of glutathione obtained for γ -glutamyl transferase in human liver, bile and serum are given in Table 3.9.

pH optimum

The influence of pH on the activity of γ -glutamyl transferase from human liver, bile and serum was studied using Tris-HCl buffer recommended by the Scandinavian method for this enzyme. The pH was adjusted to a range of values between 7.0 and 9.0 at 37°C. γ -Glutamyl transferase activity measured was plotted against the pH values, and such plots are shown in Fig. 3.28.

The pH curves obtained for our preparations of the enzyme all show a rather broad peak of activity with an optimum pH in the range between values 7.6 and 8.2.

3.6 Summary

γ -Glutamyl transferase from human liver was purified about 300 fold to a specific activity of 11.1 I.U./mg protein. The molecular weight of the enzyme, estimated before the removal of Na-deoxycholate, was about 160,000. This value probably represents the molecular weight of the enzyme-detergent complex. Upon the removal of the detergent, γ -glutamyl

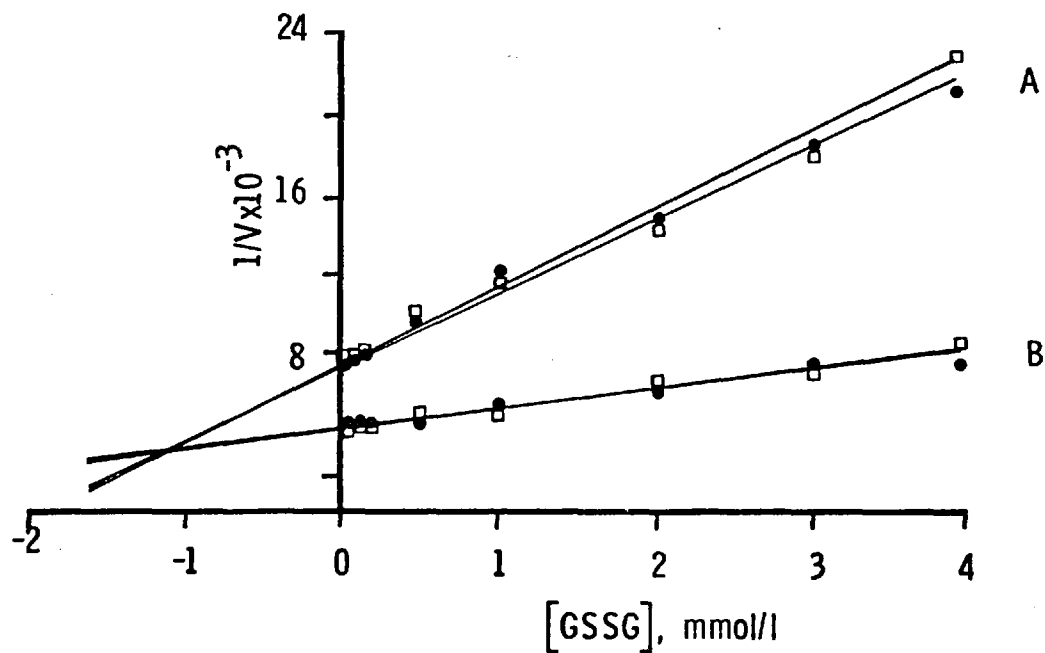
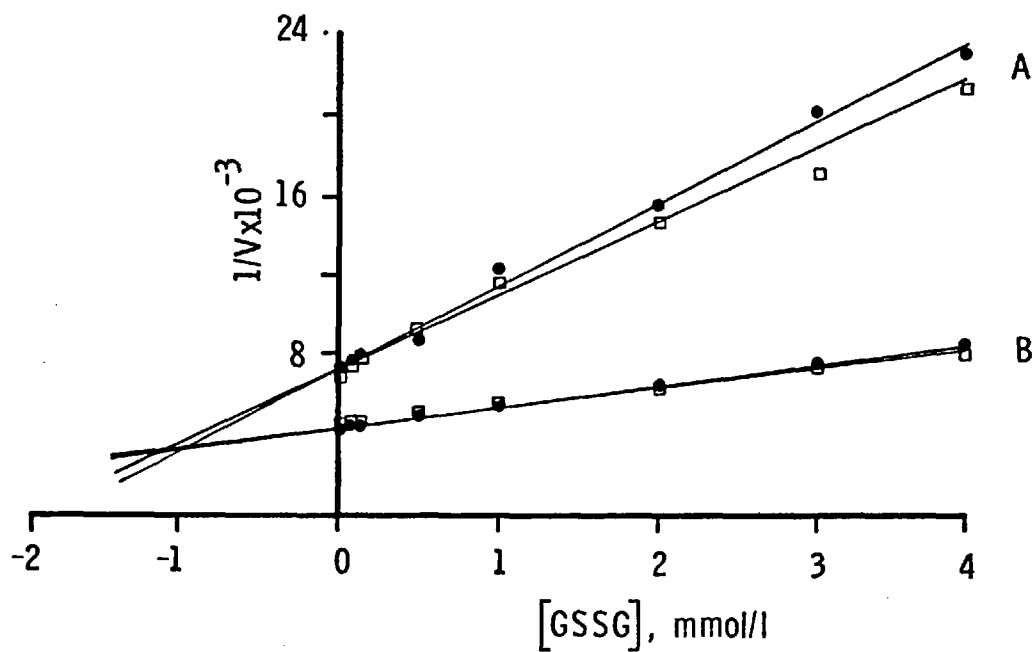


Fig. 3.26

Four replicate determinations of K_i value of B_1 -pool for the oxidized form of glutathione: A, in the presence of 1 mmol/l and B, in the presence of 5 mmol/l γ -glutamyl-p-nitroanilide.

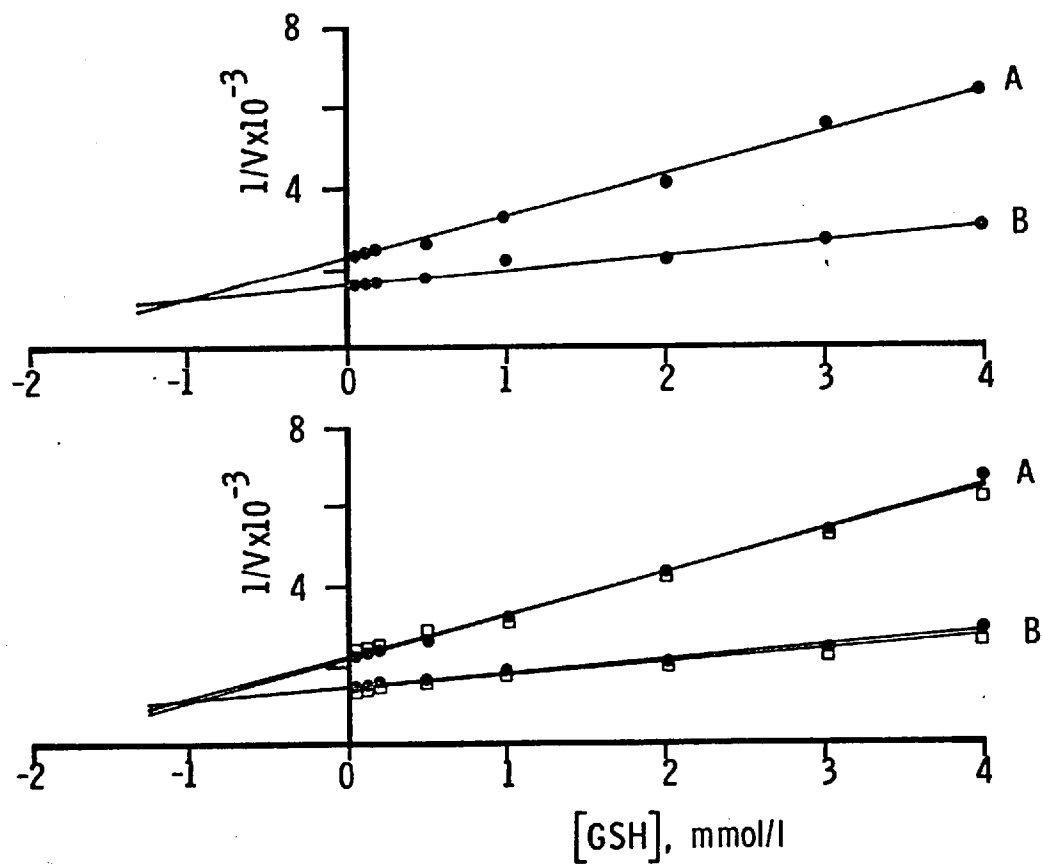


Fig. 3.27. Three replicate determinations of K_i value of γ -glutamyl transferase purified from human liver for the reduced form of glutathione: A, in the presence of 1 mmol/l and B, in the presence of 4 mmol/l of γ -glutamyl-p-nitroanilide.

Table 3.9 - Kinetic properties of γ -glutamyl transferase in human liver, bile and serum

No.	γ -glutamyl transferase preparation	K_m for γ -glutamyl-p-nitroanilide mmol/l $\pm$ S.D.	K_m for glycylglycine mmol/l $\pm$ S.D.	K_i for GSSG mmol/l $\pm$ S.D.	K_i for GSH mmol/l $\pm$ S.D.
1	Purified from human liver	1.10 ± 0.05	16.2 ± 0.6	0.94 ± 0.07	1.00 ± 0.09
2	B ₁	1.04 ± 0.02	16.75 ± 0.50	1.08 ± 0.05	1.01 ± 0.05
3	B ₃	1.02 ± 0.05	16.38 ± 0.48	1.05 ± 0.11	1.02 ± 0.03
4	S ₁	1.10 ± 0.05	16.0 ± 0.6	0.98 ± 0.03	1.01 ± 0.05
5	S ₂	1.08 ± 0.06	16.12 ± 1.18	1.02 ± 0.06	1.38 ± 0.22

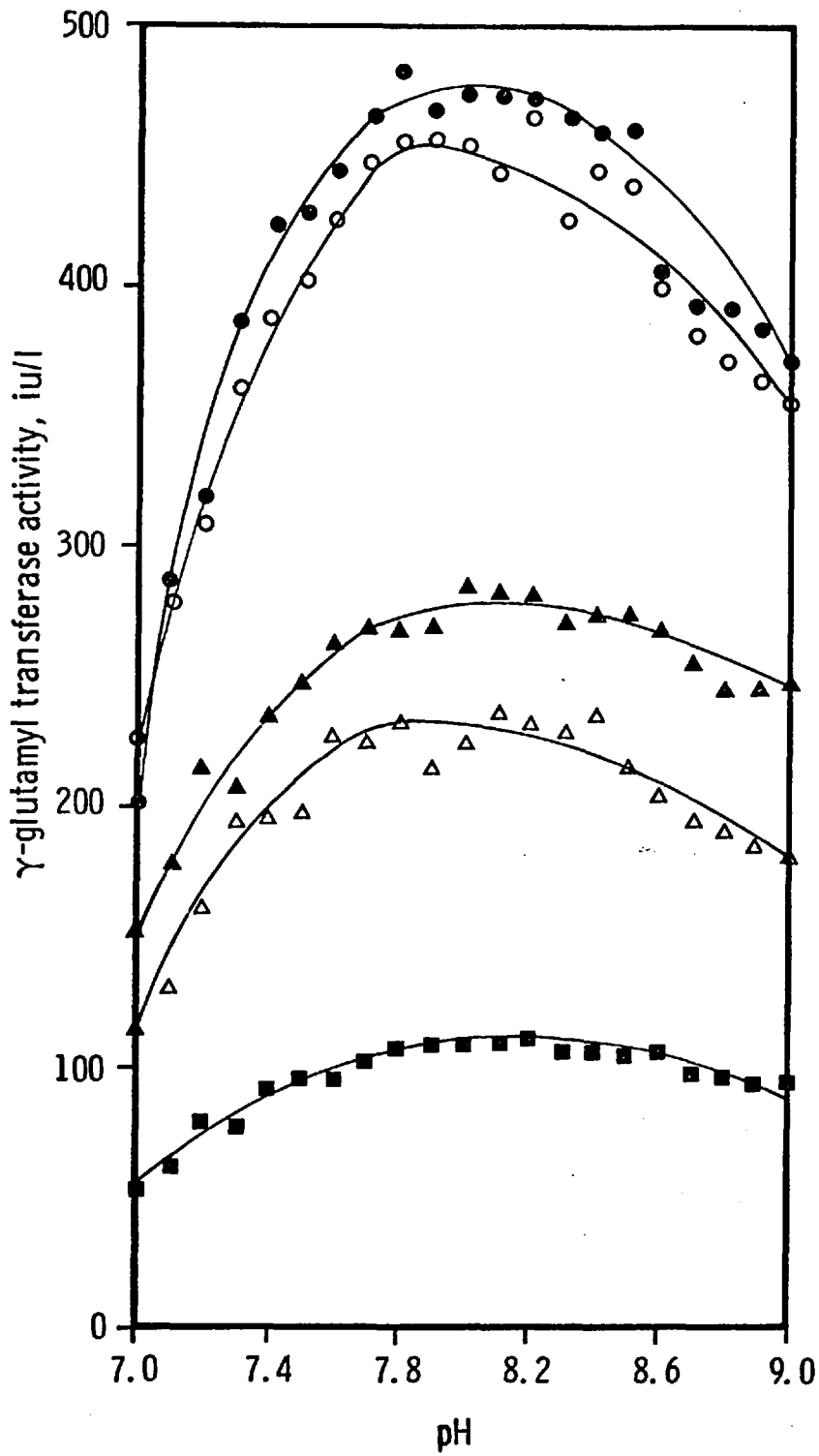


Fig. 3.28 Effect of pH on the activity of γ -glutamyl transferase fractions from human liver, bile and serum: purified liver enzyme (●—●), B1 (▲—▲), B3 (■—■), S1 (○—○) and S2 (△—△).

transferase purified from human liver aggregated and tended to precipitate out of solution rather easily. Gel-filtration of such a detergent-free enzyme preparation gave a very broad zone of activity, which suggested that complexes or aggregates of different size had been formed. The average molecular weight of the detergent-free γ -glutamyl transferase purified from human liver was estimated to be approximately 550,000.

For human bile γ -glutamyl transferase optimized conditions for measurement of the enzyme activity were found to be at the dilution of the whole bile by about 1:100. The heat stability of the biliary γ -glutamyl transferase appeared to be affected by the bile composition; the labilizing factor(s) can be dialyzed out of the bile with subsequent increase in the half-life of γ -glutamyl transferase at 60°C. In individual bile samples, the total γ -glutamyl transferase activity was represented by different molecular weight forms of the enzyme, with the distribution of activity varying between different molecular weight zones from one bile sample to another. Three fractions of γ -glutamyl transferase were identified and designated B_1 , B_2 and B_3 .

Electrophoresis carried out on 89 human serum samples has shown that serum γ -glutamyl transferase activity is heterogeneous with respect to charge. Three zones of mobility were identified in serum: the origin zone and the zones with mobilities of 0.3 - 0.5 and 0.7 - 0.9 relative to serum albumin. γ -Glutamyl transferase in a serum pool with raised total activity was shown to be heterogeneous with respect to size as well as charge. Two serum γ -glutamyl transferase fractions (S_1 and S_2) were prepared.

Fractions of the enzyme prepared from human liver, bile and serum

exhibited the following rather similar physical properties: binding to Concanavalin A, reaction with neuraminidase and stability to denaturation by heat. Their catalytic properties, which were characterized by K_m values for γ -glutamyl-p-nitroanilide and glycylglycine, K_i values for oxidized and reduced forms of glutathione and pH optimum, were found to be virtually the same.

The differences between various γ -glutamyl transferase fractions in human liver, bile and serum appear to be mainly in their size and electrophoretic mobility. These properties are examined more closely in the following chapters.

CHAPTER 4

ELUTION OF γ -GLUTAMYL TRANSFERASE FROM HUMAN LIVER TISSUE

CHAPTER 4

ELUTION OF γ -GLUTAMYL TRANSFERASE FROM HUMAN LIVER TISSUE

4.1 Introduction

The observations reported in the previous chapter showed that γ -glutamyl transferase exists in human liver and body fluids in several components, distinguishable with respect to size and net charge, but with similar, if not identical, heat-stability and catalytic properties. These observations suggested that the various forms of γ -glutamyl transferase might arise by post-translational modification of a single enzyme type, e.g., by the formation of complexes or aggregates. The experiments described in this chapter were designed to reproduce in vitro some of the multiple forms of γ -glutamyl transferase.

4.2 Incubation of human liver tissue in serum and other fluids

Methods

Human liver tissue was obtained and stored as described in Chapter 2. Samples of serum were obtained both from normal subjects and from patients with a variety of hepatobiliary diseases. γ -Glutamyl transferase activity was estimated by the method of Rosalki and Tarlow (1974).

Agarose-gel electrophoresis, location of γ -glutamyl transferase activity in the gels, scanning of the zones of the enzymic activity and determination of their electrophoretic mobilities was carried out as described in Chapter 2. The standard deviations of duplicate scans of the

electrophoretic patterns were ± 3 , ± 13 and ± 13 I.U./l for zones with average activities of 5, 61 and 471 I.U./l, respectively. Nine experiments, in which liver tissue was incubated in serum and electrophoretic scans were made, gave the following estimates of standard deviation for the percentage activities in various zones (with mean percentages in parentheses); ± 3.5 (17.1), ± 9.8 (18.8) and ± 10.8 (56.4). Values for the activity of various zones given in the results were as determined for single analyses, they were not adjusted for these estimates of precision.

Enzyme activity eluted from liver was also characterized by gel-filtration chromatography (Chapter 2).

Two types of experiment were carried out. In the first type, human liver cut into pieces was incubated at 37°C in human serum samples, 0.9% (w/v) NaCl or in Ringer phosphate-bicarbonate solution (Long, 1961), in the proportion of 1 g of liver to 4 ml of serum or protein-free solution. At the beginning of incubation and then every 24 h up to a period of 5 days, these mixtures were centrifuged at a low speed (1,700 r.p.m.) for 10 minutes and 0.2 ml samples of supernatant liquid were taken for determination of γ -glutamyl transferase activity and for electrophoresis. In order to maintain a constant volume, an equal volume of the same serum or protein-free solution was added. Corresponding serum samples incubated without liver tissue served as controls. In the second type of experiment, partially-purified γ -glutamyl transferase from human liver (see Chapter 3) was added to serum samples, 0.9% (w/v) NaCl or to Ringer solution, and incubated as described above.

All incubation mixtures contained 0.05% sodium azide to prevent

bacterial contamination; this concentration of the bacteriostatic agent was found not to affect the enzyme activity.

4.3 Release of γ -glutamyl transferase activity from the liver and its electrophoretic heterogeneity

As a preliminary to the study of the enzyme activity released from liver into serum, electrophoresis was carried out on 29 serum samples which were later used for incubation with liver tissue. The distribution of activity between zones with electrophoretic mobilities of 0, 0.3 - 0.5 and 0.7 - 0.9 (Chapter 3) for these sera is given in Table 4.1.

When liver tissue was added to serum or other fluids, γ -glutamyl transferase activity in the liquid phase gradually increased and a maximum was approached usually between 48 and 72 h after the start of incubation at 37°C (Fig. 4.1). The rate of increase of activity differed between sera, and between the protein-free solutions and serum. Sera with higher initial enzyme activities tended to elute less additional activity of γ -glutamyl transferase from liver tissue (Fig. 4.2).

In each of 29 experiments on incubation of liver tissue with serum, a band of γ -glutamyl transferase activity was detectable at the origin on electrophoresis immediately after the start of incubation. At this stage, the enzyme activity in the origin zone increased in all but three sera, with an average increase of about four-fold. After 48 h of incubation, all sera showed increased activity in this region, the average level being 19 times greater than in the corresponding region before incubation (Table 4.2). Activity was also increased in the zone with mobility 0.3 - 0.5 immediately after the beginning of incubation in 23 out of the

TABLE 4.1 - Distribution of γ -glutamyl transferase activity between zones after electrophoresis of 29 serum samples.

Serum No.	Total activity of serum (A) and distribution in zones (I.U./l)				
	A	0	0.3-0.5	0.7-0.9	>1.0
1	28	1.5	9	17.5	0
2	8	3	3	2	0
3	9	1	3	5	0
4	7	1	1	5	0
5	27	2	0	25	0
6	18	6	0	12	0
7	42	2	1	39	0
8	195	4	16	175	0
9	120	1	8	111	0
10	91	2	18	71	0
11	107	6	22	79	0
12	77	5	11	61	0
13	390	50	87	253	0
14	195	3	24	168	0
15	125	2	12	111	0
16	116	4	20	92	0
17	30	-	-	-	0
18	251	7	20	224	0
19	400	7	36	357	0
20	118	5	3	110	0
21	56	4.6	0.4	51	0
22	167	0.3	7.7	159	0
23	92	3	6	83	0
24	386	17	69	300	0
25	283	7	76	200	0
26	71	-	-	-	0
27	283	2	0	281	0
28	128	1	1	126	0
29	381	0	12	369	0
Average	151.8	5.4	17.3	129.1	0

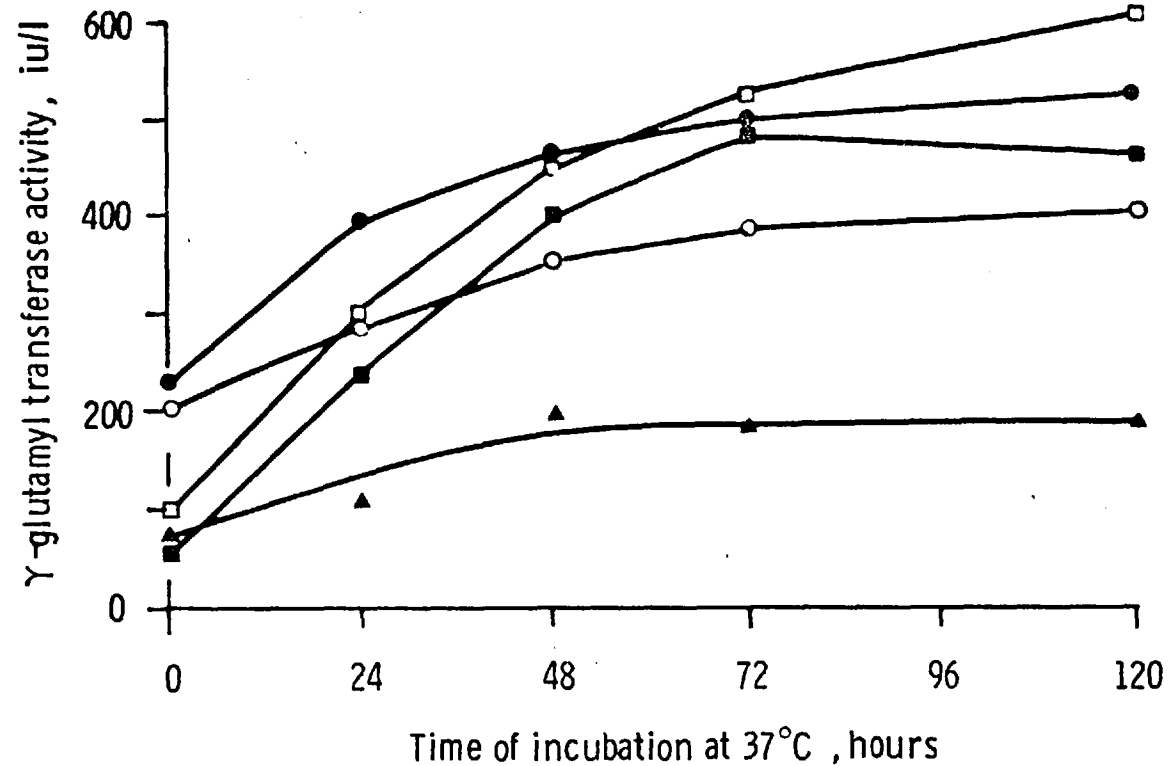


Fig. 4.1

Increase in activity of γ -glutamyl transferase in the liquid phase during incubation of human liver tissue in a pool of normal serum (□), a pool of sera with high γ -glutamyl transferase activity (●), a single specimen of serum of activity 195 I.U./l (○), sodium chloride solution (■), and Ringer solution (▲). The "zero-time" activity was measured immediately after adding the liver tissue.

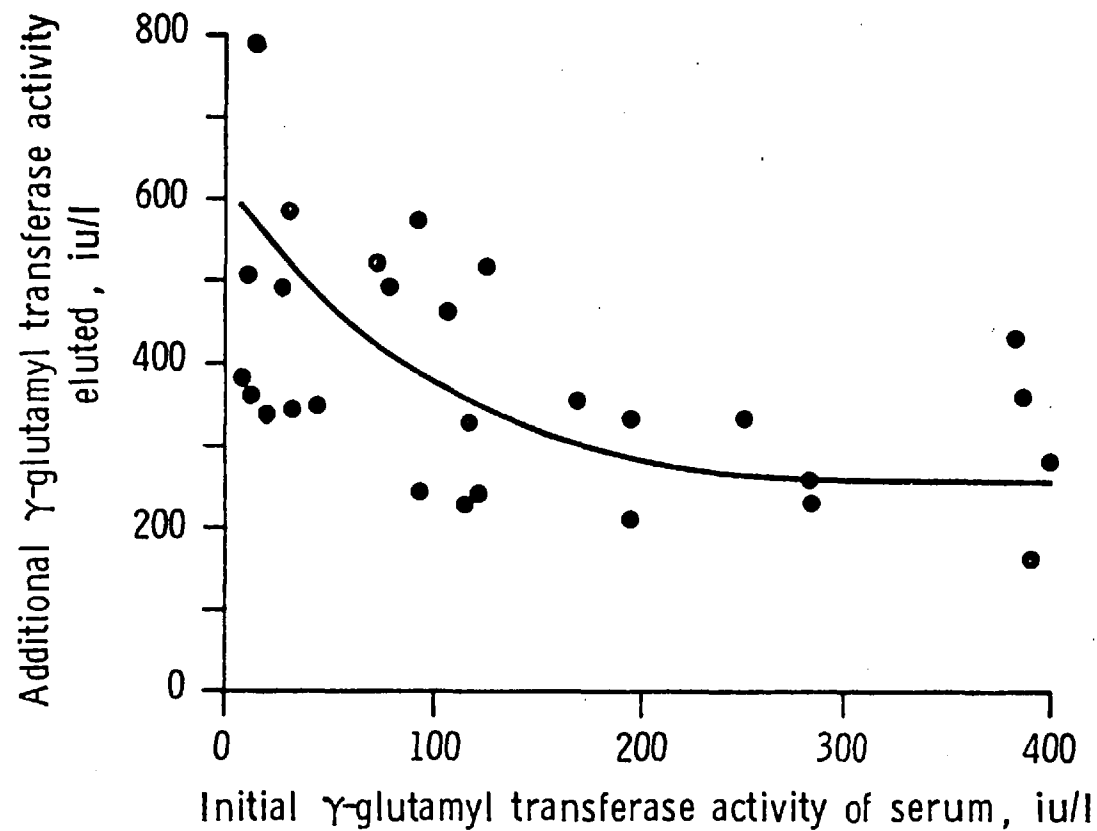


Fig. 4.2

Relationship between the increase in γ -glutamyl transferase activity in the liquid phase and the initial activity of the serum, after incubation of human liver tissue for 5 days at 37°C in various serum samples.

TABLE 4.2 - Changes in total γ -glutamyl transferase activity and activities of zones separated by electrophoresis before and after incubation of a single human liver in various sera.

Serum No.	Total activity shortly after beginning of incubation (A) and distribution in zones (I.U./l)					Total activity after 48 h incubation at 37°C (B) and distribution in zones (I.U./l)				
	A	0	0.3-0.5	0.7-0.9	>1.0	B	0	0.3-0.5	0.7-0.9	>1.0
1	98	27	0	71	0	475	114	0	297	64
2	48	15	6	27	0	363	50	0	251	62
3	42	16	7	19	0	274	52	0	188	34
4	35	7	6	22	0	247	27	0	198	22
5	72	12	9	51	0	429	73	0	319	37
6	48	3	0	45	0	275	34	204	0	37
7	63	9	8	46	0	268	60	0	208	0
8	232	19	24	189	0	473	151	0	246	76
9	121	4	14	103	0	305	53	0	181	71
10	135	28	28	79	0	490	102	0	324	64
11	135	34	71	30	0	327	164	0	163	00
12	106	29	20	57	0	379	87	0	254	38
13	390	30	217	143	0	471	75	0	264	132
14	204	12	29	163	0	358	69	0	241	48
15	200	26	26	148	0	497	82	0	288	127
16	149	25	20	104	0	322	106	0	156	60
17	58	8	13	37	0	306	109	0	197	0
18	279	47	22	210	0	471	127	0	221	123
19	400	16	0	384	0	600	234	0	279	87
20	139	10	0	129	0	324	59	0	214	51
21	122	51	0	71	0	469	145	0	324	0
22	214	14	80	120	0	325	61	0	264	0
23	116	8	14	94	0	262	52	0	210	0
24	465	25	168	272	0	671	517	0	154	0
25	307	6	96	205	0	366	38	0	134	194
26	102	12	12	78	0	361	47	0	224	90
27	269	15	30	224	0	453	43	0	247	163
28	177	11	23	143	0	442	31	0	210	201
29	474	25	46	403	0	661	57	0	219	385
Average	179.3	18.8	34.1	126.4	0	402.2	97.2	7.0	223.3	74.7

29 sera, this type of activity averaging twice the pre-incubation level for all samples. After 48 h, however, activity in this zone was not measurable, except in one serum, either because of a decrease in its activity or because it had become obscured by faster zones with stronger enzyme activity. Twenty out of 29 sera showed an increased activity in the fastest zone (electrophoretic mobility 0.7 - 0.9) at the beginning of incubation but the average increase was only about 6%. After 48 h at 37°C, the activity in this zone was above the initial level in 22 sera, with an average increase of nearly 2 times (Table 4.2).

The most pronounced change in electrophoretic pattern after 48 h of incubation was the appearance in 22 sera of another zone with anodal mobility greater than that of albumin (Table 4.2). This zone constituted approximately 25% of the average total activity of the sera in which it appeared, and in some sera, it accounted for the greatest part of the total γ -glutamyl transferase activity. Prolonged incubation of some samples in which this fast-moving zone was not clearly present after 48 h caused this zone to become more prominent (Fig. 4.3). The appearance of densitometric scans of enzyme activity after electrophoresis of some sera in which the fast-moving zone was seen after 48 h suggested that it may have originated from splitting of zones present at an earlier stage of incubation, or from other alterations in their mobilities (Figs. 4a and b), especially in those cases in which the increase in total activity of the serum during the later stages of incubation was less than the activity in the fastest zone. Incubation at 37°C of serum controls without added liver produced no marked changes in electrophoretic pattern although slight increases in anodal mobility of the faster zones were observed in a few samples.

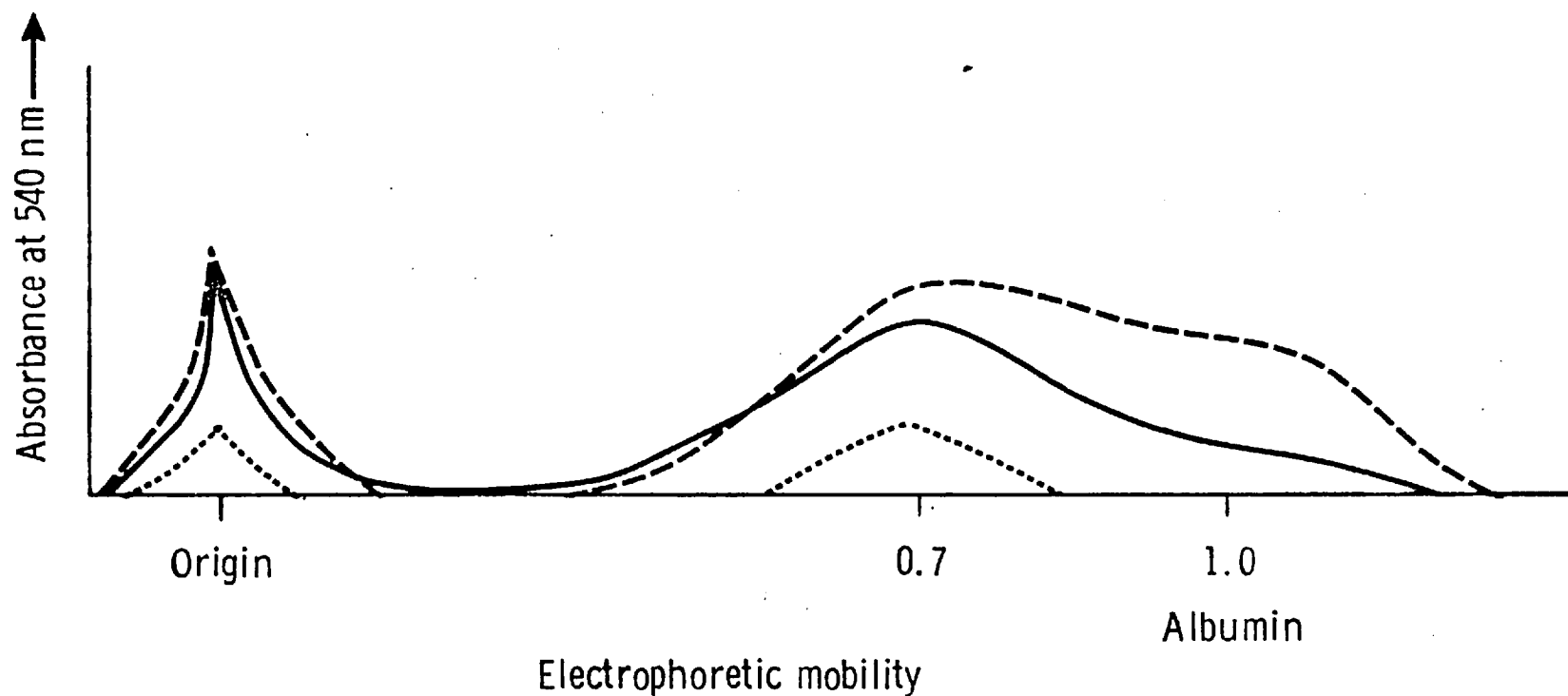


Fig. 4.3

Distribution of γ -glutamyl transferase activity amongst zones separated by agarose gel electrophoresis after incubation of human liver tissue in serum at 37°C, immediately after beginning incubation (dotted line), after 48 hours incubation (solid line), and after 5 days (dashed line).

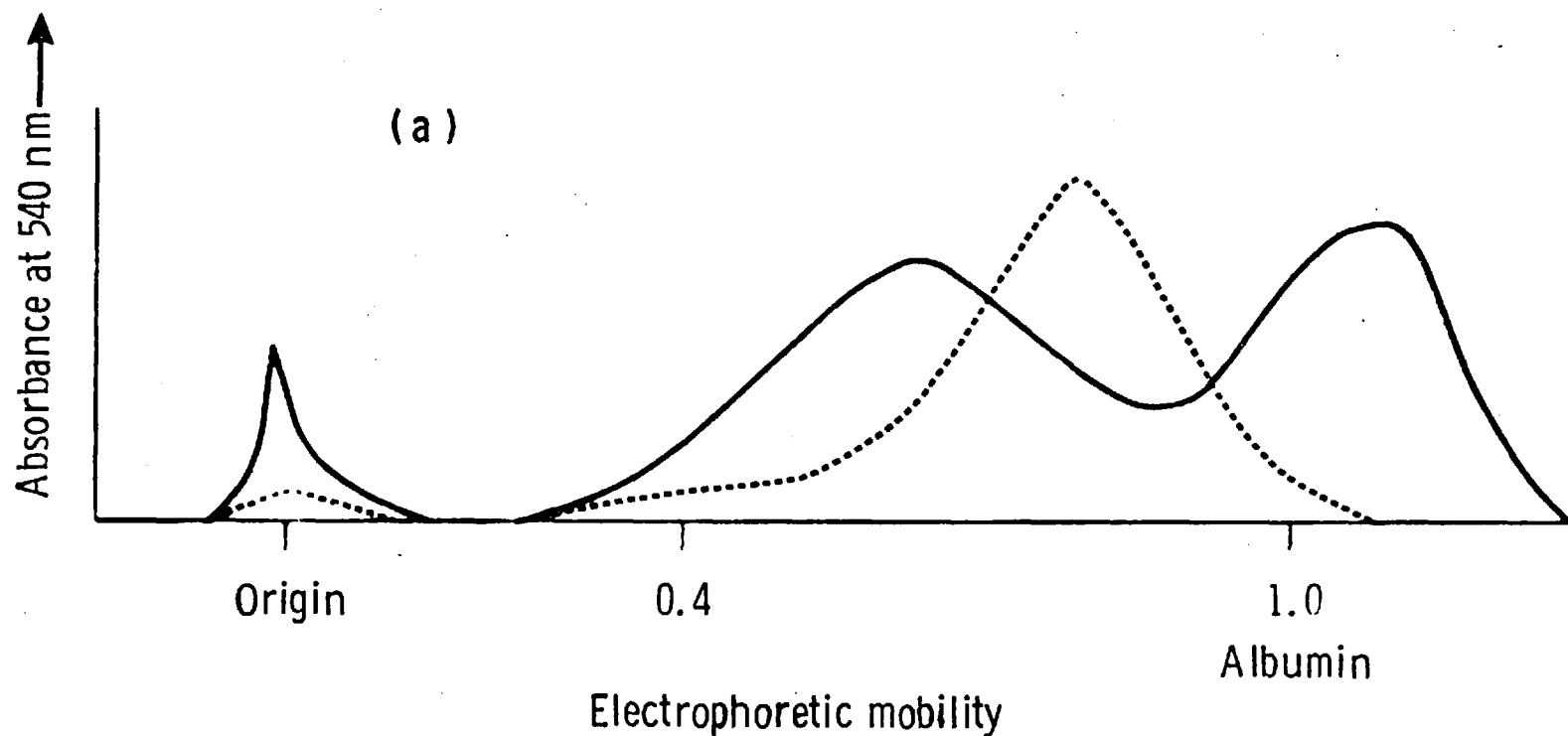


Fig. 4.4a

Distribution of γ -glutamyl transferase activity amongst zones separated by electrophoresis in agarose gel after incubation of human liver tissue in serum, immediately after the start of incubation (dotted line), and after 48 hours at 37°C (solid line).

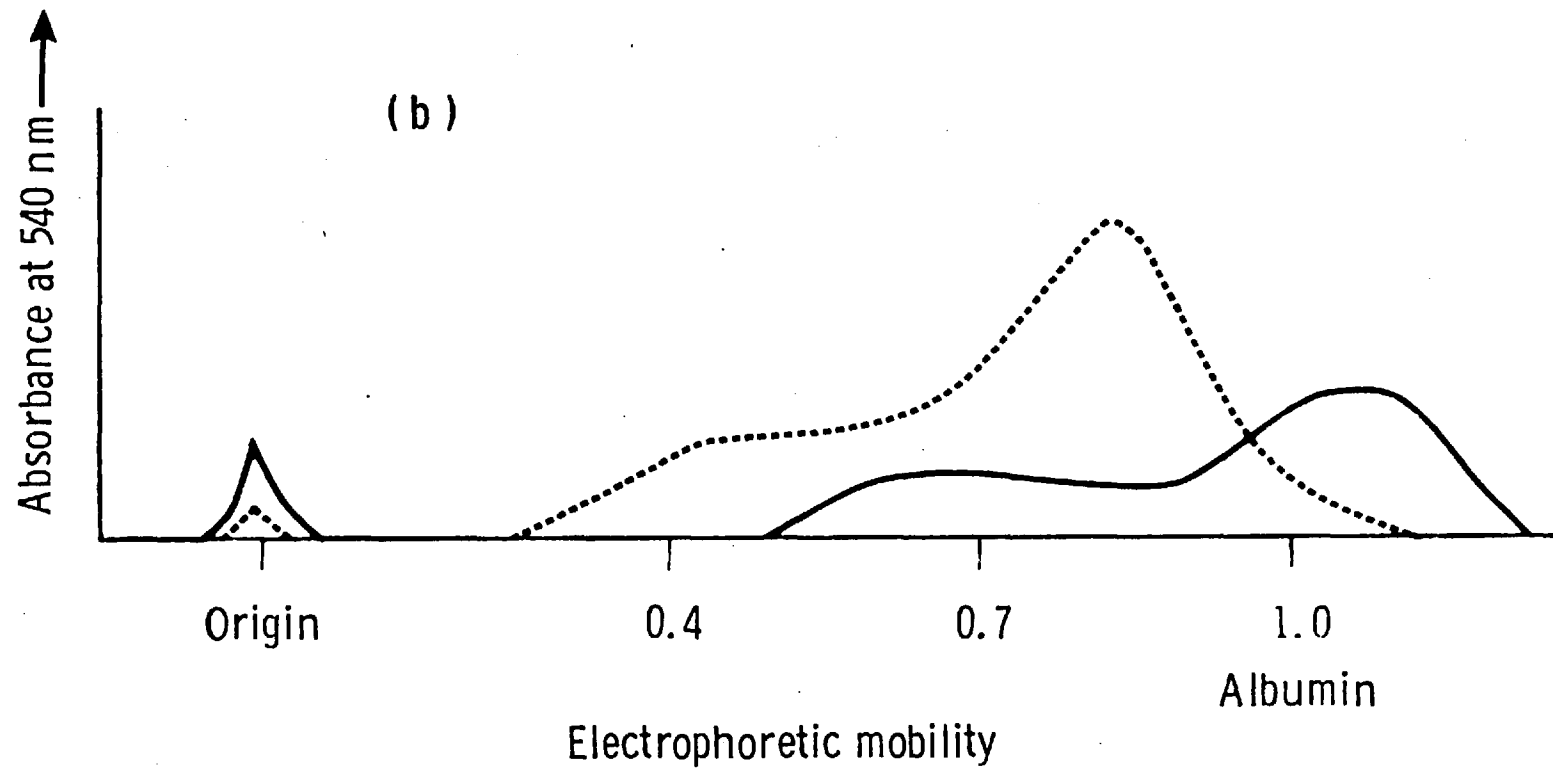


Fig. 4.4b

Distribution of γ -glutamyl transferase activity amongst zones separated by electrophoresis in agarose gel after incubation of human liver tissue in serum, immediately after the start of incubation (dotted line), and after 48 hours at 37°C (solid line).

When liver tissue was incubated in either saline or Ringer solution at 37°C, two zones of γ -glutamyl transferase activity appeared, one at the origin and the other one with a mean electrophoretic mobility of approximately 0.6 that of albumin (Fig. 4.5), i.e., slightly less anodal than the main band present in native sera. Both zones were detectable right after the start of incubation and became stronger as incubation progressed, without marked changes in electrophoretic mobility. Zones with mobility greater than 0.7 relative to albumin were not produced in these experiments.

Partially purified liver γ -glutamyl transferase (Chapter 3) had an anodal electrophoretic mobility of about 0.7 - 0.8 that of albumin, irrespective of whether it was dissolved in 0.9% NaCl or Ringer solution, or in various samples of serum. Incubation at 37°C did not change the mobility of the zone corresponding to added partially purified enzyme significantly, although sometimes a slight increase in anodal mobility was observed.

Discussion

These experiments demonstrated that forms of γ -glutamyl transferase released from liver tissue into serum in vitro resembled to some extent forms occurring in native serum as far as their electrophoretic mobility was concerned.

γ -Glutamyl transferase activity which stays at the origin during electrophoresis is increased by incubation of liver tissue, but not partially purified γ -glutamyl transferase, in serum or in protein-free solutions. The zone with electrophoretic mobility of 0.7 - 0.9, which is

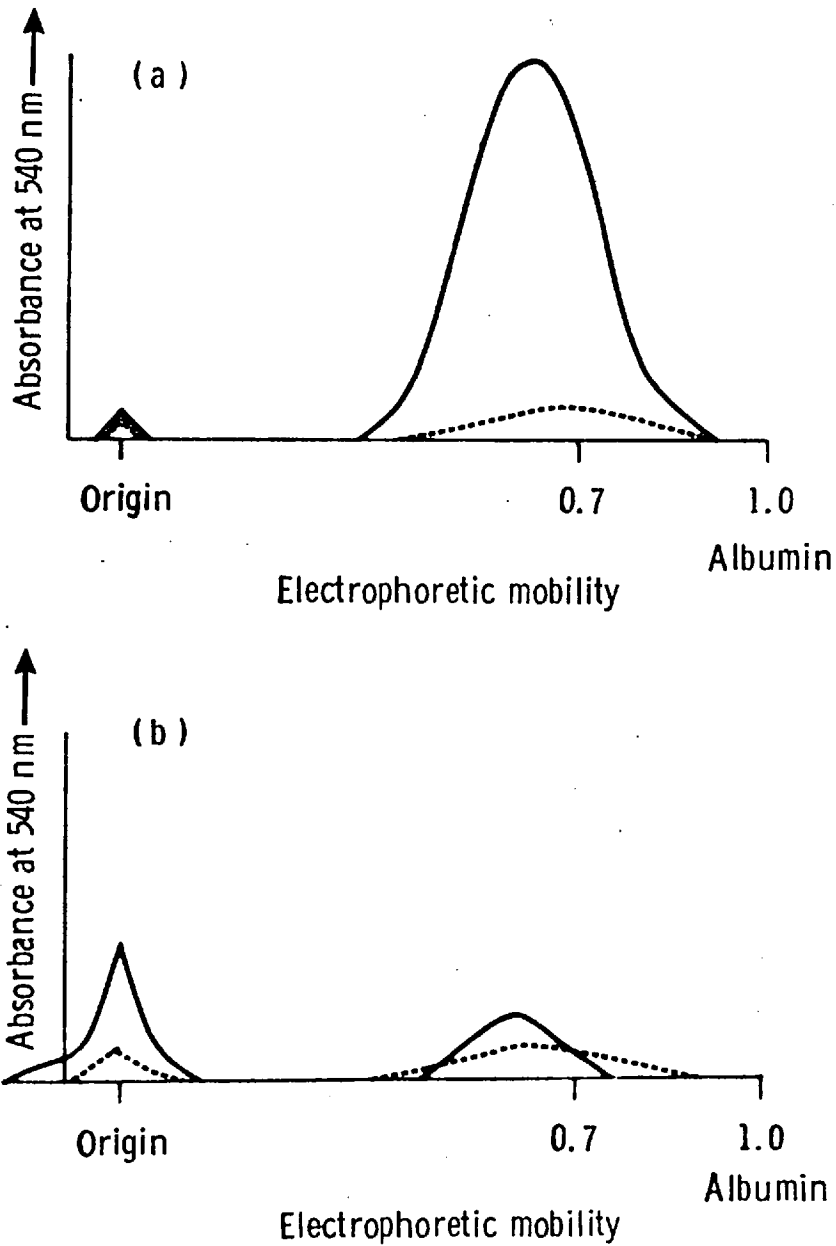


Fig. 4.5 Distribution of γ -glutamyl transferase activity amongst zones separated by agarose gel electrophoresis, immediately after the start of incubation of liver tissue (dotted line), and after 48 hours incubation at 37°C (solid line), in (a) sodium chloride solution and (b) Ringer solution.

predominant in serum, can also be reproduced by incubating liver tissue with serum samples, although the experimentally-produced increase in activity was less than might have been expected in view of the prominence of this zone in serum samples.

A zone of γ -glutamyl transferase activity with intermediate mobility (0.3 - 0.5) is frequently present in serum (Chapter 3), and was also present in increased amounts soon after the beginning of incubation of liver tissue in serum. However, this zone was never seen when liver tissue was incubated with protein-free solutions, nor when the partially purified enzyme was added to serum or other fluids. Therefore, serum and liver tissue together seem necessary to produce an increased activity in this zone, although the increase was transient in these experiments. A further implication of these experiments is therefore that the electrophoretic mobility of γ -glutamyl transferase eluted from liver can be modified to some extent by the serum into which it is released, but that the partially purified enzyme is not susceptible to this process. Other investigators have also provided evidence that the electrophoretic properties of γ -glutamyl transferase can be modified by interaction with other components of serum (Von Freise, Magersted and Schmidt, 1976), and that purification can affect the ability of the enzyme to take part in such interactions (Huseby, 1978).

Furthermore, evidence suggestive of interconversion of electrophoretically separable components of γ -glutamyl transferase in serum is provided by the observations that prolonged incubation gives rise to new zones, or to a re-distribution of activity between different zones. These apparent modifying effects of serum on the electrophoretic pattern of

γ -glutamyl transferase eluted from liver, particularly on longer incubation, together with the lower amounts of activity eluted by sera with initially high γ -glutamyl transferase activities, may indicate the presence of binding sites for the enzyme in serum which can be saturated with enzyme in its native state but not in its purified form.

4.4 Gel-filtration of γ -glutamyl transferase fractions obtained by incubation of liver tissue in vitro

Since the studies reported in Chapter 3 had shown naturally-occurring γ -glutamyl transferase activity in serum to be heterogeneous with respect to size as well as charge, the enzyme fractions obtained by incubation of liver tissue in various fluids were characterized by gel-filtration as well as by electrophoretic mobility. This technique also allowed further γ -glutamyl transferase fractions to be prepared in sufficient quantities for their properties to be compared with those of apparently analogous naturally-occurring fractions.

For the preparation of these fractions, pieces of human liver tissue were incubated in a pool of normal human serum at 37°C, in the proportion of 1 g of liver to 4 ml of serum pool. To prevent bacterial growth, sodium azide (0.1% w/v) was added to the incubation mixture. After 48 h incubation, the mixture was centrifuged at low speed, the supernatant liquid was collected and filtered through glass wool. This filtrate was applied on to the Sepharose 6B column pre-equilibrated with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl.

The total γ -glutamyl transferase activity of the incubation liquid separated into two activity peaks: the one of greater molecular

size eluted usually between fractions No. 21 - 29 (63 - 87 ml effluent volume) and the smaller one between fractions No. 39 - 49 (117 - 147 ml effluent volume). The fractions comprising these two components were designated LS_2 and LS_1 , respectively. Column fractions containing either LS_1 or LS_2 -enzyme activities were pooled and concentrated with "Carbowax" 20m for use in further experiments. A typical elution profile of the incubation liquid (liver in serum) from the Sepharose 6B column is shown in Fig. 4.6.

Examples of changes in the distribution of γ -glutamyl transferase activity between different molecular weight forms in the liquid during incubation of liver tissue in serum are shown in Figs. 4.7a and b.

Enzyme from liver was prepared by incubating the tissue in 0.9% (w/v) NaCl in the same way as with serum pool. The supernatant liquid after low-speed centrifugation gave just one peak of γ -glutamyl transferase activity on gel-filtration through the Sepharose 6B column. This peak of activity (designated LN_1) was eluted between fractions No. 37 - 44 (111 - 132 ml effluent volume), with the maximum activity around fraction No. 41 (Fig. 4.8). The active fractions were pooled and concentrated with "Carbowax" 20m.

4.5 Properties of LS_1 , LS_2 and LN_1 γ -glutamyl transferase preparations

(a) Physical properties

Determination of molecular weights and electrophoretic mobility, affinity chromatography on Concanavalin A covalently bound to Sepharose 4B, effect of neuraminidase action, and heat stability experiments on

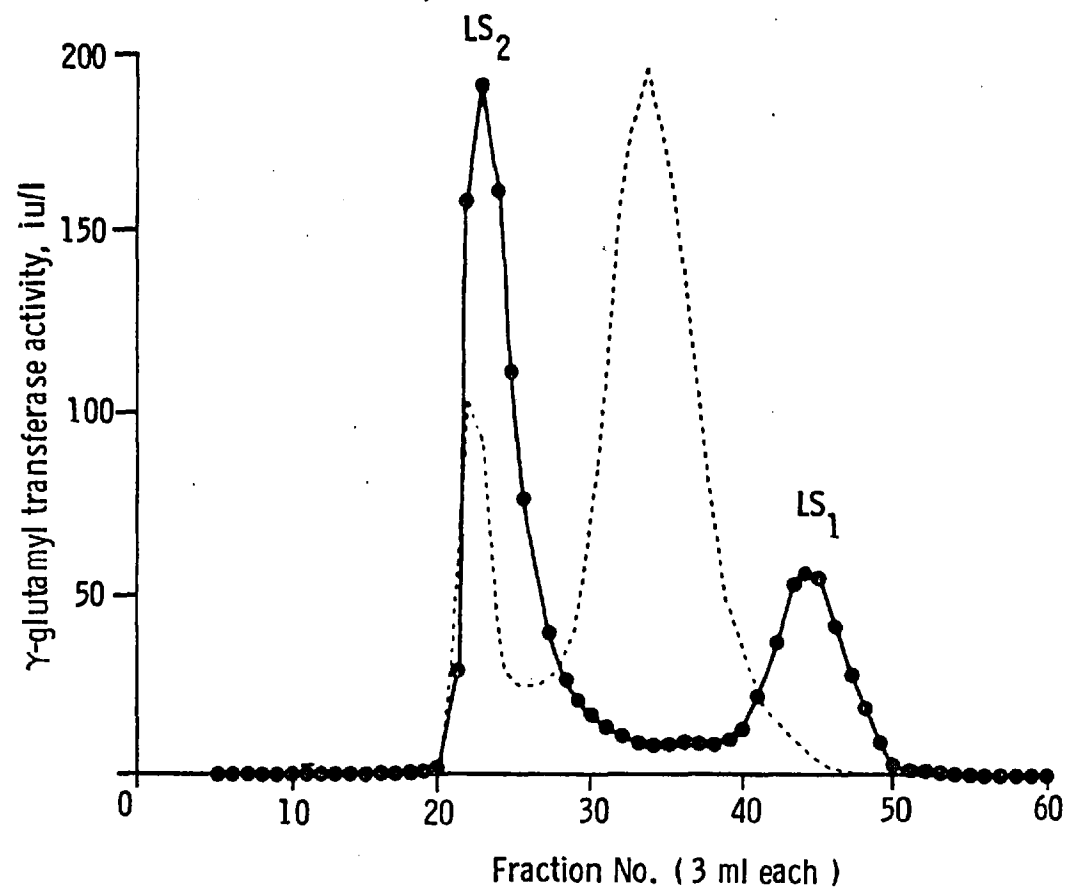


Fig. 4.6 Sepharose 6B gel-filtration of the incubation liquid when liver tissue was incubated in normal serum pool for 48 hours at 37°C; 8 ml sample with total γ -glutamyl transferase activity of 218 I.U./l was applied; broken line represents the elution position of the enzyme activity in "high γ GT serum pool".

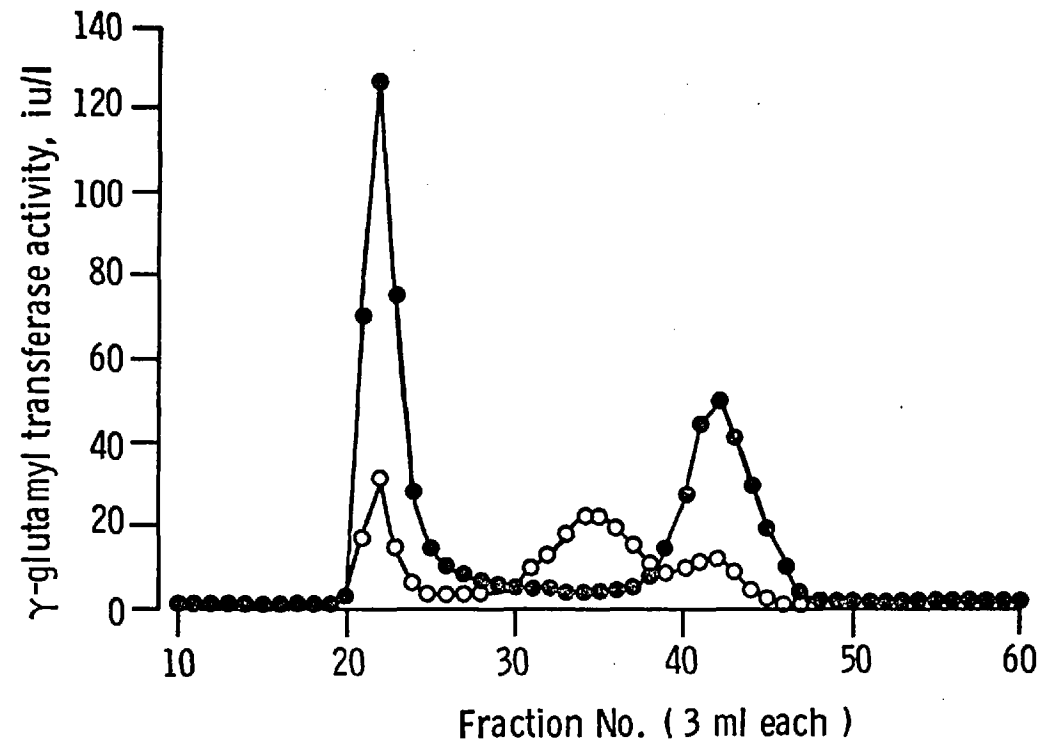


Fig. 4.7a.

Sepharose 6B gel-filtration of the incubation liquid when liver tissue was incubated in "high γ -glutamyl transferase serum pool": distribution of γ -glutamyl transferase activity at the beginning (○—○) and after 48 hours incubation at 37°C (●—●); 3 ml samples of 211 I.U./l and 340 I.U./l, respectively, were applied to the column.

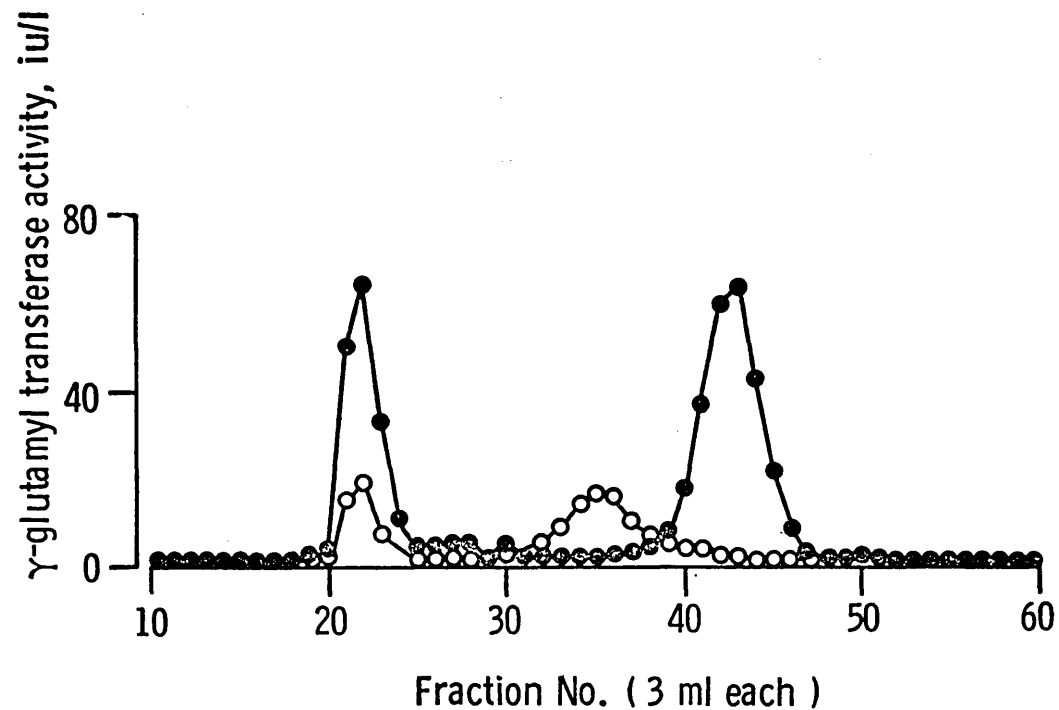


Fig. 4.7b.

Sephacrose 6B gel-filtration of the incubation liquid when liver tissue was incubated in a patient's serum sample: distribution of γ -glutamyl transferase activity at the beginning ($\circ$ — $\circ$) and after 48 hours incubation at 37°C ($\bullet$ — $\bullet$); 1.3 ml sample with activity of 274 I.U./l and 2.3 ml sample with activity of 576 I.U./l, respectively, were applied to the column.

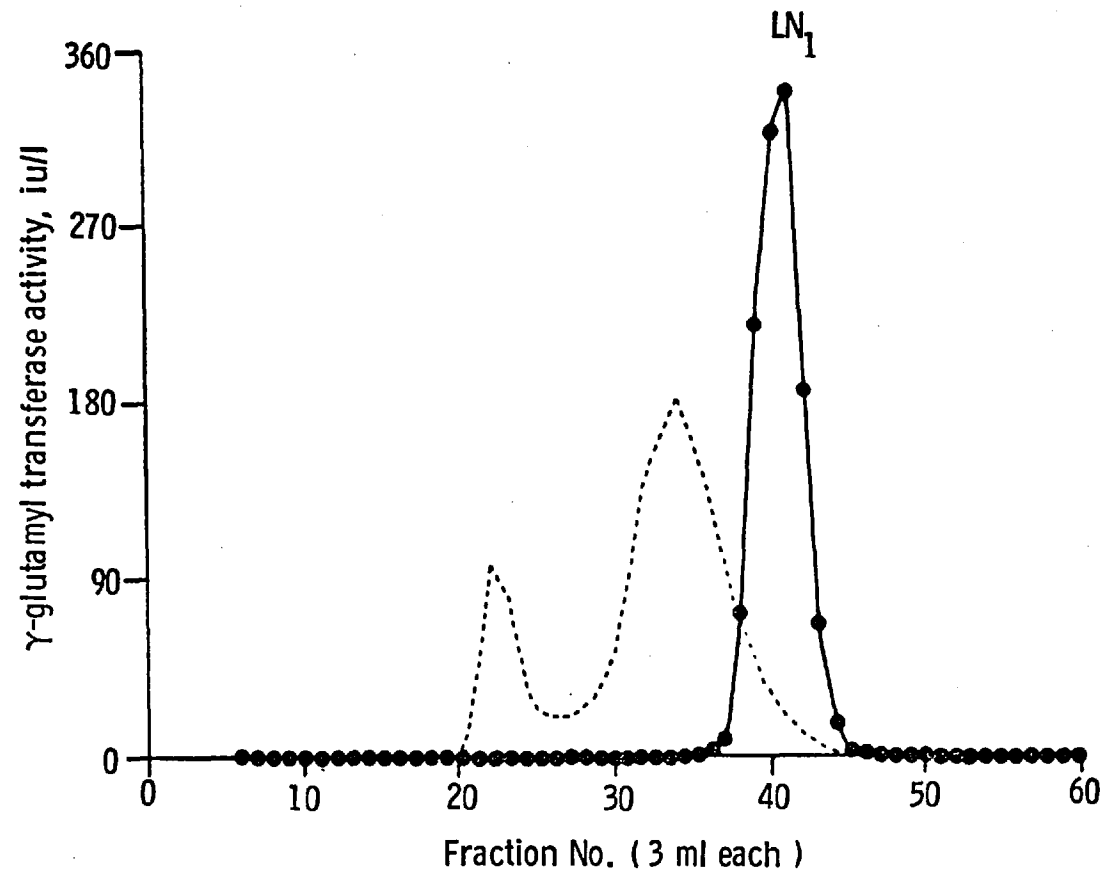


Fig. 4.8.

Sephacrose 6B gel-filtration of the incubation liquid when liver tissue was incubated in saline solution: 3 ml sample with γ -glutamyl transferase activity of 1,394 I.U./l was applied; broken line represents the elution position of the enzyme activity in "high γ -glutamyl transferase serum pool".

γ -glutamyl transferase fractions eluted from liver by serum and 0.9% NaCl were carried out as described for the enzyme in human liver, bile and serum in Chapter 3. The results obtained are summarized in Tables 4.3, 4.4, 4.5 and 4.6.

As can be seen from Table 4.3, the molecular weights of LS_1 and LN_1 fractions of γ -glutamyl transferase as well as their electrophoretic mobilities were similar. The LS_2 fraction which had a molecular weight of over 1×10^6 , was heterogeneous on electrophoresis, i.e., part of its activity remained at the origin and part had a mobility of 0.6 that of serum albumin. With respect to size and charge, the artificially prepared LS_2 fraction of γ -glutamyl transferase resembled the B_3 and S_2 fractions of this enzyme which occur naturally in human bile and serum (Chapter 3).

Affinity chromatography of γ -glutamyl transferase fractions prepared by incubation of liver tissue in vitro (Table 4.4) shows that most of the enzyme activity (between 70 and 90% of the total activity) binds to Concanavalin A.

Treatment with neuraminidase resulted in slower-moving bands of activity in the case of LS_1 and LN_1 preparations. However, neuraminidase had no effect on the electrophoretic mobility of the LS_2 fraction of γ -glutamyl transferase (Table 4.5).

The stability of the fractions of the enzyme prepared by incubating liver tissue in vitro to inactivation by heat was rather similar. This is shown by their half-lives at 60°C (Table 4.6).

Table 4.3 Estimated molecular weights and electrophoretic mobilities of γ -glutamyl transferase fractions obtained by incubation of liver tissue in vitro.

No.	γ -Glutamyl transferase preparation	Molecular weight	Molecular weight determined by gel-filtration on:	Electrophoretic mobility (Albumin = 1.0)
1	LS ₁	82,000	Sepharose 6B and Sephadex G-200	0.5 - 0.7
2	LS ₂	>1,000,000	Sepharose 6B (excluded)	0 and 0.6
3	LN ₁	82,000	Sepharose 6B and Sephadex G-200	0.6 - 0.7

Table 4.4 Affinity chromatography of γ -glutamyl transferase fractions prepared by incubation of liver tissue in vitro.

No.	γ -Glutamyl transferase preparation	γ -Glutamyl transferase bound to Concanavalin A		γ -Glutamyl transferase not bound to Concanavalin A	
		% of total activity	Electrophoretic mobility	% of total activity	Electrophoretic mobility
1	LS ₁	85.6	0.6	14.4	0; 0.6
2	LS ₂	70.0	0; 0.6	30.0	0; 0.8
3	LN ₁	89.7	0; 0.6	10.3	0; 0.66

Table 4.5 - Effect of neuraminidase on γ -glutamyl transferase fractions obtained by incubation of liver tissue in vitro.

No.	Enzyme preparation	γ -Glutamyl transferase activity (I.U./l) and % original activity in brackets:			Electrophoretic mobility	
		Untreated	Incubated controls	After treatment	Before treatment	After treatment, mean of 4 experiments $\pm$ S.D.
1	LS ₁	204	194 (95.1)	174 (85.3)	0.5 - 0.7	0 $\pm$ 0; 0.19 $\pm$ 0.05
2	LS ₂	368	286 (77.7)	333 (90.5)	0; 0.6	0 $\pm$ 0; 0.58 $\pm$ 0.01
3	LN ₁	735	663 (90.2)	590 (80.3)	0.6 - 0.7	0.15 $\pm$ 0.02

Table 4.6 - Heat-stability of γ -glutamyl transferase fractions prepared by incubation of liver tissue in vitro.

No.	Enzyme preparation	Half-life at 60°C (mean of 4 experiments ± S.D.), mins.
1	LS ₁	10.8 ± 1.0
2	LS ₂	8.8 ± 0.9
3	LN ₁	8.0 ± 0.2

(b) Catalytic properties

K_m values for γ -glutamyl-p-nitroanilide and glycylglycine and K_i values for oxidized and reduced forms of glutathione of the LS₁, LS₂ and LN₁ forms of γ -glutamyl transferase were determined in the same way as for the enzyme in human liver, bile and serum (Chapter 3). The results are summarized in Table 4.7.

γ -Glutamyl transferase activity of LS₁, LS₂ and LN₁ pools was measured in buffers with pH ranging from 7.0 to 9.0, and such activity values were plotted versus the pH values (Fig. 4.9). As with the enzyme in human liver, bile and serum, the pH curves obtained showed a broad peak of activity with the pH optimum between values 7.6 and 8.2.

Table 4.7 - Kinetic properties of γ -glutamyl transferase fractions obtained by incubation of liver tissue in vitro.

No.	γ -Glutamyl transferase preparation	K_m for γ -glutamyl-p-nitroanilide, mmol/l (mean of 3 experiments $\pm$ S.D.)	K_m for glycylglycine, mmol/l (mean of 4 experiments $\pm$ S.D.)	K_i for GSSG, mmol/l (mean of 3 experiments $\pm$ S.D.)	K_i for GSH, mmol/l (mean of 3 experiments $\pm$ S.D.)
1	LS ₁	1.25 $\pm$ 0.05	15.4 $\pm$ 1.9	1.06 $\pm$ 0.14	1.10 $\pm$ 0.20
2	LS ₂	1.20 $\pm$ 0.09	15.9 $\pm$ 0.6	0.80 $\pm$ 0.04	1.23 $\pm$ 0.18
3	LN ₁	1.17 $\pm$ 0.12	16.0 $\pm$ 0.8	1.03 $\pm$ 0.03	1.02 $\pm$ 0.05

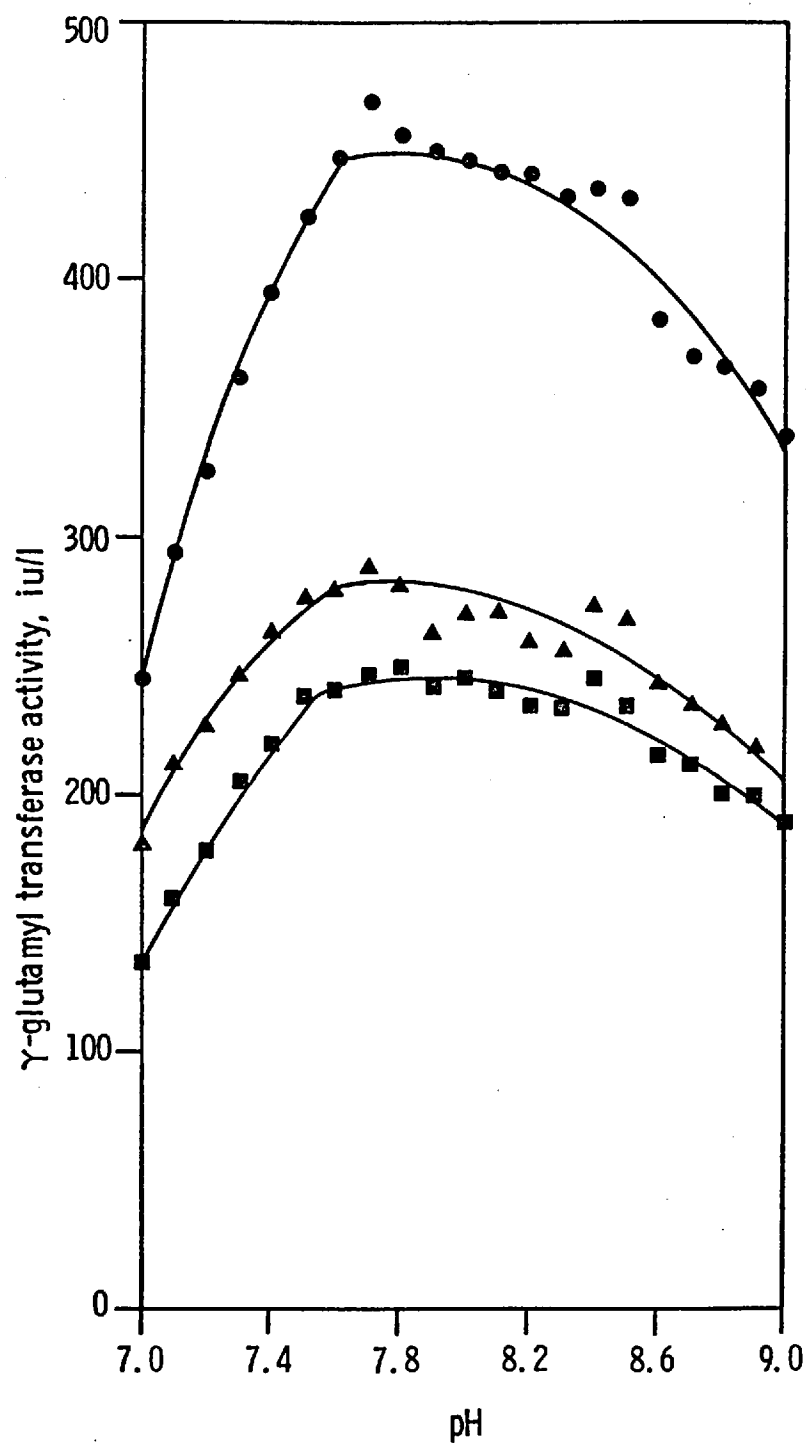


Fig. 4.9 Effect of pH on γ -glutamyl transferase activity in the enzyme fractions eluted from human liver tissue by serum (LS₁, $\blacktriangle$ — $\blacktriangle$ and LS₂, $\bullet$ — $\bullet$) and saline solution (LN₁, $\blacksquare$ — $\blacksquare$).

As can be seen from Table 4.7, the catalytic properties of γ -glutamyl transferase fractions obtained by incubation of liver tissue in serum or saline were very similar.

4.6 Discussion

Incubation of liver tissue in serum or in sodium chloride solution thus resulted in the recognition of three further forms of γ -glutamyl transferase, designated LS_1 , LS_2 and LN_1 . The catalytic characteristics of these fractions were similar to those found earlier for naturally occurring forms of the enzyme, and the new fractions did not differ markedly between themselves in these respects. This observation therefore confirms the conclusion reached by studying the naturally occurring forms, that differences in catalytic characteristics are unlikely to contribute to an understanding of the multiple forms of γ -glutamyl transferase in serum, bile or liver.

As with the naturally-occurring forms of γ -glutamyl transferase, the artificially-prepared LS_1 , LS_2 and LN_1 forms display considerable electrophoretic heterogeneity. This is no doubt due to a large extent to their glycoprotein character, which is shown by their affinity for Concanavalin A, and (except for the LS_2 fraction) to the presence in their molecules of sialic acid residues which can be removed by neuraminidase. Forms of γ -glutamyl transferase differing in apparent molecular weight are obtained by incubation of liver tissue in serum.

The electrophoretic and gel-filtration properties of the LS_1 , LS_2 and LN_1 forms of γ -glutamyl transferase are summarized diagrammatically in Fig. 4.10. This Figure also shows the corresponding characteristics of the naturally-occurring forms as previously determined.

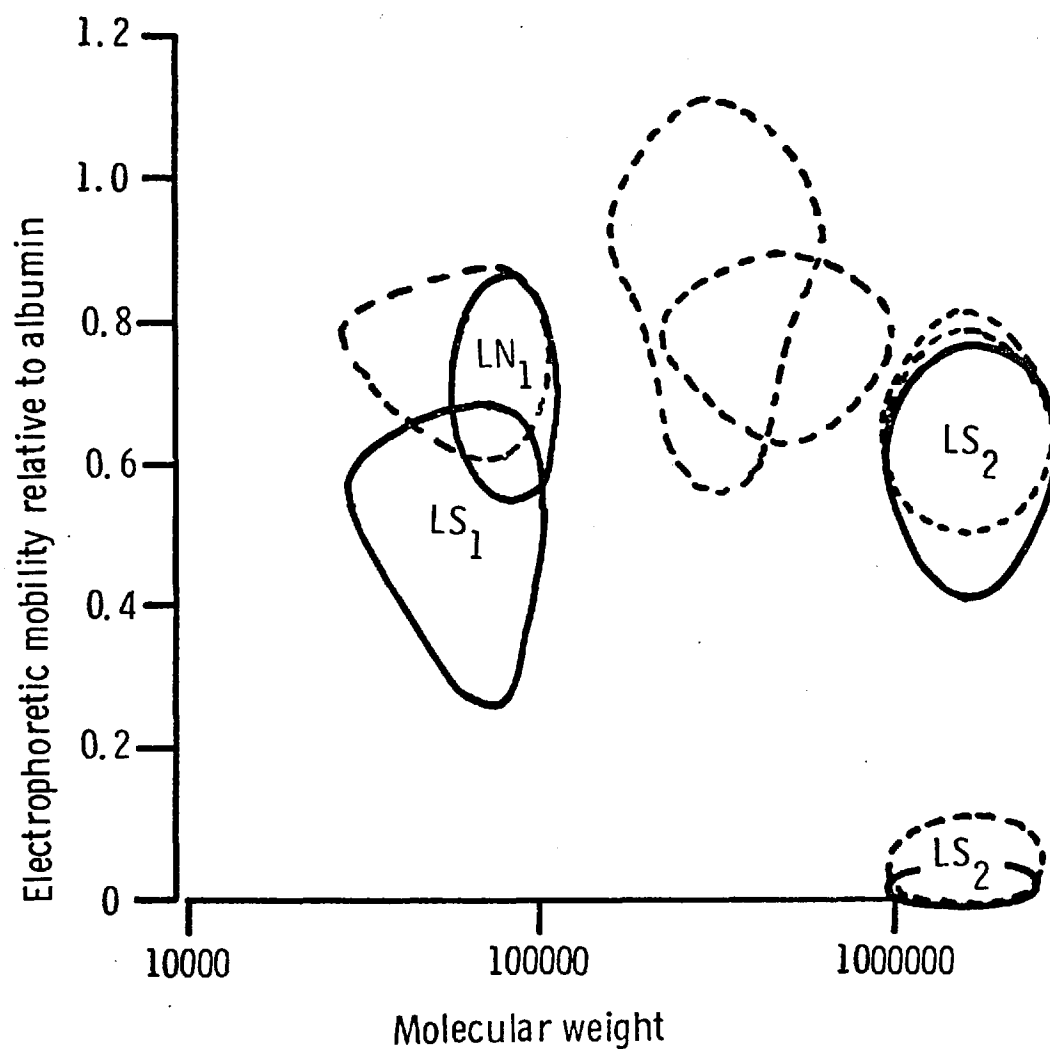


Fig. 4.10 Diagrammatic presentation of electrophoretic and gel-filtration properties of the artificially prepared forms of γ -glutamyl transferase. Dotted outlines show the corresponding characteristics of the naturally-occurring forms of the enzyme.

CHAPTER 5

EXPERIMENTS ON INTERCONVERSION OF VARIOUS γ -GLUTAMYL TRANSFERASE PREPARATIONS

CHAPTER 5

EXPERIMENTS ON INTERCONVERSION OF VARIOUS γ -GLUTAMYL TRANSFERASE PREPARATIONS

5.1 Introduction

The main differences between the naturally occurring in human liver, bile and serum and artificially-produced fractions of γ -glutamyl transferase described in Chapters 3 and 4 appear to be mainly in their size and electric charge; these properties are outlined diagrammatically in Fig. 5.1. The apparent similarities of some artificially-produced and naturally-occurring forms, and the differences between some of the fractions in both categories, particularly with respect to size (Fig. 5.1), suggested that the relationships between them might be clarified by experiments designed to convert one form into another, or to identify components common to some or all of the fractions. Experiments of this kind are now described.

5.2 Experiments on interconversion of various γ -glutamyl transferase preparations

In interconversion experiments, the enzyme preparations from human liver (L- γ GT), bile (B_1 , B_2 and B_3), serum (S_1 and S_2) and also from liver tissue incubated with serum (LS_1 and LS_2) or physiological saline solution (LN_1) were used. Molecular weight and electrophoretic mobility estimations were carried out as described earlier in the thesis (Chapters 2 and 3).

a) Digestion with trypsin

Each of the γ -glutamyl transferase preparations was incubated with trypsin at 37°C for 24 h in the proportion of 1 mg of trypsin to 5 mg of

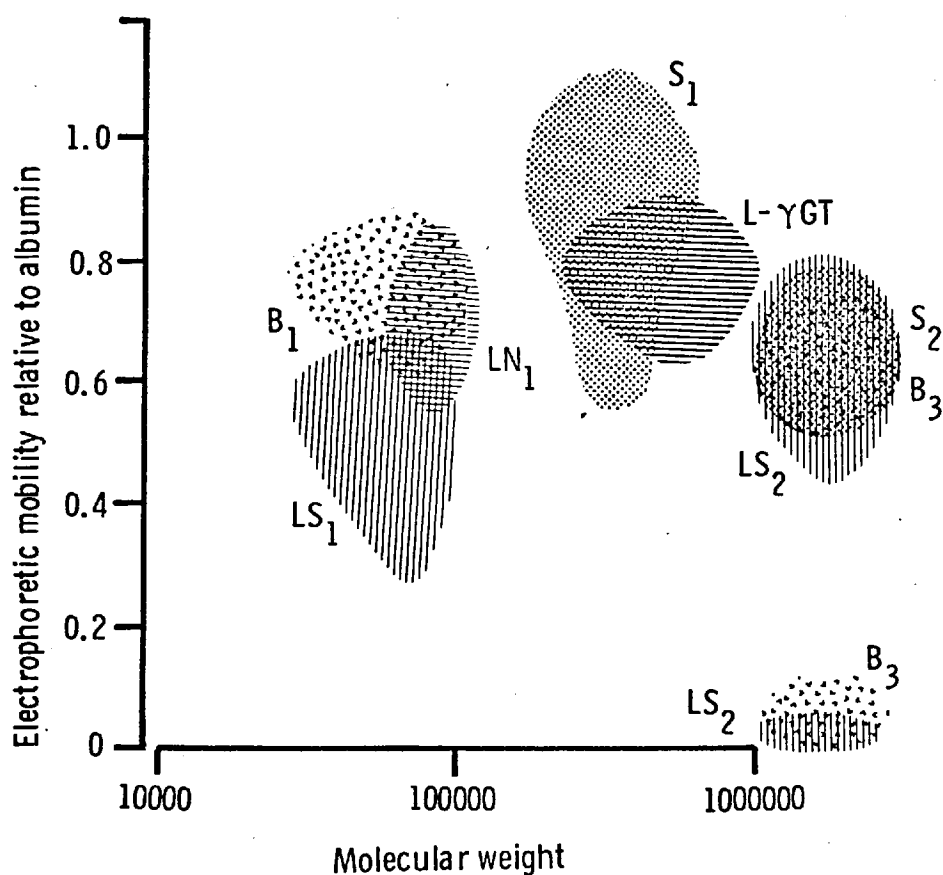


Fig. 5.1 Diagrammatic presentation of the naturally-occurring forms of γ -glutamyl transferase, in human liver (L- γ GT), bile (B₁ and B₃) and serum (S₁ and S₂), and forms artificially prepared by incubation of liver tissue in serum (LS₁ and LS₂) and physiological saline solution (LN₁).

protein in a sample. Corresponding controls were incubated in the same way but without the addition of trypsin. The enzyme activities in all samples before and after 24 h incubation at 37°C were measured. After 24 h, γ -glutamyl transferase activity in nearly all trypsinized and control samples was found to be lower than at the beginning of incubation. Trypsin-treated samples lost more activity than controls.

All trypsinized and control samples were subjected to gel-filtration on a Sepharose 6B column (1.5 x 95 cm) which had been pre-equilibrated with marker proteins. Molecular weights of the peaks of eluted enzyme activity were estimated, the active fractions were pooled, concentrated with "Carbowax" 20m and their mobilities on agarose-gel were determined.

An example of a gel-filtration pattern of one of the γ -glutamyl transferase preparations treated with trypsin (LS₂) is shown in Fig. 5.2. Results of this experiment are given in Table 5.1.

As can be seen from this table, incubation with trypsin did not have any effect on the molecular weights and electrophoretic mobility of B₁, LS₁ and LN₁ γ -glutamyl transferase. For all other preparations, the action of trypsin produced a smaller sized fragment with molecular weight of about 82,000; i.e., the same as B₁, LS₁ and LN₁. However, the electrophoretic mobility of this fragment was not exactly the same for different samples. The largest molecular weight fractions of the enzyme (B₂, B₃, S₂ and LS₂) were not converted into a smaller molecular weight form fully, and part of the enzyme activity of these samples treated with trypsin still eluted from the column with the exclusion peak (M.W. >1,000,000). Similarly, the action of trypsin on the purified liver enzyme (L- γ GT)

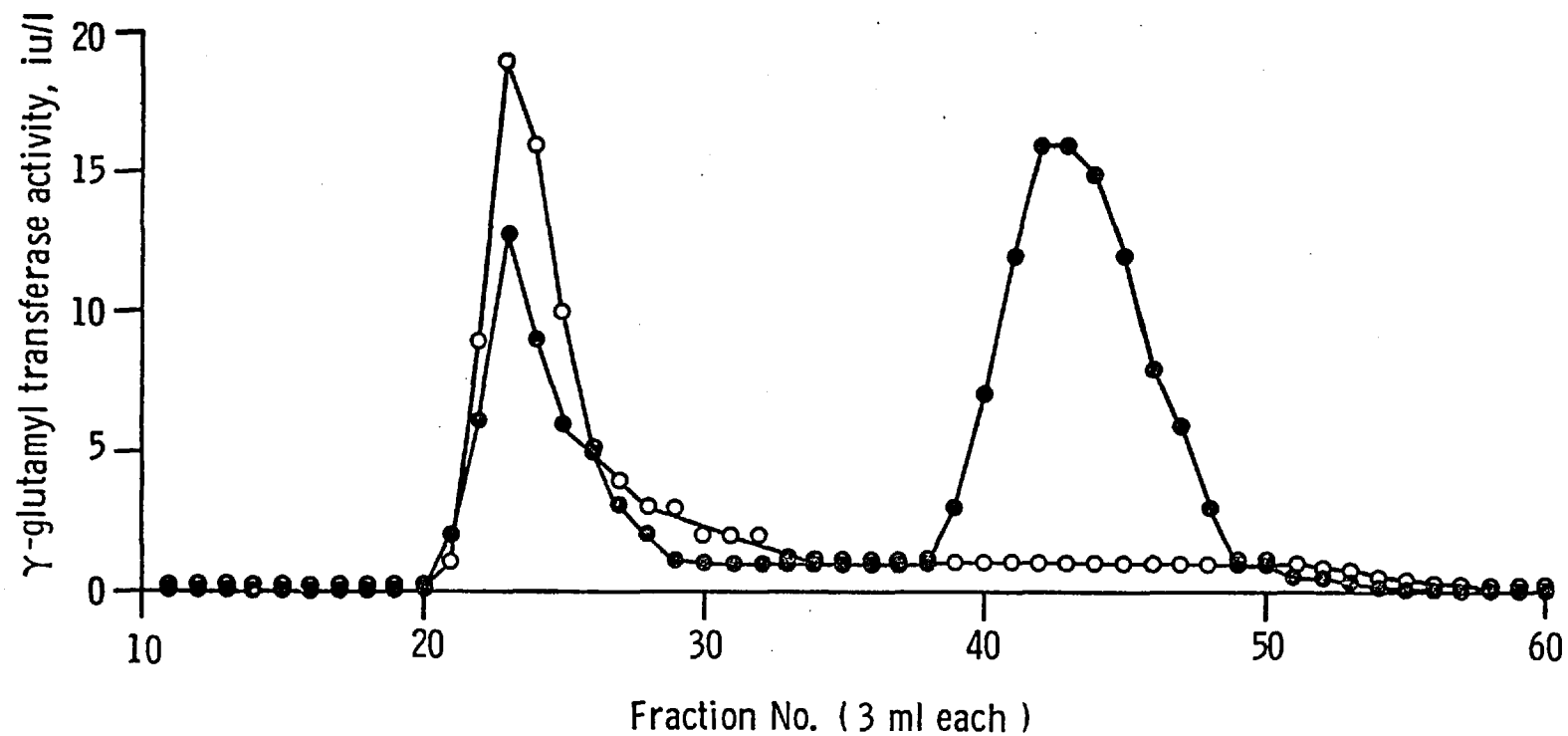


Fig. 5.2.

Sephacrose 6B gel-filtration of LS₂-pool: incubated with 1mg/5mg protein trypsin (●—●) and corresponding control (○—○): 1.5 ml sample with activity of 272 I.U./l and 0.8 ml sample with activity of 383 I.U./l, respectively, were applied to the column.

Table 5.1 - Effect of digestion with trypsin (1 mg/5 mg protein) on various γ -glutamyl transferase preparations

No.	Enzyme preparation	γ -Glutamyl transferase activity I.U./l			Distribution of recovered activity between zones after gel-filtration		Electrophoretic mobility		Recovery of activity from the column, %
		Before treatment	After incubation with trypsin (% of initial activity remaining)	After incubation in controls	Peak I, M.W. (% of total activity eluted)	Peak II, M.W. (% of total activity eluted)	Peak I	Peak II	
1	L- γ GT	558	475 (85.0)	543 (97.0)	82,000 (68.2)	550,000 (31.8)	0.73	0.73	82.1
2	B ₁	771	771 (100)	746 (96.8)	82,000 (100)	---	0.78	---	71.1
3	B ₂	407	404 (99.3)	407 (100)	82,000 (81.0)	>1,000,000 (19.0)	0.78	0	88.5
4	B ₃	1,006	960 (95.4)	1,006 (100)	82,000 (52.6)	>1,000,000 (47.4)	0.77	0; 0.6	85.0
5	S ₁	460	359 (78.0)	456 (99.0)	82,000 (17.5)	>1,000,000 (82.5)	1.3	0.76	98.1
6	S ₂	286	238 (83.0)	262 (92.0)	82,000 (34.0)	>1,000,000 (66.0)	0.8	0.7	91.6
7	LS ₁	160	116 (73.0)	160 (100)	82,000 (100)	---	0.5	---	100
8	LS ₂	412	272 (66.0)	383 (93.0)	82,000 (69.4)	>1,000,000 (30.6)	0.6	0.6	100
9	LN ₁	614	582 (95.0)	606 (99.0)	82,000 (100)	---	0.7	---	78.6

only partially converted it into a smaller molecular weight form (M.W. 82,000). The proportion of the activity converted into a smaller molecular weight form (M.W. 82,000) for different enzyme preparations is given in Table 5.1.

Gel-filtration patterns of γ -glutamyl transferase preparations incubated for 24 h at 37°C (controls) usually did not change significantly. In the case of control samples of bile fractions, however, appearance of the small molecular weight peak of activity (i.e., the same as in trypsin-treated samples) was observed (Fig. 5.3). This may represent the result of proteolytic activity in such samples during incubation at 37°C.

Changes in molecular weight and electrophoretic mobility produced by trypsinization are summarized diagrammatically in Fig. 5.4.

b) Extraction with sodium-deoxycholate

Sodium deoxycholate (Na-DOC) was added to all γ -glutamyl transferase preparations to a final concentration of 0.5% (w/v) and the samples were incubated at 37°C overnight. Enzyme activities were determined before and after incubation. Na-DOC-treated samples were then applied onto the Sepharose 6B column and eluted with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl and 0.05% Na-DOC. Active column fractions were concentrated with "Carbowax" 20m and their mobilities following agarose-gel electrophoresis were determined.

Gel-filtration patterns of three of the treated with 0.5% Na-DOC γ -glutamyl transferase preparations (B_1 , S_1 and LN_1) are shown in Figs. 5.5, 5.6 and 5.7. Changes in the enzyme activity following incubation with Na-DOC, distribution of activity in different zones after gel-filtration and their electrophoretic mobilities are given in Table 5.2.

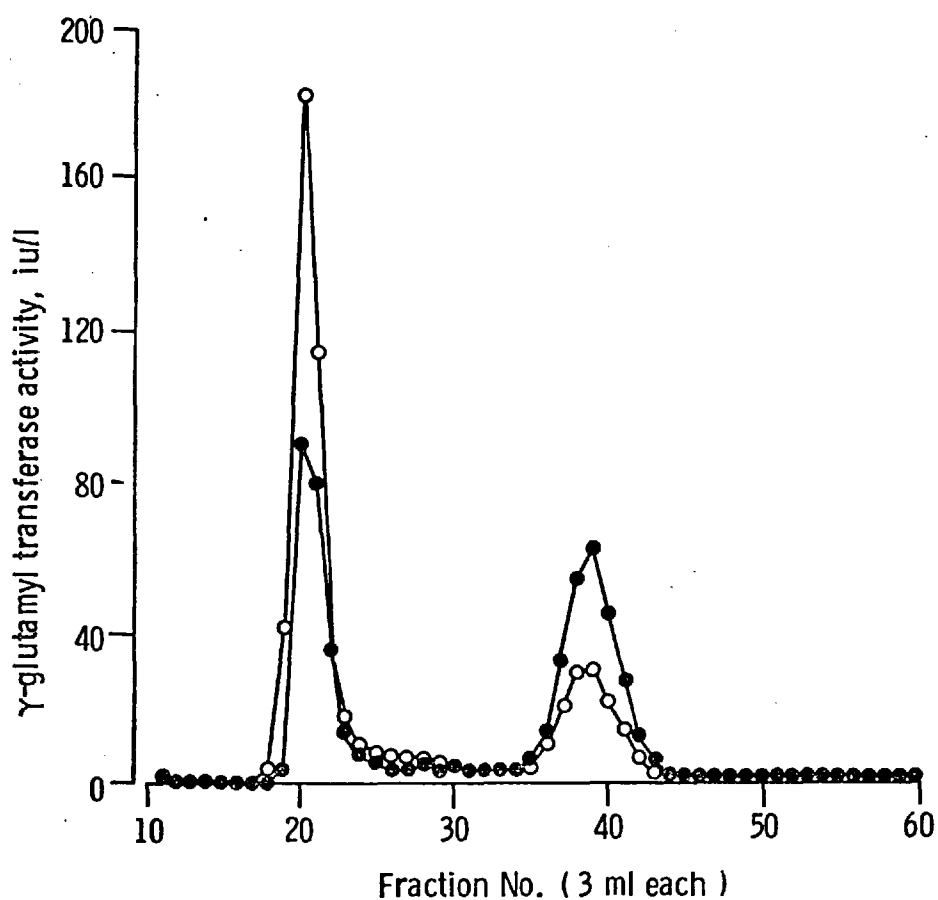


Fig. 5.3 Sepharose 6B gel-filtration of B_3 -fraction of γ -glutamyl transferase activity: treated with 1 mg/5 mg protein trypsin (●—●) and corresponding control sample (○—○); 1.85 ml samples with activity of 960 I.U./l and 1,118 I.U./l, respectively, were applied to the column.

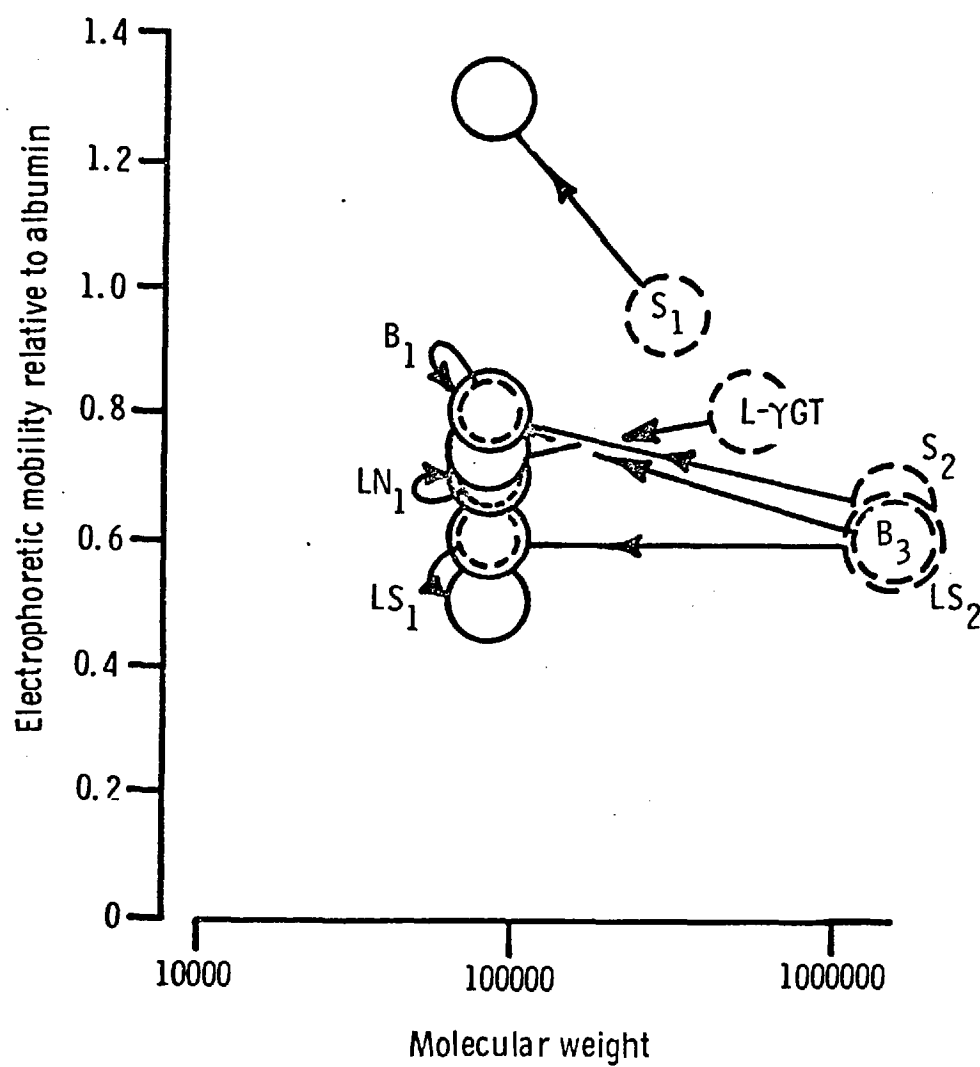


Fig. 5.4 Effect of trypsin (1 mg/5 mg protein) on the multiple forms of γ -glutamyl transferase: dotted outlines represent the position of the fractions before treatment and full lines represent their position after trypsinization.

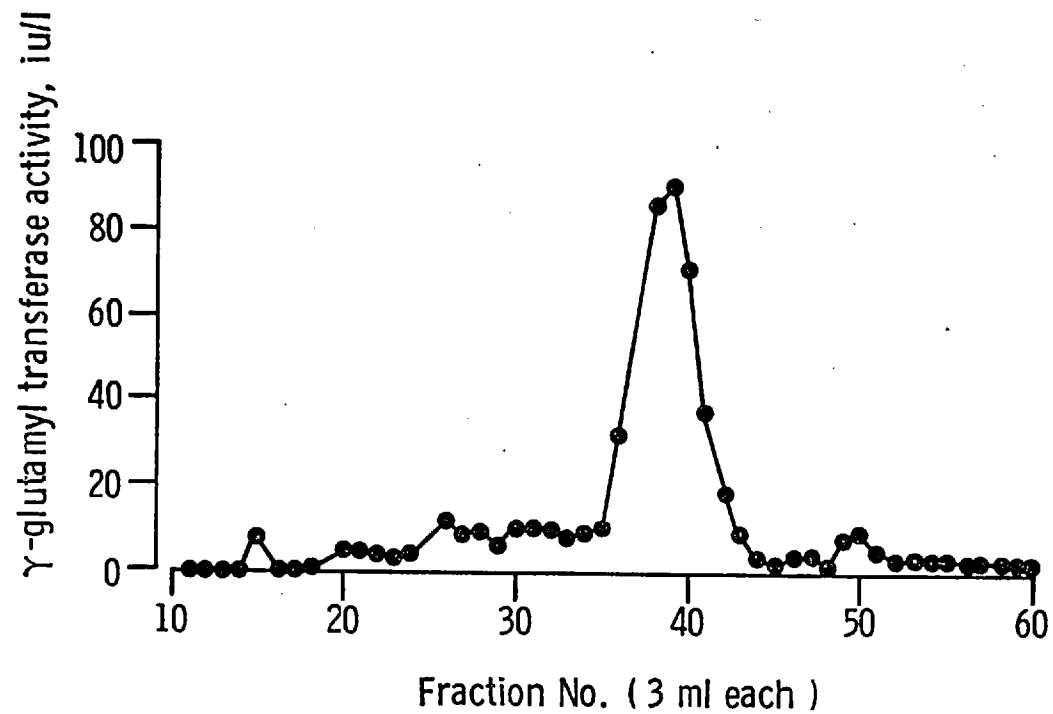


Fig. 5.5 Sepharose 6B gel-filtration of B₁-pool treated with 0.5% Na-deoxycholate: 3.9 ml sample with total γ-glutamyl transferase activity of 297 I.U./l was applied to the column, 0.05% w/v Na-deoxycholate was present in the eluent buffer.

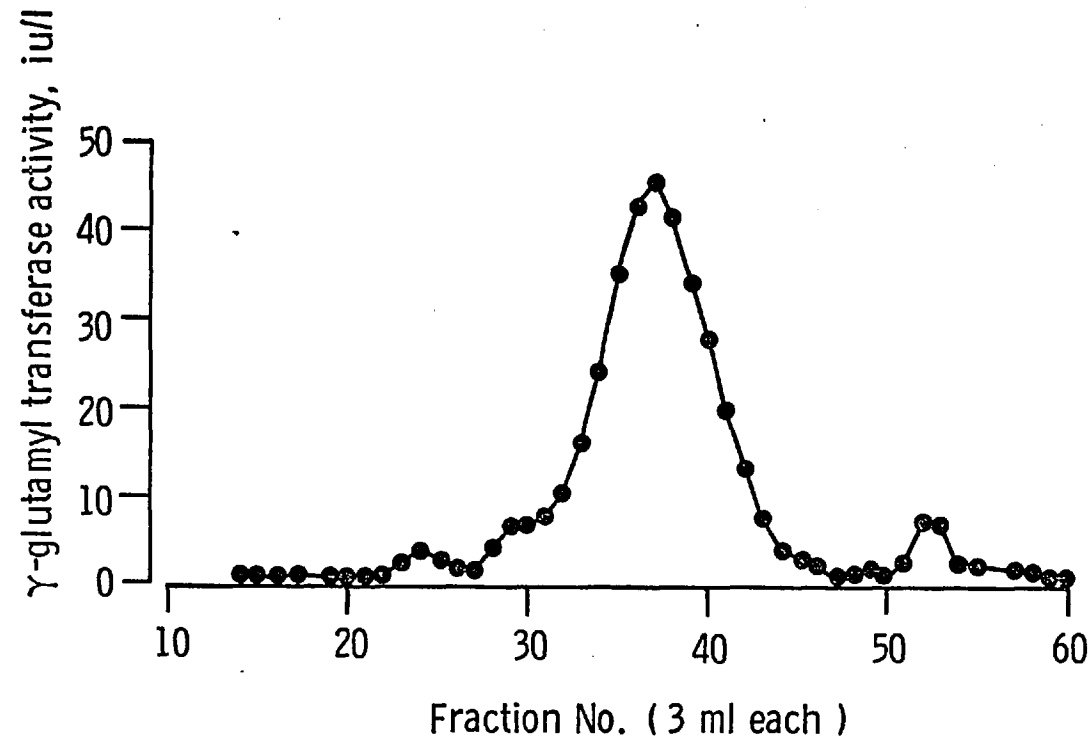


Fig. 5.6

Sepharose 6B gel-filtration of S₁-pool treated with 0.5% Na-deoxycholate: 3 ml sample with total γ -glutamyl transferase activity of 267 I.U./l was applied to the column, 0.05% Na-deoxycholate was present in the eluent buffer.

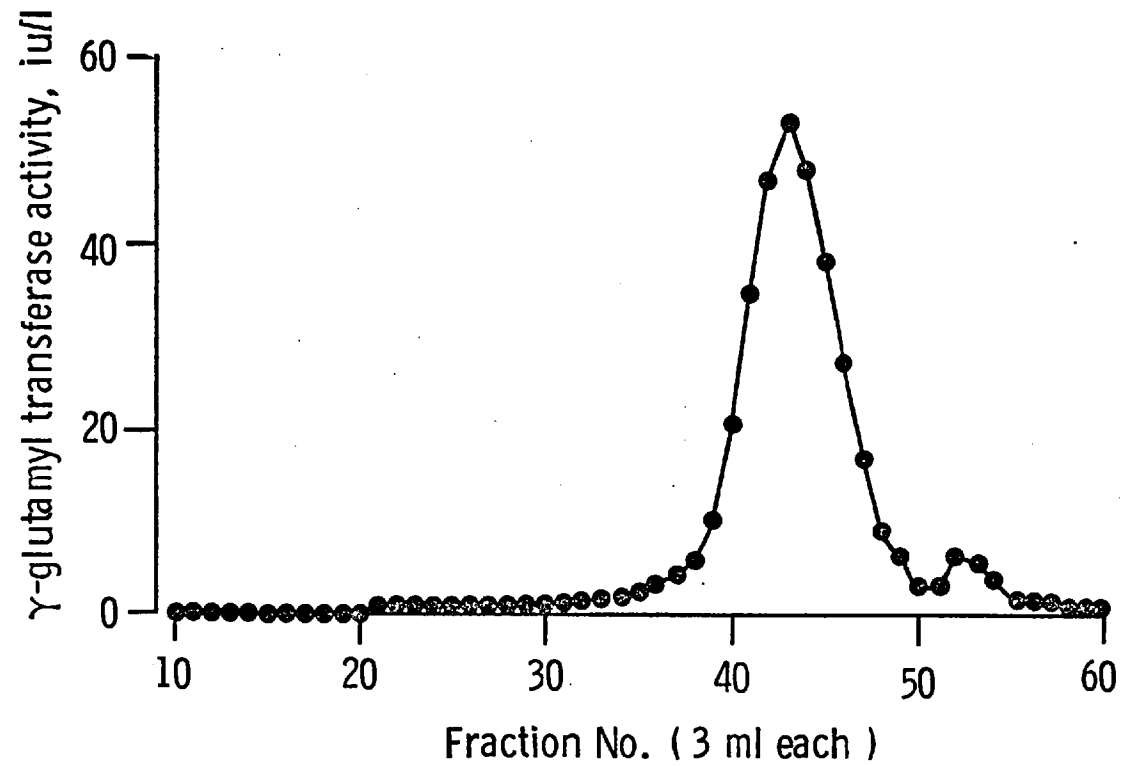


Fig. 5.7

Sepharose 6B gel-filtration of LN₁-pool treated with 0.5% Na-deoxycholate: 5.9 ml sample with total γ -glutamyl transferase activity of 136 I.U./l was applied to the column, 0.05% Na-deoxycholate was present in the eluent buffer.

Table 5.2 - Extraction of γ -glutamyl transferase fractions with sodium deoxycholate (0.5%, w/v)

No.	Enzyme preparation	Before treatment	After the extraction (% of initial activity)	Distribution of recovered activity between zones after gel-filtration; % of total eluted activity in brackets			Electrophoretic mobility		
				Peak I M.W.	Peak II M.W.	Peak III M.W.	Peak I	Peak II	Peak III
1	L- γ GT	1,424	400 (28.1)	10,000 (12.1)	450,000 (87.9)	---- (0)	0	0.7	----
2	B ₁	887	257 (33.5)	14,500 (5.7)	88,000 (72.5)	450,000 to >1,000,000 (21.8)	0	0.8	0; 0.8
3	B ₂	460	215 (46.7)	14,500 (8.3)	88,000 (60.1)	450,000 to >1,000,000 (31.6)	0	0.8	0
4	B ₃	1,056	791 (74.9)	14,500 (2.6)	88,000 (38.7)	88,000 to 1,000,000 (45.0) >1,000,000 (13.7)	0	0.8	0
5	S ₁	418	406 (97.1)	12,000 (6.45)	160,000 (89.55)	>1,000,000 (4.0)	0	0.95	---
6	S ₂	194	152 (78.4)	11,000 (28.0)	300,000 (53.3)	>1,000,000 (18.7)	0	0; 0.7	0
7	LS ₁	105	76 (72.4)	11,000 (10.7)	45,000 (89.3)	---- (0)	0	0.5	---
8	LS ₂	218	130 (59.6)	11,000 (16.0)	130,000 (65.7)	>1,000,000 (18.3)	0	0; 0.7 1.0	0
9	LN ₁	422	136 (32.2)	12,000 (8.1)	45,000 (91.9)	--- (0)	0	0.7	---

As can be seen from this table, treatment with 0.5% Na-DOC changed the molecular weights of all γ -glutamyl transferase preparations. However, two effects can be distinguished. First, the molecular weights of all the treated fractions have been generally reduced. Second, a small molecular weight fraction of enzymic activity (M.W. 10,000 - 14,500) was produced in all cases, which on average represented about 11% of the total activity. A fragment of this size was produced by all the preparations irrespective of their source and initial molecular weight. The molecular weights of intermediate peaks of the enzyme, between the lightest molecular weight form and the exclusion-limit form, varied from one enzymic preparation to another. Their electrophoretic mobilities were also somewhat different.

To confirm that the activity measured in the small molecular weight peak (average apparent molecular weight approximately 12,800) was true γ -glutamyl transferase activity and not just a result of hydrolysis of the substrate (γ -glutamyl-p-nitroanilide) by a hydrolase, the rate of the transpeptidation reaction of the γ -glutamyl moiety to the acceptor (glycylglycine) was determined at several different concentrations of glycylglycine. As the concentration of the acceptor increased, the rate of the reaction increased as well, and the Michaelis constant for glycylglycine was found to be 17.0 ± 1.0 mmol/l. This K_m value agrees quite well with the K_m values for glycylglycine of various γ -glutamyl transferase preparations from human liver, bile and serum (Table 3.9). Thus this experiment provided some evidence that the small molecular weight peak was indeed a form of γ -glutamyl transferase.

It is possible that the action of sodium-deoxycholate causes the two subunits of which the enzyme molecule is believed to consist to

dissociate, and that the activity in the small molecular weight peak may represent the light subunit of the enzyme.

Changes in molecular weight and electrophoretic mobility produced by treatment with Na-deoxycholate are summarized diagrammatically in Figs. 5.8 and 5.9.

c) Butanol/di-iso-propyl ether extraction

A mixture of n-butanol and di-iso-propyl ether (DIPE) in the proportion of butanol:DIPE = 40:60 was used in this type of experiment. n-Butanol/DIPE mixture in this ratio is thought to remove triglyceride, cholesterol and phospholipids from their complexes with proteins (lipo-proteins) (Cham and Knowles, 1976).

To extract lipids, 4 ml of each γ -glutamyl transferase preparation was shaken with 8 ml of butanol/DIPE mixture for 15 mins, then centrifuged for 10 mins at 1,700 RPM. The lower (aqueous) layer containing γ -glutamyl transferase activity was dialyzed against several changes of 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl in order to get rid of any traces of the organic solvent. Enzyme activity was measured before and after the extraction. Each extracted sample was subjected to gel-filtration on the Sepharose 6B column. Active fractions were concentrated and their electrophoretic mobilities estimated as in previous experiments.

An example of the Sepharose 6B gel-filtration profile of one of the extracted by n-butanol/DIPE preparations (S_1) is shown in Fig. 5.10.

A summary of the results obtained in this experiment is presented in Table 5.3 and, diagrammatically, in Fig. 5.11.

As can be seen from Table 5.3, extraction with n-butanol/DIPE

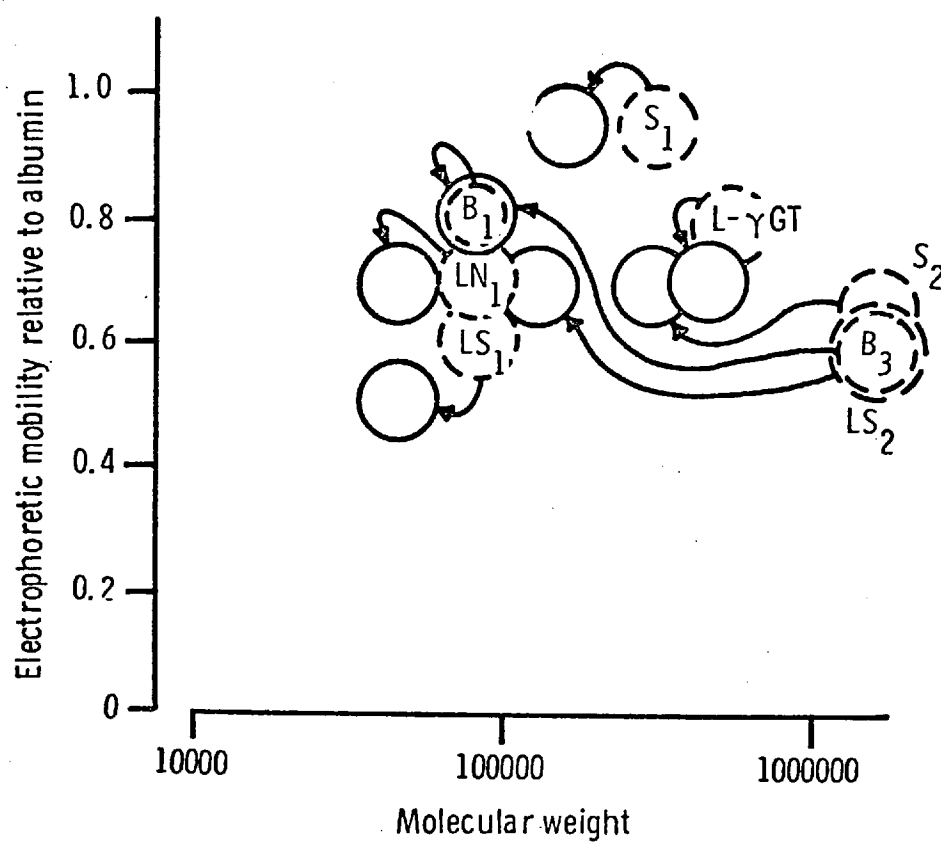


Fig. 5.8

Effect of Na-deoxycholate on the multiple forms of γ -glutamyl transferase: dotted outlines represent the position of the fractions before treatment and full lines represent the position of the fractions after the treatment.

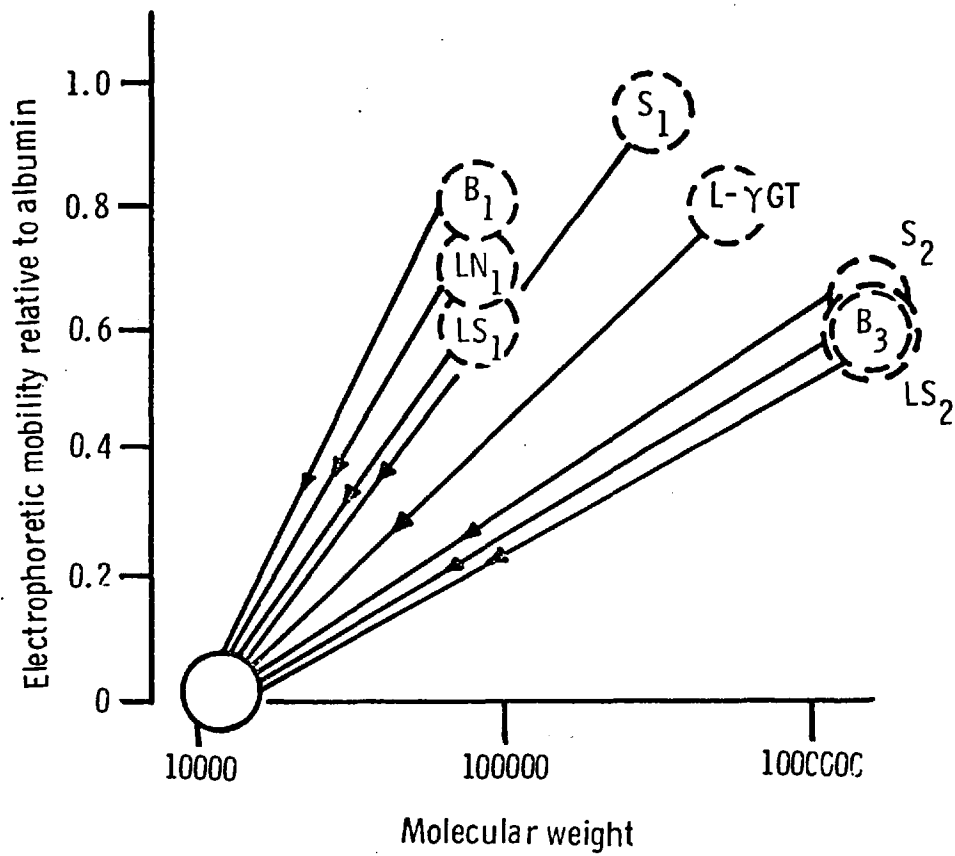


Fig. 5.9 Effect of Na-deoxycholate on the multiple forms of γ -glutamyl transferase: production of the smallest molecular weight form of the enzyme.

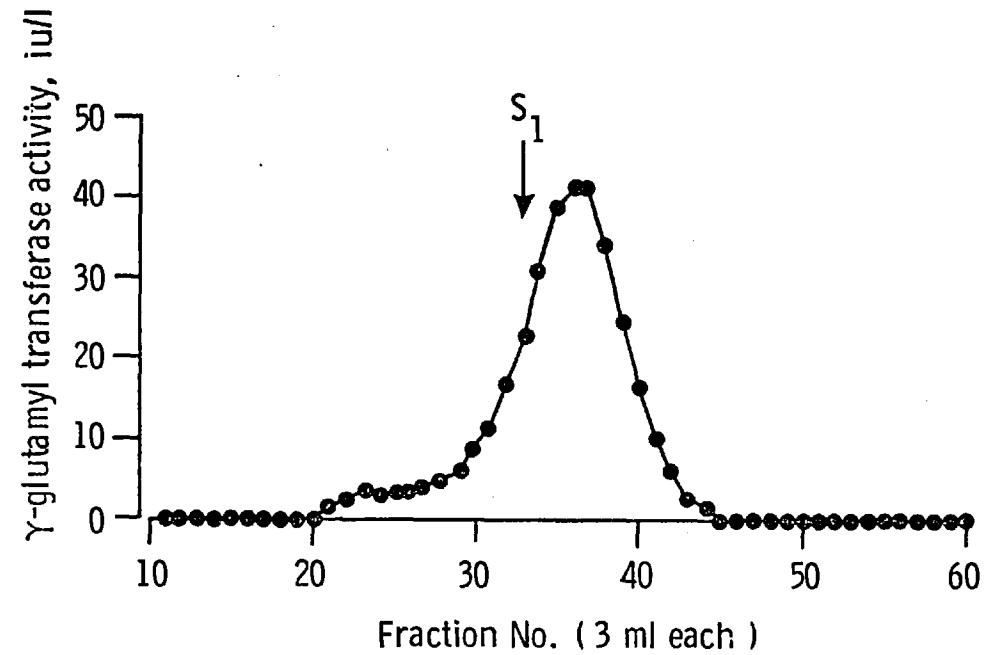


Fig. 5.10. Sepharose 6B gel-filtration of the S₁-fraction extracted with n-butanol/DIPE (v:v = 40:60): 3.6 ml sample with total γ-glutamyl transferase activity of 309 I.U./l was applied; the position of the S₁-peak before treatment is shown by the arrow.

Table 5.3 - Extraction of γ -glutamyl transferase fractions with butanol/di-iso-propyl ether.

No.	Enzyme preparation	γ -Glutamyl transferase activity, I.U./l		Distribution of recovered activity between zones after gel-filtration; % of total eluted activity in brackets		Electrophoretic mobility	
		Before treatment	After the extraction (% of initial activity)	Peak I M.W.	Peak II M.W.	Peak I	Peak II
1	L- γ GT	455	388 (85.3)	62,000 (16.1)	~550,000 (83.9)	0.7	0.7
2	B ₁	622	509 (85.0)	82,000 (96.1)	>1,000,000 (3.9)	0.8	0; 0.8
3	B ₂	226	192 (76.5)	82,000 (68.6)	>1,000,000 (31.4)	0.8	0
4	B ₃	497	257 (51.7)	82,000 (27.1)	>1,000,000 (72.9)	0.8	0
5	S ₁	394	309 (78.4)	185,000 (94.8)	>1,000,000 (5.2)	0.9	0.6
6	S ₂	173	53 (30.6)	175,000 (76.1)	>1,000,000 (23.9)	0.9	-
7	LS ₁	147	140 (95.2)	57,000 (95.6)	>1,000,000 (4.4)	0.5	0.6
8	LS ₂	333	90 (27.0)	240,000 (80.4)	>1,000,000 (19.6)	0.66	0.5
9	LN ₁	691	606 (87.7)	72,000 (100)	----- (0)	0.8	-

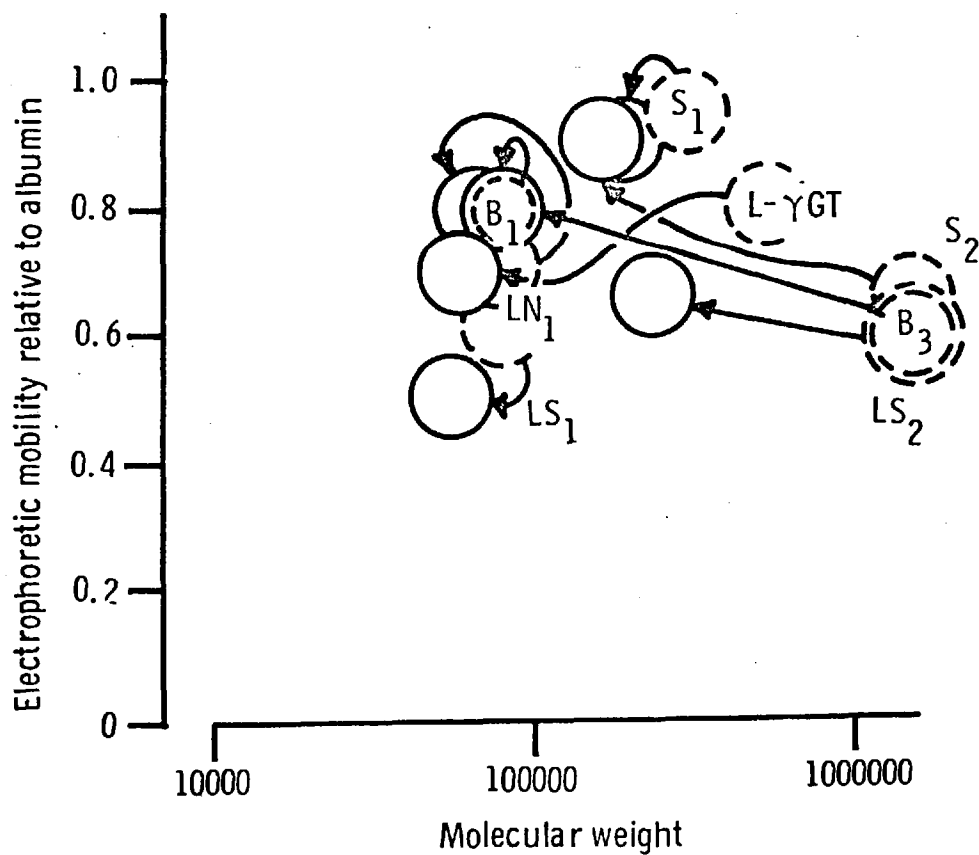


Fig. 5.11 Effect of extraction with n-butanol/di-iso-propyl ether mixture (v:v = 40:60) on multiple forms of γ -glutamyl transferase: dotted outlines show the position of fractions before treatment and solid outlines show the position of fractions after the extraction.

reduced the molecular weights of nearly all γ -glutamyl transferase fractions. In the case of very high molecular weight preparations (S_2 , LS_2 and B_3) the size of quite a large proportion of the activity (between about 20 and 73%) was unaltered by the treatment. Butanol/DIPE extraction of the two serum fractions of the enzyme gave rise to a fraction of activity with an average molecular weight of about 180,000 and electrophoretic mobility of 0.9 that of albumin. Most of the LS_2 -type of activity (80%) was converted into a fraction with a molecular weight of approximately 240,000.

d) Freezing and thawing with 1 mol/l NaCl

Freezing and thawing of the enzyme preparations was done by adding solid sodium chloride with mixing to the samples of the enzyme to make a final concentration of 1 mol/l. The samples were then placed in the deep-freezer at -20°C . After they were completely frozen, they were left to thaw at room temperature. This freezing and thawing procedure was repeated 4 - 6 times for each γ -glutamyl transferase preparation.

The enzyme activity before and after the freezing-thawing experiment was measured and samples were subjected to gel-filtration on the Sepharose 6B column. Gel-filtration patterns after freezing and thawing of two of the enzyme fractions (L - γ GT and LN_1) are shown in Fig. 5.12 and the results obtained for all the preparations are summarized in Table 5.4.

Freezing and thawing in the presence of 1 mol/l NaCl did not change the molecular size of L - γ GT, B_1 , LS_2 and LN_1 fractions; with B_2 , B_3 , S_1 and LS_1 fractions, smaller size fragments of γ -glutamyl transferase were produced. The decrease in molecular weights of some fractions may be the

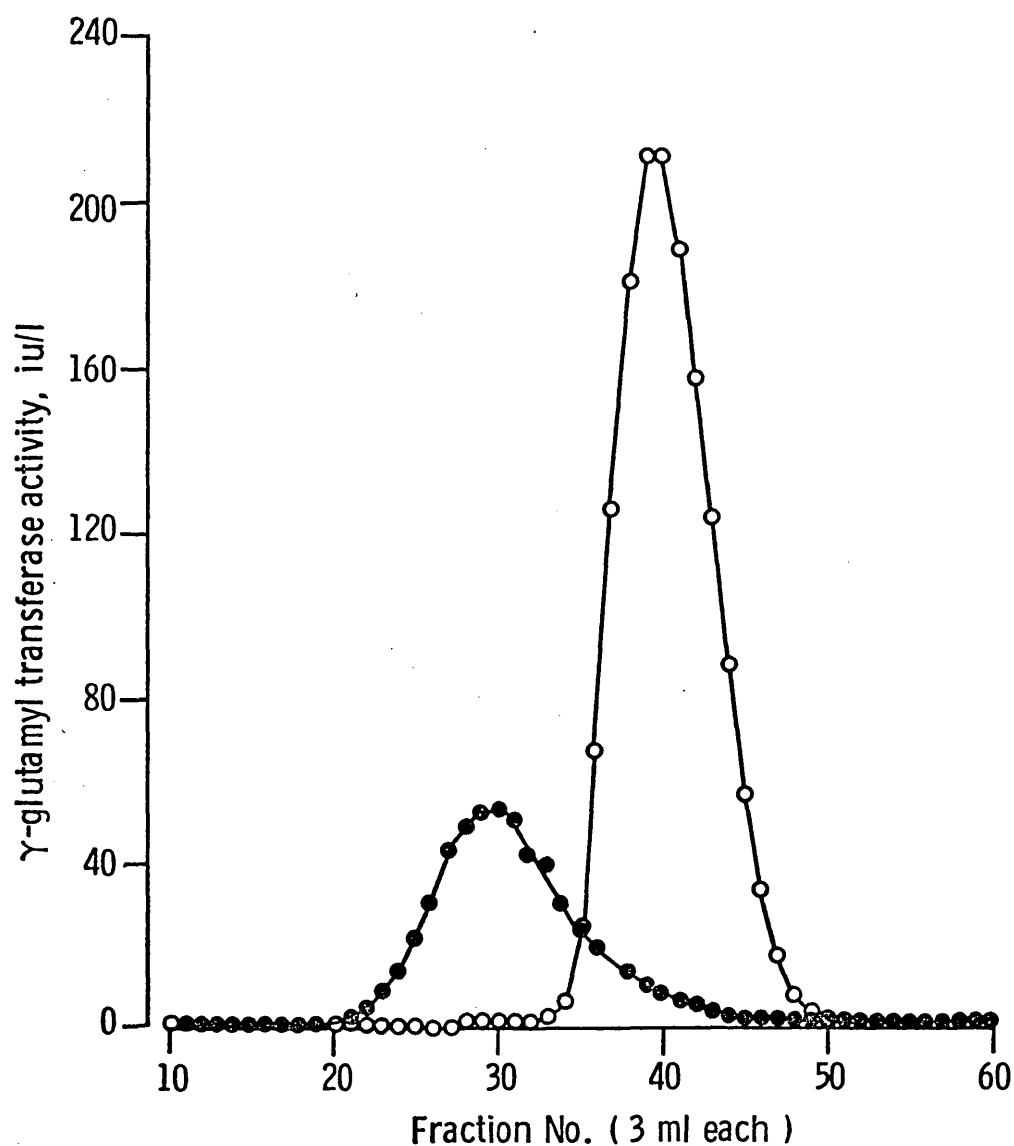


Fig. 5.12 Sepharose 6B gel-filtration of L- γ GT (●—●) and LN1 (○—○) fractions of γ -glutamyl transferase frozen and thawed in the presence of 1 mol/l NaCl.

Table 5.4 - Freezing and thawing of γ -glutamyl transferase preparations in the presence of 1 mol/l NaCl.

No.	Enzyme preparation	γ -Glutamyl transferase activity, I.U./l		Distribution of recovered activity between zones after gel-filtration; % of total eluted activity in brackets		Electrophoretic mobility	
		Before treatment	After freezing-thawing with 1 mol/l NaCl (% of initial activity)	Peak I M.W.	Peak II M.W.	Peak I	Peak II
1	L- γ GT	380	395 (103.9)	-- (0)	~500,000 (100)	---	0.8
2	B ₁	593	628 (106.0)	~95,000 (95.2)	>1,000,000 (4.8)	0.8	0; 0.8
3	B ₂	218	204 (93.6)	~95,000 (76.6)	>1,000,000 (23.4)	0.8	0; 0.8
4	B ₃	537	579 (108.0)	~95,000 (18.2)	>1,000,000 (81.8)	0.8	0; 0.8
5	S ₁	465	461 (99.1)	~270,000 (97.0)	>1,000,000 (3.0)	0.6; 0.96	---
6	S ₂	187	222 (118.7)	--- (0)	>1,000,000 (100)	---	0.6
7	LS ₁	140	184 (131.4)	36,000 (100)	--- (0)	0.5	---
8	LS ₂	303	344 (113.5)	--- (0)	>1,000,000 (100)	---	0.6
9	LN ₁	625	658 (105.3)	82,000 (100)	--- (0)	0.75	---

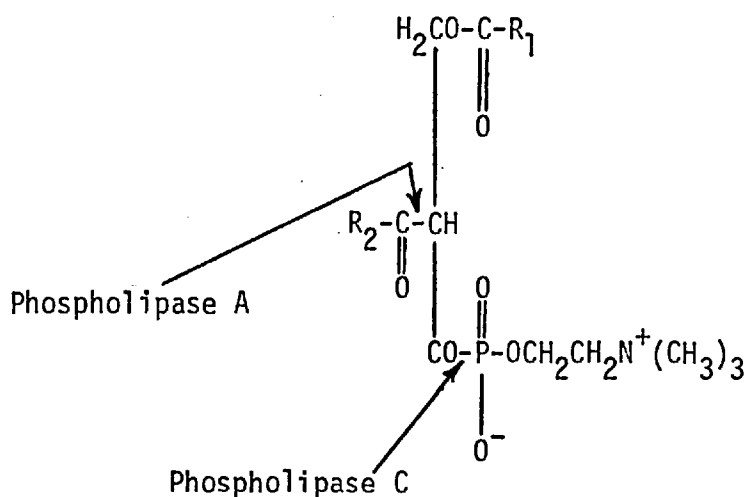
result of the denaturing effect of the treatment. At the same time, γ -glutamyl transferase in nearly all the fractions was activated by sodium chloride, the average activation for the nine preparations of the enzyme being 8.8%.

e) Effect of phospholipases C and A

The large molecular weight (M.W. >1,000,000) preparations of γ -glutamyl transferase B_3 , S_2 and LS_2 , may represent a membrane-associated form of the enzyme. They therefore probably contain phosphoglycerides, which are known to be structural components of cellular membranes. The enzyme purified from human liver (L- γ GT) and the smaller molecular weight fraction of the enzyme in serum could also be associated with lipids or lipoproteins. An attempt was therefore made to modify the molecular organization of the membrane-associated forms of the enzyme by removing the lipid component from them by the action of phospholipases, and to see whether such treatment would affect the size and electrical charge of these and also of the S_1 and L- γ GT preparations.

Phospholipases are enzymes catalyzing hydrolysis of phosphoglycerides. The most abundant phosphoglycerides in higher plants and animals are: phosphatidyl ethanolamine (cephalin) and phosphatidyl choline (lecithin). Phospholipase C (lecithinase C, EC 3.1.4.3) hydrolyzes the bond between phosphoric acid and glycerol, while phospholipases of the A class remove the fatty acid from the second position in the molecule of a phosphoglyceride. Their action can be illustrated as follows:

Phosphatidyl choline (lecithin)



(R_1 and R_2 are fatty acid residues)

Phospholipase C, Type I, from *Cl. Welchii* was used in this experiment. γ -Glutamyl transferase preparations (L - γ GT, B_3 , S_1 , S_2 and LS_2) were incubated with 5 mU/ml phospholipase C for 24 h at 37°C . Corresponding controls were incubated in the same way, but without any phospholipase added to them. The enzyme activities in all samples were measured before and after incubation at 37°C . All incubated samples were subjected to gel-filtration on a Sepharose 6B column (1.5 x 95 cm) to determine their molecular weights, and electrophoretic mobilities of active fractions were determined following agarose-gel electrophoresis (Table 5.5).

Treatment with phospholipase C (5 mU/ml) did not affect the enzyme activity significantly (as compared with the incubated controls). For all the treated fractions, molecular weights remained the same as in

Table 5.5 - Effect of Phospholipase C (5 mU/ml) on some γ -glutamyl transferase preparations

No.	Enzyme preparation	γ -Glutamyl transferase activity, I.U./l			Molecular weight of phospholipase C-treated samples after gel-filtration on Sepharose 6B		Electrophoretic mobility	
		Before incubation	After 24 h at 37°C in controls in treated samples		Peak I (controls)	Peak II (controls)	Peak I (controls)	Peak II (controls)
1	L- γ GT	628	628 (100)	628 (100)	---	$\sim$ 550,000 (550,000)	0.5 (0.64)	0.6 (0.74)
2	B ₃	398	398 (100)	395 (99.2)	---	>1,000,000	---	0; 0.7 (0; 0.7)
3	S ₁	384	384 (100)	356 (92.7)	$\sim$ 300,000 (300,000)	>1,000,000 (>1,000,000)	0; 0.8 (0.7; 1.3)	0 (0)
4	S ₂	535	531 (99.3)	497 (92.9)	---	>1,000,000 (>1,000,000)	---	0; 0.7 (0; 0.7)
5	LS ₂	198	187 (94.4)	195 (98.5)	---	>1,000,000 (>1,000,000)	---	0; 0.7 (0; 0.6)

the corresponding controls. Electrophoretic mobilities of the fractions with molecular weights over 1×10^6 remained more or less the same as in the incubated controls. However, the mobilities of the purified liver γ -glutamyl transferase treated with phospholipase C and the S_1 -fraction were lower than in the corresponding controls.

Elution patterns of one of the enzyme preparations from the Sepharose 6B column in this experiment are shown in Fig. 5.13.

A higher concentration of phospholipase C (45 mU/ml) was tried on one of the high-molecular weight fractions of γ -glutamyl transferase, B_3 -pool, but no change in its molecular weight could be detected and its mobility after electrophoresis on agarose gel was only slightly different from that of the control sample (0 and 0.6 for the phospholipase C-treated sample and 0 and 0.7 for the control).

Phospholipase A_2 (lecithinase A_2 , phosphatide 2-acyl-hydrolase, EC 3.1.1.4, from bovine pancreas, suspension in 3.2 mol/l $(\text{NH}_4)_2\text{SO}_4$ solution, pH approximately 5.5), was added to S_2 -pool to give a final concentration of 218 mU/ml. The mixture was incubated for 24 h at 37°C ; γ -glutamyl transferase activity at the beginning of incubation was 404 I.U./l, after the incubation with phospholipase A_2 it decreased to 380 I.U./l (94% of the initial activity). As with phospholipase C-treated S_2 preparation, the molecular weight of the fraction determined by gel-filtration on Sepharose 6B column remained unchanged; the electrophoretic mobility was also the same, i.e., a small part of the fraction's activity stayed at the origin while most of the activity had a mobility of 0.74 that of albumin.

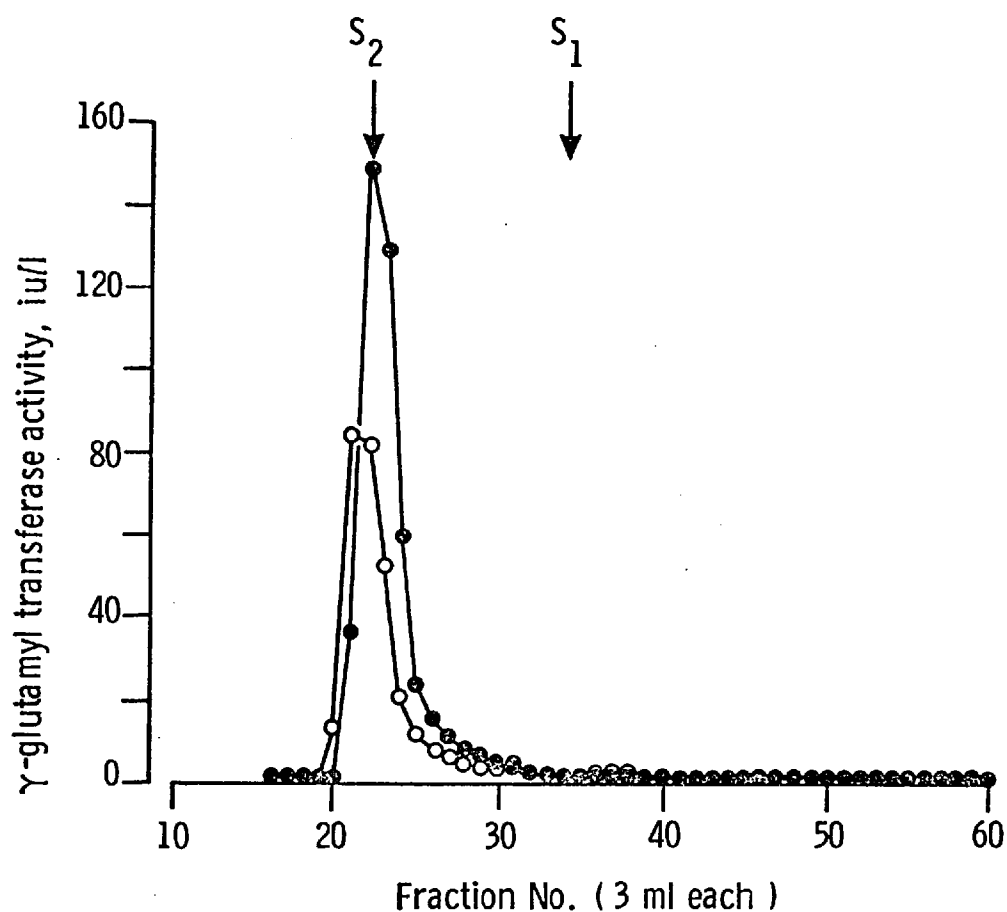


Fig. 5.13

Sephacrose 6B gel-filtration of S_2 -pool treated with 5mU/ml phospholipase C (●—●) and corresponding control (○—○): 2.85 ml sample with γ -glutamyl transferase activity of 497 I.U./l and 1.85 ml sample with activity of 531 I.U./l, respectively, were applied to the column.

These experiments with phospholipases C and A₂ show that even if the high molecular weight fractions of γ -glutamyl transferase (M.W. $>1 \times 10^6$) are associated with membranes, the hydrolysis of phospholipids does not lead to a detectable dissociation of our enzyme from phospholipase C and A₂-modified membranes. However, treatment of the enzyme purified from human liver and the S₁-preparation with phospholipase C does not change their molecular weights noticeably, but alters their charge on electrophoresis.

5.3 Summary

Experiments on the interconversion of the naturally-occurring and experimentally-produced fractions of γ -glutamyl transferase included proteolytic treatment with trypsin, extraction with sodium deoxycholate and with butanol/di-iso-propyl ether mixture, freezing and thawing in the presence of 1 mol/l NaCl and treatment with phospholipases C and A.

Trypsinization of the larger molecular weight forms gave rise to a smaller size fragment of activity with an apparent molecular weight of 82,000. Proteolytic treatment of the small molecular weight forms (B₁, LS₁ and LN₁) did not change their size. This fragment of molecular weight of approximately 82,000 therefore is common to all the preparations of γ -glutamyl transferase from human liver, bile and serum. The proteolyzed forms of the enzyme in various fractions, however, had different mobilities on electrophoresis. Such differences in charge may be caused by the association of these proteolyzed forms with some small molecular weight compounds, which alter the behaviour of the enzyme on electrophoresis.

Extraction with Na-deoxycholate and butanol/di-iso-propyl ether

changed the molecular weights of nearly all the preparations of γ -glutamyl transferase. The effect of both treatments was rather similar, except that extraction with detergent of all enzyme fractions produced a small molecular weight form of the enzyme (average M.W. approximately 13,000), which may represent the light subunit of γ -glutamyl transferase.

Freezing and thawing with 1 mol/l NaCl and treatment with phospholipases C and A did not seem to produce any marked changes in the size and charge of the enzyme fractions.

CHAPTER 6

GEL-FILTRATION ANALYSIS OF SAMPLES OF HUMAN SERUM AND BILE

CHAPTER 6

GEL-FILTRATION ANALYSIS OF SAMPLES OF HUMAN SERUM AND BILE

6.1 Introduction

Qualitative and quantitative analysis of multiple forms of lactate dehydrogenase, creatine kinase and alkaline phosphatase in serum have greatly added to the scope and value of diagnostic enzymology. It is therefore not surprising that much effort has been directed towards extending the clinical usefulness of γ -glutamyl transferase by a similar approach.

Zone electrophoresis has proved to be in general the most useful single technique in isoenzyme analysis, and many studies on the electrophoretic heterogeneity of γ -glutamyl transferase in serum have been reported, with paper, cellulose acetate, or agar (or agarose), starch or polyacrylamide gels as the supporting media (Kokot and Kuska, 1965; Kokot et al., 1965; Szewczuk, 1966; Orłowski and Szczeklik, 1967; Rutenburg, Smith and Fischbein, 1967; Jacyszyn and Laursen, 1968; Kokot and Kuska, 1968; Lehman, Prentice and Rosalki, 1970; Rosalki et al., 1970; Miyazaki and Okumura, 1972; Azzopardi and Jayle, 1973; Igartua, Domecq and Findor, 1973; Hetland, Andersson and Gerner, 1975; Burlina, 1978; Kok et al., 1978; Wenham, Price and Sammons, 1979). All these studies have demonstrated the considerable heterogeneity of the enzyme in sera, with up to seven zones of activity being reported. However, no clear correlations between electrophoretic patterns and specific diseases appear to have emerged from these studies, and a

similar absence of such correlations was found in the present work with regard to the electrophoretic patterns of γ -glutamyl transferase in 89 serum specimens reported in Chapter 3.

γ -Glutamyl transferase activity in serum has also been shown to be heterogeneous with respect to apparent molecular size. Orłowski and Szczeklik (1967) and Jacyszyn and Laursen (1968) found three peaks of enzyme activity following gel-filtration on Sephadex G-200. Each peak was eluted approximately with one of the protein peaks, and the highest activity was found in the smallest molecular weight peak of the enzyme. Kokot and Kuska (1968) also observed three peaks of γ -glutamyl transferase in serum of a healthy person, but the largest percentage of activity both in normal serum and in serum of patients with liver diseases was found in the protein peak which eluted first. At least three peaks of activity were observed in another study (Orłowski, Szczeklik and Kołaczowska, 1965) and the distribution of activity was found to be somewhat different in different diseases. However, Ideo and Ronchi (1972) demonstrated only one peak of γ -glutamyl transferase activity in serum of 21 patients with various liver diseases, this peak being eluted with the first protein fraction. They therefore suggested that γ -glutamyl transferase is a rather homogeneous enzyme with molecular weight of about 200,000, explaining the different results obtained by other workers by the formation of multiple molecular forms due to aggregation phenomena and/or the association of enzyme molecules with different serum protein fractions. Gel-filtration performed on a column of Ultrogel Aca 34 caused γ -glutamyl transferase activity in normal human serum pool to separate into three fractions, the first one being eluted in the void

volume and the last one a little before serum albumin (Huseby, 1978).

Although these various studies, like separations dependent on differences in net charge, failed to reveal any obvious clinically-useful interpretations, it was felt that analysis on the basis of size differences might offer some possibility of testing certain theories of the origin of γ -glutamyl transferase fractions in serum, such as the hypothesis that the enzyme may be released in the form of membrane fragments under certain circumstances (De Broe, Borgers and Wieme, 1975).

In earlier experiments (Chapter 3) the activity in "high γ -glutamyl transferase serum pool" was separated into two peaks (S_1 and S_2) by gel-filtration on Sepharose 6B. Molecular weights of these fractions were estimated to be approximately 300,000 for S_1 -peak and over 1,000,000 for S_2 -peak. In the work described in the present chapter, therefore, an attempt was made to study the distribution of the enzyme activity between these two forms of the enzyme in sera of patients with raised total γ -glutamyl transferase activity in order to establish whether any correlation exists between this distribution and the nature of the underlying disease.

The heterogeneity with respect to size of γ -glutamyl transferase in human bile has also been noted (Wenham, Price and Sammons, 1978; and Chapter 3). The tendency for the distribution of activity between the different-sized fractions to vary from one bile sample to another was also briefly reported in Chapter 3. This variation was further examined in bile samples obtained from several patients at intervals during percutaneous intrahepatic decompression, and the results are given in this chapter.

6.2 Gel-filtration analysis of serum samples

Materials and Methods

Serum samples with raised γ -glutamyl transferase activities were selected from those submitted to the laboratory for routine estimation of enzymes.

Gel-filtration was performed on a small Sepharose 6B column (0.5 x 43 cm). Sepharose 6B was equilibrated and eluted with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl; 45 - 50 l ml fractions were collected using LKB 2112 Radirac Fraction Collector, at a flow rate of 6 ml/h. 0.4 to 3.0 ml samples of serum were applied onto the column; the higher the total γ -glutamyl transferase activity in the serum was, the smaller the volume of the sample was needed for accurate estimation of the enzyme activity in the eluted fractions.

Recovery of the serum enzyme activity after gel-filtration on the Sepharose 6B column ranged between 67.7% and 231.6%, the mean value for 83 sera being 142%. A similar procedure was used for gel-filtration analysis of bile samples. Recovery of the enzyme activity after gel-filtration of bile samples on the same column ranged between 67% and 115.5%, the mean value for 27 samples being 86.1%.

γ -Glutamyl transferase activity was measured by the Scandinavian method, as described in "Materials and Methods", Chapter 2.

Results

After gel-filtration on Sepharose 6B, the total activity of γ -glutamyl transferase in all serum samples usually separated into two activity peaks, the apparent molecular weights of which were the same as

for the S_1 and S_2 peaks obtained from "high γ -glutamyl transferase serum pool" (Chapter 3). However, the distribution of activity between these two main zones was different for various patient's sera (examples are shown in Figs. 6.1a, b and c). For gel-filtration of each serum specimen, total γ -glutamyl transferase activity applied to the column and total activity eluted from the column were calculated. The enzyme activities in S_1 (lower) and S_2 (higher) molecular weight peaks were estimated separately and the value of S_1/S_2 ratio was calculated for all serum samples. Altogether, about 90 gel-filtration experiments were performed with sera of patients with a variety of diseases.

The percentage of activity present in the form of the high molecular weight peak of the enzyme was independent of the total activity of the serum (Fig. 6.2). However, the S_1/S_2 ratio was somewhat different for different serum samples with similar total γ -glutamyl transferase activities (Fig. 6.3). Therefore an attempt was made to group the results obtained on the basis of the patients' diseases. The following groupings were adopted, although this can only be regarded as an arbitrary classification in view of the small numbers of patients and the somewhat limited and provisional clinical details available.

- | | | |
|-----------|---|---|
| Group I | - | patients with alcoholism and/or cirrhosis
(17 specimens) |
| Group II | - | patients with malignant diseases thought to
involve the liver (38 specimens) |
| Group III | - | patients with other diseases (21 specimens) |

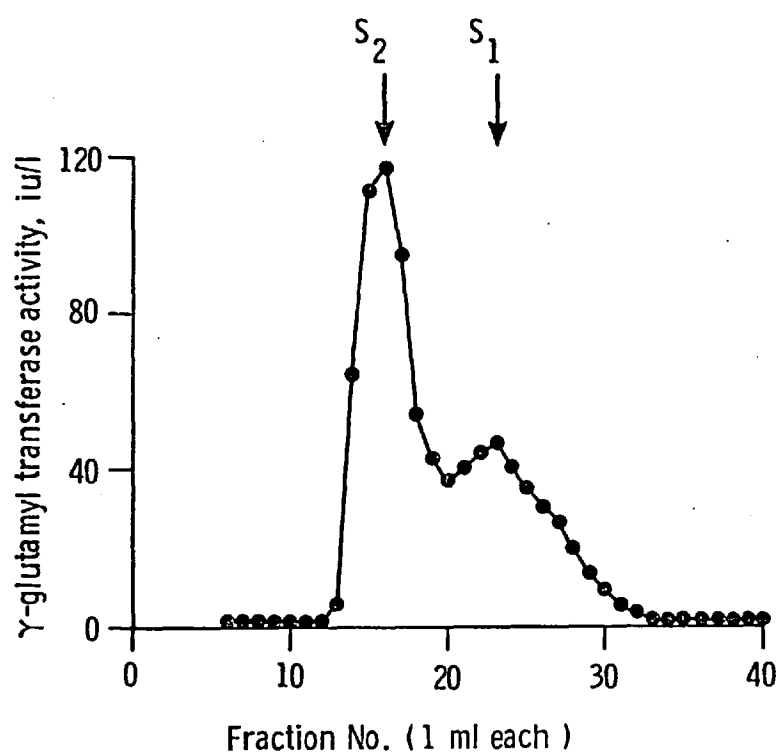


Fig. 6.1a.

Sepharose 6B gel-filtration of individual patients' serum samples: an example where most of the γ -glutamyl transferase activity eluted in the S_2 -peak (0.6 ml of serum with activity of 1,015 I.U./l was applied to the column).

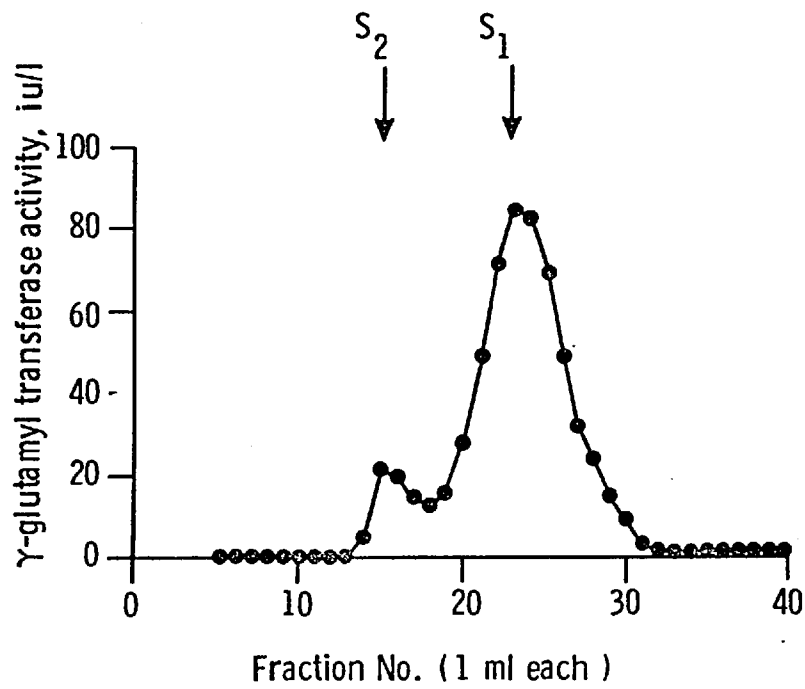


Fig. 6.1b.

Sepharose 6B gel-filtration of individual patients' serum samples: an example where most of the γ -glutamyl transferase activity eluted in the S_1 -peak (0.6 ml of serum with activity of 480 I.U./l was applied to the column).

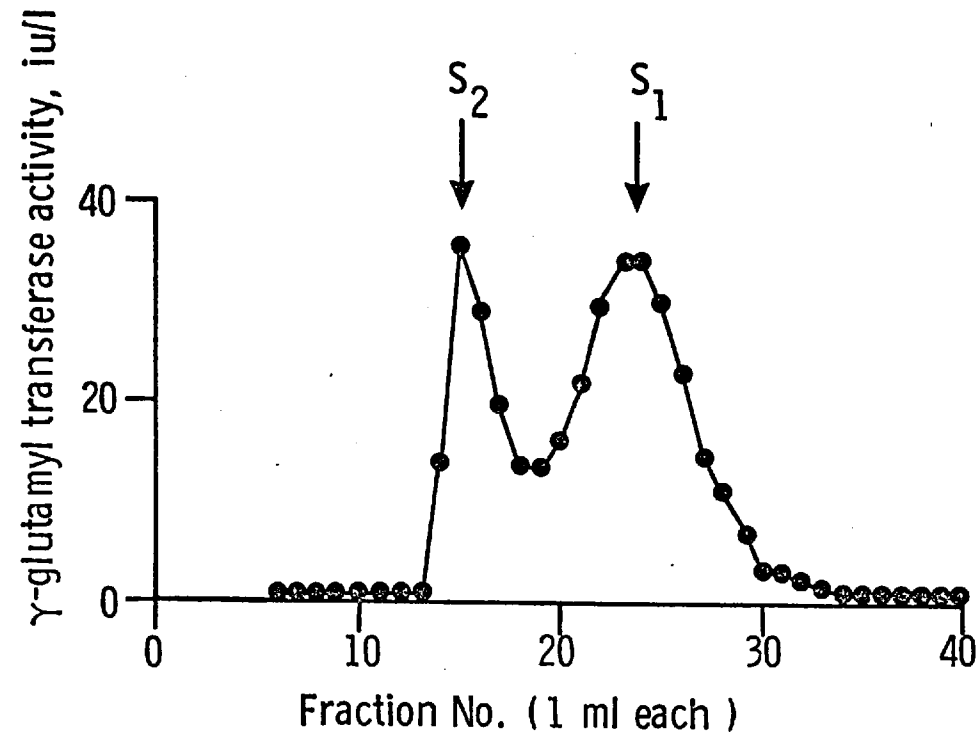


Fig. 6.1c.

Sephacrose 6B gel-filtration of individual patients' serum samples: an example where γ -glutamyl transferase activity is more or less equally distributed between the S_1 - and S_2 -peaks (0.45 ml of serum with activity of 380 I.U./l was applied to the column).

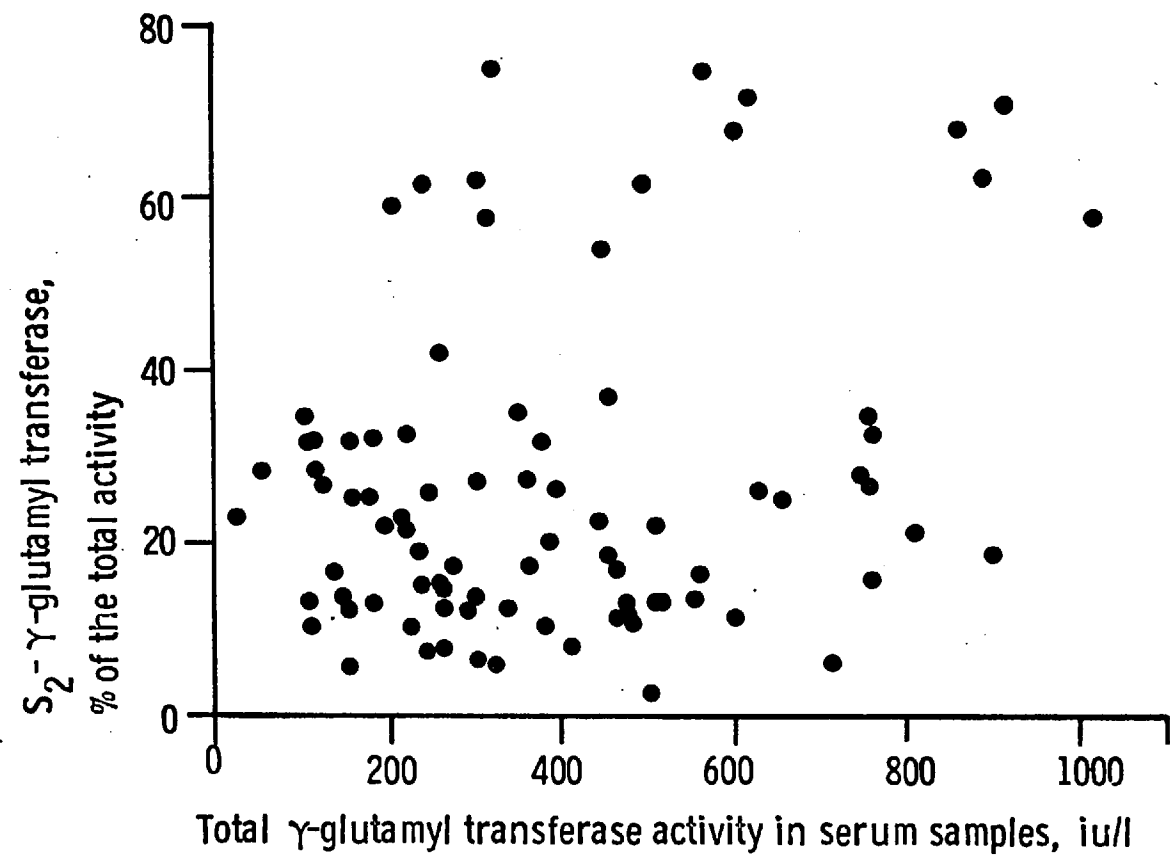


Fig. 6.2.

Sephacrose 6B gel-filtration of individual patients' serum samples: relationship between the proportion of the S₂- fraction and the total γ- glutamyl transferase activity in serum.

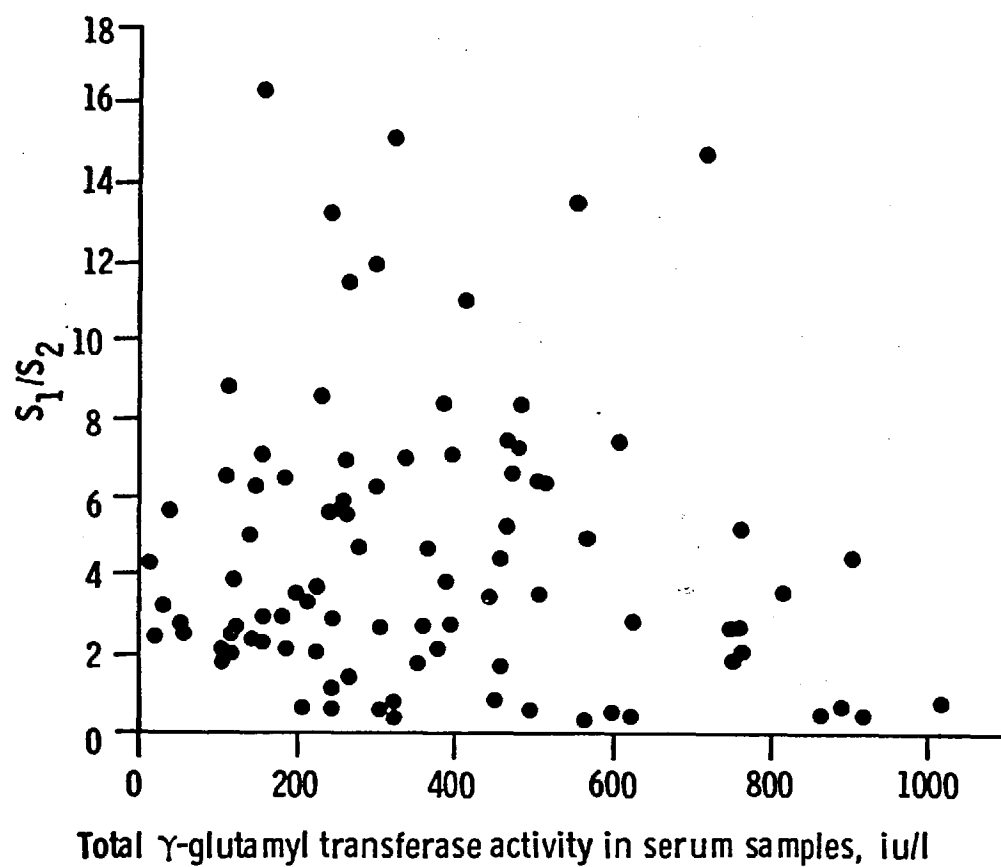


Fig. 6.3.

Sephacrose 6B gel- filtration of the individual patients' serum samples: relationship between the S_1/S_2 ratio and total γ -glutamyl transferase activity in serum.

Results of gel-filtration analysis of individual patients' serum samples classified into the three groups are presented in Tables 6.1, 6.2 and 6.3, respectively.

γ -Glutamyl transferase activity in the sera of the 17 patients in Group I was 5 - 17 times the upper limit of normal, with an average activity of 432 I.U./l. After gel-filtration on Sepharose 6B, most of the enzyme activity (on average about 89% of the applied activity) was eluted in the S_1 -peak, with S_2 -type of the enzyme representing about 11% of the applied activity. The values of S_1/S_2 ratios for the 17 serum samples in this group ranged from 5.19 minimum (patient No. 17) to 14.69 maximum (patient No. 16), with a mean value of 8.09.

γ -Glutamyl transferase activity in the sera of the 38 patients in Group II varied between 111 I.U./l and 1,015 I.U./l, with a mean value of 427 I.U./l. After gel-filtration, on average, there was about two times as much activity in the S_1 -peak as in the S_2 -peak (mean $S_1/S_2 = 2.00$), but between different sera the S_1/S_2 ratio ranged from values below 1 to values as high as 8.80 (patient No. 23). While the average γ -glutamyl transferase activity in this group was comparable to that of Group I, the distribution of activity between the peaks after gel-filtration was different. On the whole, sera of patients with malignant diseases seemed to have a bigger proportion of the peak with molecular weight of over 1×10^6 (S_2) than sera of patients in Group I, although there was some overlapping between the groups. This difference is reflected in their respective mean ratios of S_1/S_2 , the mean value for Group I being more than four times that of Group II.

Table 6.1 - Sepharose 6B gel-filtration of serum samples from patients in Group I.

Patient No.	γ -Glutamyl transferase activity in serum, I.U./l	γ -Glutamyl transferase activity after gel-filtration in:		S ₁ /S ₂	Age and sex of patient; diagnosis
		S ₁ -peak (% of total)	S ₂ -peak (% of total)		
1	326	93.8	6.2	15.1	M 1923 Alcoholic cirrhosis of the liver
2	303	86.2	13.8	6.27	M 1940 Chronic alcoholism
3	465	83.9	16.1	5.23	M 1928 Alcoholic cirrhosis
4	415	91.6	8.4	10.95	M 1937 Alcoholic liver disease
5	485	89.2	10.8	8.27	M Alcoholic
6	480	88.0	12.0	7.30	F 1925 Alcoholic cirrhosis
7	604	88.2	11.8	7.47	F 1947 Alcoholic hepatitis
8	516	86.5	13.5	6.38	M 1933 Alcohol-induced hyperlipidaemia
9	386	89.4	10.6	8.43	M 1920 Alcoholic hepatitis
10	297	87.5	12.5	7.02	M 1928 Alcohol abuse, no liver cirrhosis
11	266	87.4	12.6	6.95	M 1924 Cirrhosis (?)
12	247	93.0	7.0	13.22	F 1911 Cirrhosis of the liver
13	266	92.0	8.0	11.49	M 1938 Alcoholic liver disease with delerium tremens
14	555	93.1	6.9	13.47	M 1944 Alcoholism; Wermicke's encephalopathy
15	260	85.0	15.0	5.65	F 1919 Alcohol abuse and hypertension
16	713	93.6	6.4	14.69	F Alcoholic liver disease
17	759	83.8	16.2	5.19	F 1937 Alcoholic hepatitis
Mean values	432	89.0	11.0	8.09	

Table 6.2 - Sepharose 6B gel-filtration of serum samples from patients in Group II.

Patient No.	γ -Glutamyl transferase activity in serum, I.U./l	γ -Glutamyl transferase activity after gel-filtration in:		S_1/S_2	Age and sex of patient; diagnosis
		S_1 -peak (% of total)	S_2 -peak (% of total)		
1	914	29.1	70.9	0.41	M 1907 Ca of gall bladder with liver metastases
2	325	25.2	74.8	0.34	M 1933 Malignant histocytosis, chronic liver disease
3	1,015	42.4	5.76	0.74	M 1907 Ca of gall bladder with liver metastases
4	245	38.4	61.6	0.63	M 1932 Ca of common bile duct, obstructive jaundice
5	620	28.5	71.5	0.40	M 1917 Liver adenocarcinoma
6	860	32.4	67.6	0.48	F 1913 Ca of breast, with liver metastase
7	568	25.1	74.9	0.33	M 1927 Lymphoma
8	888	38.1	61.9	0.62	F 1931 Ca of breast with liver metastases
9	223	67.4	32.6	2.07	F 1901 Ca of breast, metastases in lungs and chest wall
10	564	83.3	16.7	5.00	F Ca of cervix
11	395	73.8	26.2	2.82	M Ca bronchus
12	756	65.2	34.8	1.88	F 1925 Ca of breast, anaemia
13	125	73.2	26.8	2.73	M 1966 AML
14	352	64.8	35.2	1.84	M 1942 CGL - blast crisis
15	812	78.5	21.5	3.66	M 1938 Phaeochromocytoma, removed by operation in 1975.

16	760	67.3	32.7	2.06	F 1911 Ca (?)
17	470	88.2	11.8	7.45	F 1955 T-cell chronic lymphocytic leukaemia
18	458	63.0	37.0	1.70	F 1920 Leiomyosarcoma of left thigh with lung and bone metastases
19	306	72.9	27.1	2.69	F 1911 Ca of breast with liver metastases Paget's disease of bone
20	380	68.3	31.7	2.15	M 1924 Ca of bronchus
21	279	82.5	17.5	4.71	F 1933 Multiple liver secondaries with no obvious primary
22	180	74.6	25.4	2.93	F 1930 CGL
23	112	89.8	10.2	8.80	M 1917 Carcinoid syndrome with widespread carcinoid metastases
24	148	86.3	13.7	6.32	F 1927 Ca of oesophagus
25	240	84.9	15.1	5.61	F 1935 Ca of breast
26	757	73.4	26.6	2.76	F 1918 Ca of breast
27	509	77.7	22.3	3.49	M 1956 Hodgkin's disease
28	222	78.4	21.6	3.62	M 1945 Lymphoma
29	238	80.8	19.2	4.21	M 1935 Ca of larynx
30	161	74.9	25.1	2.98	M 1920 Ca of bronchus
31	258	85.4	14.6	5.86	M 1956 Hodgkin's disease
32	900	81.5	18.5	4.42	M 1928 Ca of lung
33	365	72.7	27.3	2.66	F 1956 CGL - blast crisis
34	246	74.2	25.8	2.88	M 1925 Ca of bronchus

Continued

35	111	86.8	13.2	6.57	F 1956 Hodgkin's disease
36	187	67.9	32.1	2.12	M 1921 Ca of pancreas; obstructive jaundice
37	116	68.0	32.0	2.12	M 1922 Ca of bladder
38	161	68.1	31.9	2.14	F 1920 Ca of ovary
Mean values	427	66.7	33.3	2.00	

Group III consisted of patients that could not be included in either of the first two groups, and γ -glutamyl transferase activity in their sera varied from normal to values as high as 748 I.U./l. Three bone marrow transplants and one renal transplant patient had S_1/S_2 ratios of less than unity. The same was observed for one patient with myelofibrosis (No. 5 in Table 6.3), and one with a diagnosis of hepatitis undergoing renal function studies (No. 13). The rest of the patients in this group had S_1/S_2 values ranging from about 2.0 to 7.16.

6.3 Detection of hepatic metastases

Early detection of metastatic spread of cancer is a very important aspect of clinical enzymology. Liver is known to be one of the organs most susceptible to the development of metastases, and increases in serum γ -glutamyl transferase, alkaline phosphatase and lactate dehydrogenase are frequently associated with metastatic liver cancer. γ -Glutamyl transferase activity in serum is a sensitive indicator of hepatic involvement in malignant disease (see Chapter 1). Great sensitivity has recently been claimed for measurement of a high molecular weight form of alkaline phosphatase in serum (Crofton, 1980). Since it is probable that the high molecular weight form of this enzyme and of γ -glutamyl transferase are identical, the advantages of measuring the high molecular weight form of alkaline phosphatase should also be shown by measurements of the corresponding component of serum γ -glutamyl transferase.

Samples analyzed: Blood samples were obtained from all patients admitted to the Department of Surgery because of known cancer. Samples were also obtained from such patients admitted to other departments of

Table 6.3 - Sepharose 6B gel-filtration of serum samples from patients in Group III.

Patient No.	γ -Glutamyl transferase activity in serum, I.U./l	γ -Glutamyl transferase activity after gel-filtration in:		S_1/S_2	Age and sex of patient; diagnosis
		S_1 -peak (% of total)	S_2 -peak (% of total)		
1	600	32.6	67.4	0.48	M 1956 Blast cells in marrow and spleen, bronchopneumonia; B.M. transplant
2	320	42.6	57.4	0.74	M 1956 Aplastic anaemia, B.M. transplant
3	448	46.0	54.0	0.85	Bone marrow transplant
4	307	38.1	61.9	0.62	Renal transplant
5	210	40.8	59.2	0.69	Myelofibrosis
6	250	78.8	21.2	3.72	AA +ve
7	25	95.6	4.4	22.0	AA +ve
8	40	66.6	33.4	1.99	AA +ve
9	748	72.9	27.1	2.69	M 1932 Schizophrenic; operated for gall bladder stone
10	365	82.4	17.6	4.69	M 1926 DVT (had oesophagus operation, normal benign structure, 1979)
11	445	77.6	22.4	3.46	F 1929 Primary biliary cirrhosis, chronic active hepatitis
12	388	79.4	20.6	3.85	M 1948 Marrow failure, liver biopsy showed foci necrosis; anaemia
13	495	38.5	61.5	0.63	F 1922 Hepatitis, renal function studies

Continued

14	138	83.2	16.8	4.94	M 1945 Hypertension with allergy to thiazides
15	511	86.5	13.5	6.40	M 1919 Anorexia? nausea?
16	458	81.6	18.4	4.44	F 1912 (1) Confusion and abdominal pain porphyria; (2) probable gastro-enteritis
17	119	71.7	28.3	2.54	Hypertension
18	200	78.0	22.0	3.56	M 1901 Gallstones (removed by operation)
19	155	87.7	12.3	7.16	F 1924 Stricture of the common bile duct
20	111	68.3	31.7	2.16	F 1914 Severe aortic stenosis. ? Mitral stenosis
21	184	86.7	13.3	6.51	F 1931 Liver failure of unknown etiology

the hospital. Of these patients, only those fulfilling the requirements for inclusion in either of the following two groups were used for this study.

- Group I - cases without liver metastases, where the diagnosis had been verified either at operation or at post-mortem examination and the liver had been found to contain no malignant growth.

- Group II - cases of either liver metastases or primary liver cancer, where the diagnosis had been verified by laparotomy or at post mortem examination.

Samples were obtained from 17 cases, of which 6 belonged to Group I and 11 to Group II. The tumours were mostly cancer of the colon, of the breast and of the gallbladder. Two cases of primary liver cancer were included in Group II.

Methods

γ -Glutamyl transferase and alkaline phosphatase activities were determined by the Scandinavian methods recommended for these enzymes (see Chapter 2). Gel-filtration on Sepharose 6B column was performed as described in the previous section of this chapter.

Results

γ -Glutamyl transferase and alkaline phosphatase activity in each serum sample was measured both in the whole serum and in the fractions

obtained after gel-filtration of the sample on Sepharose 6B column. γ -Glutamyl transferase activity in serum usually separated into two activity peaks; the same was observed for alkaline phosphatase activity, the first eluted peak of which always coincided with the first peak of γ -glutamyl transferase (M.W. $>1,000,000$). The second peak of alkaline phosphatase activity however eluted a little after the S_1 -peak of γ -glutamyl transferase. To avoid confusion, these enzyme activity peaks were called S_1 - γ GT and S_2 - γ GT for γ -glutamyl transferase and S_1 -ALP and S_2 -ALP for alkaline phosphatase (S_2 - γ GT and S_2 -ALP being the exclusion volume activity peaks).

Activities eluted in each of these peaks together with the S_1/S_2 ratios were calculated for each of the two enzymes separately. A typical Sepharose 6B column elution profile for serum γ -glutamyl transferase and alkaline phosphatase is given in Fig. 6.4. Results of gel-filtration and activity measurements for the two enzymes in serum of patients of Group I are presented in Table 6.4 and those of Group II, in Table 6.5.

As can be seen from Table 6.4, γ -glutamyl transferase activity in serum of patients with cancer but no liver metastases was within normal limits, the average level of activity for 6 patients being 19 I.U./l. The contribution of the S_2 -form of γ -glutamyl transferase to the total activity in serum varied between about 15% and 29% (Fig. 6.5a), with the mean value of 21%. The S_1/S_2 ratio for γ -glutamyl transferase in serum of this group of patients did not seem to depend on the total enzyme activity (Fig. 6.6a). Average alkaline phosphatase activity in Group I was 182 I.U./l; but the high molecular weight form of this enzyme (S_2 -ALP)

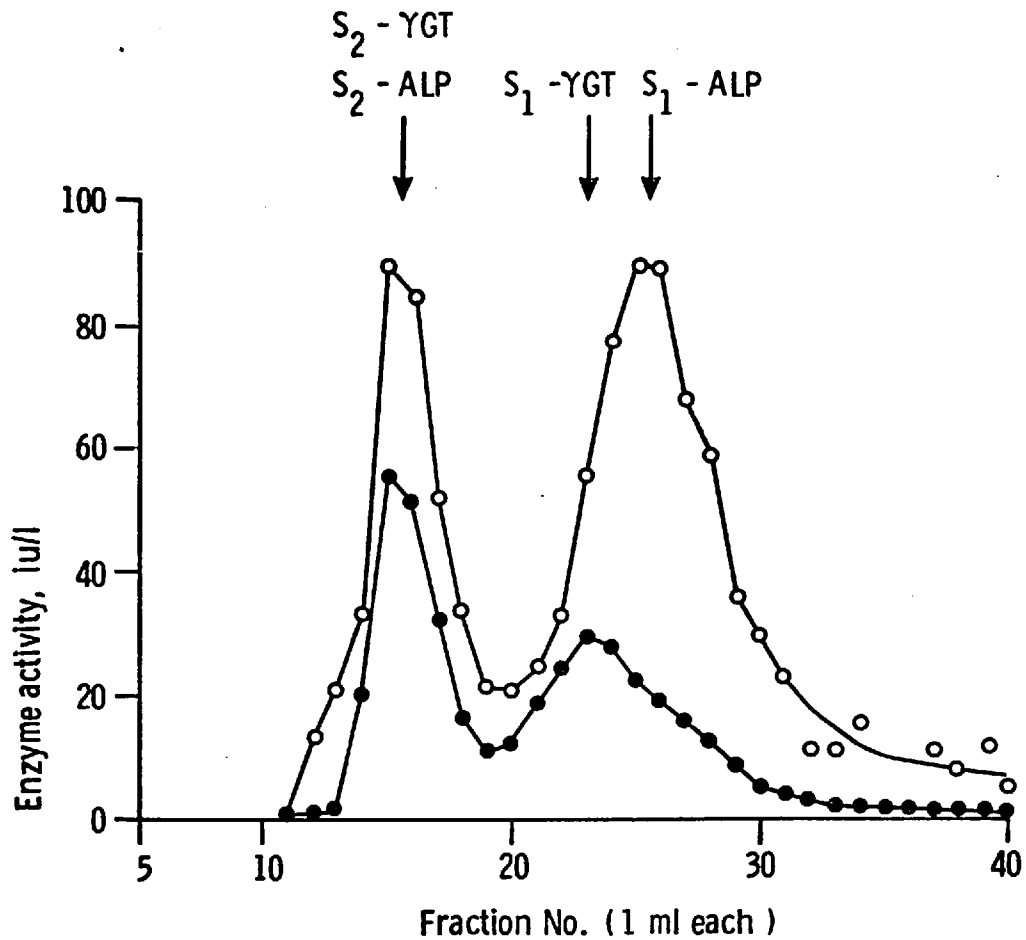


Fig. 6.4

Sepharose 6B gel-filtration of a serum sample in liver metastases study: elution profile of γ -glutamyl transferase (●—●) and alkaline phosphatase (○—○) activities; 1.6 ml of serum with total activities of 246 I.U./l and 478 I.U./l, respectively, was applied.

Table 6.4 - Sepharose 6B gel-filtration of serum samples from cancer patients without liver metastases

Patient No.	γ -Glutamyl transferase activity			Alkaline phosphatase activity			S_1/S_2 γ GT	S_1/S_2 ALP
	in serum I.U./l	distribution in peaks after gel-filtration, % of total activity		in serum I.U./l	distribution in peaks after gel-filtration, % of total activity			
		S_1 - γ GT	S_2 - γ GT		S_1 -ALP	S_2 -ALP		
1	18	70.8	29.2	186	92.0	8.0	2.42	11.48
2	10	81.3	18.7	166	92.9	7.1	4.35	13.13
3	41	85.1	14.9	186	90.0	10.0	5.73	9.04
4	18	73.7	26.3	263	93.7	6.3	2.80	14.77
5	16	83.1	16.9	102	89.6	10.4	4.92	8.65
6	9	79.8	20.2	188	97.6	2.4	3.96	40.32
Mean values	19	79.0	21.0	182	92.6	7.4	4.03	16.2

Table 6.5 - Sepharose 6B gel-filtration of serum samples from patients with either primary or secondary liver cancer.

Patient No.	γ -Glutamyl transferase activity			Alkaline phosphatase activity			$S_1/S_2\gamma\gamma\text{GT}$	S_1/S_2 ALP	Primary site of cancer
	in serum I.U./l	distribution in peaks after gel-filtration, % of total activity		in serum I.U./l	distribution in peaks after gel-filtration, % of total activity				
		S_1 - γGT	S_2 - γGT		S_1 -ALP	S_2 -ALP			
1	123	79.6	20.4	474	76.9	23.1	3.91	3.33	Colon
2	147	70.2	29.8	826	96.0	4.0	2.35	23.80	Colon
3	53	72.8	27.2	249	89.4	10.6	2.68	8.47	Colon
4	246	52.2	47.8	478	64.0	36.0	1.09	1.78	Colon
5	65	89.2	10.8	190	92.5	7.5	8.22	12.26	Colon
6	84	50.5	49.5	865	67.7	32.3	1.02	2.09	Colon
7	650	27.2	72.8	1,797	73.3	26.7	0.37	2.74	Liver
8	620	28.5	71.5	---	---	---	0.40	---	Liver
9	914	29.1	70.9	---	---	---	0.41	---	Gall bladder
10	860	32.4	67.6	---	---	---	0.48	---	Breast
11	888	38.1	61.9	---	---	---	0.62	---	Breast
Mean values	423	51.8	48.2	697	80.0	20.0	1.95	7.78	

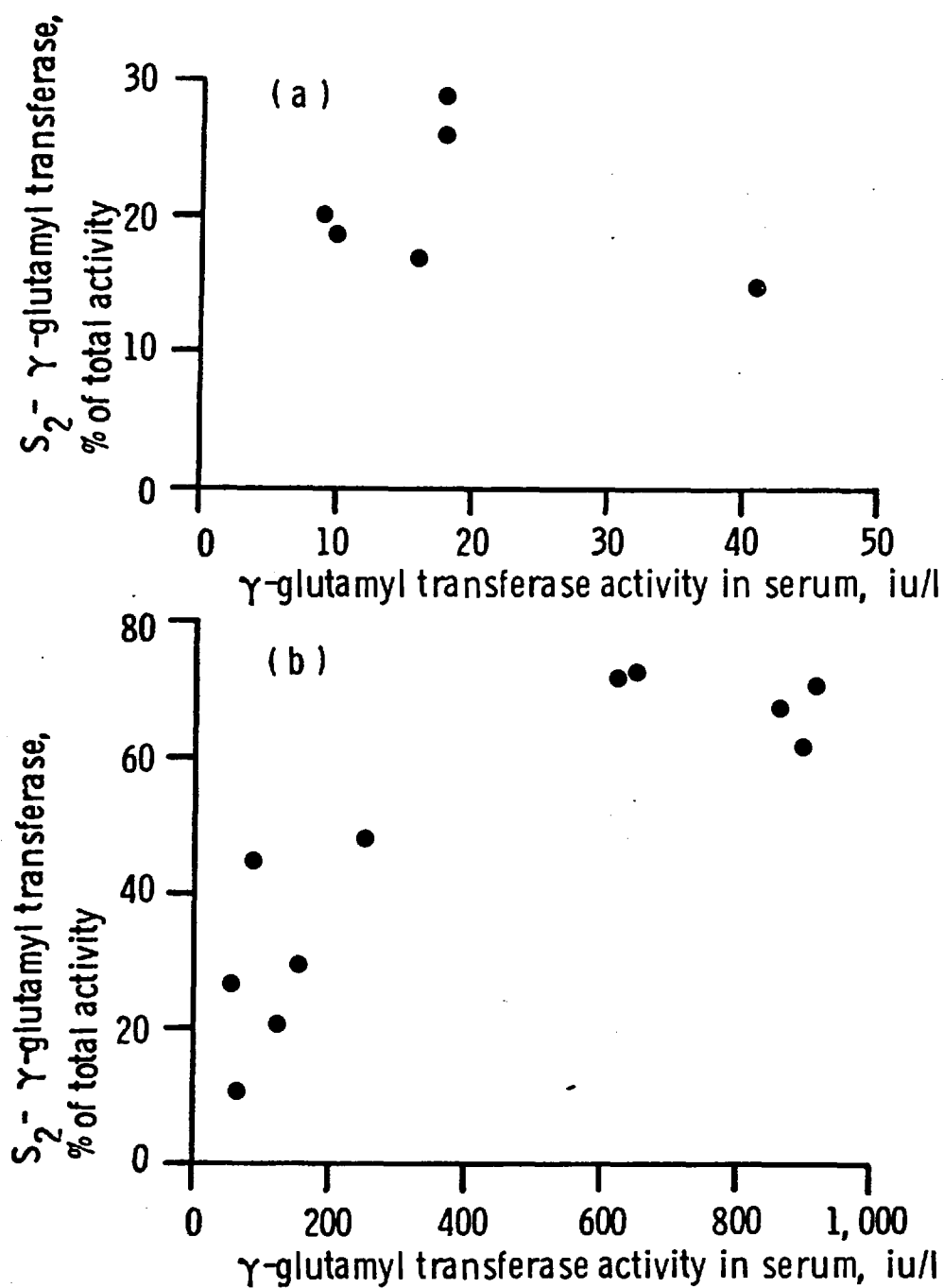


Fig. 6.5

Relationship between the proportion of S₂-form of γ-glutamyl transferase and total activity in serum samples of cancer patients with: (a) no liver metastases and (b) either primary or secondary liver cancer.

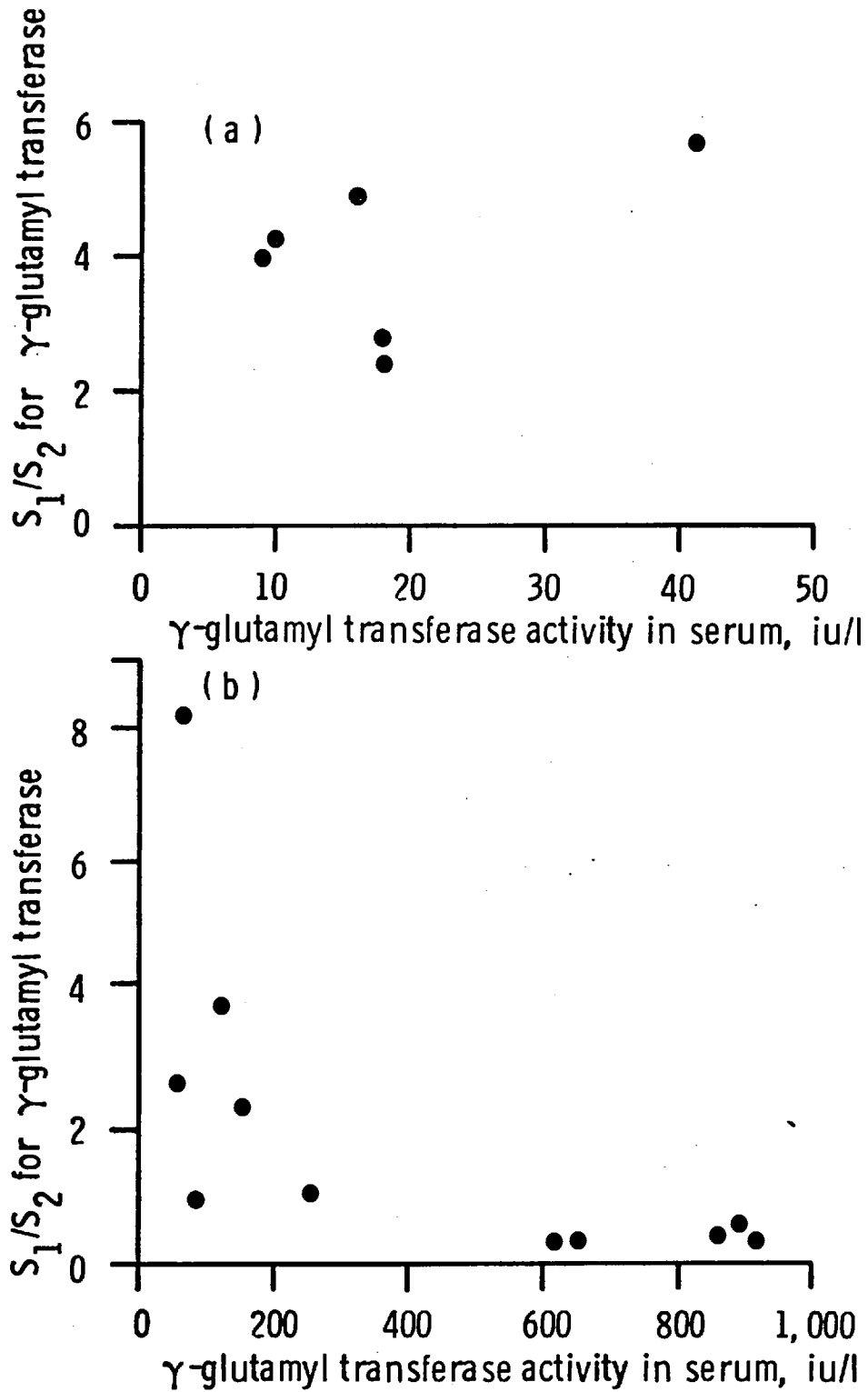


Fig. 6.6

Relationship between the S_1/S_2 ratio and total γ -glutamyl transferase activity in serum samples of cancer patients with: (a) no liver metastases and (b) either primary or secondary liver cancer.

constituted a smaller proportion of the total activity, compared with the S_2 -form of γ -glutamyl transferase (Fig. 6.7a). The S_1/S_2 ratio for alkaline phosphatase was therefore higher than such a ratio for γ -glutamyl transferase; it ranged between values of about 9 to over 40 (Fig. 6.8a) with the mean value of 16.2.

For patients with either primary or secondary liver cancer, γ -glutamyl transferase activity in serum was usually elevated, though in one case (patient No. 3, Table 6.5) it was just on the border-line. The mean γ -glutamyl transferase and alkaline phosphatase activities for this group were 423 I.U./l and 697 I.U./l, respectively. The proportion of the high molecular weight form of γ -glutamyl transferase increased with an increase of the total activity of this enzyme in serum (Fig. 6.5b). This was reflected in progressive lowering of the S_1/S_2 ratio for γ -glutamyl transferase with elevation of the total activity of the serum enzyme (Fig. 6.6b). The mean value of the S_1/S_2 ratio for γ -glutamyl transferase in Group II was 1.95, i.e. about four times lower than in cancer patients without liver metastases. The lowest values of 0.37 and 0.40 of this ratio were observed in two cases of primary liver cancer (patients No. 7 and 8, Table 6.5). Two other patients with the values of such ratio below unity died soon after the test was carried out.

For alkaline phosphatase, the proportion of the high molecular weight form in serum of patients in Group II also seemed to increase with increase in the total activity of this enzyme (Fig. 6.7b), but such an increase is not very certain, and could probably be explained by a smaller number of cases in which gel-filtration of alkaline phosphatase activity was carried out (7 cases). The S_1/S_2 ratio for this enzyme varied between values of about 2 to 24 (Fig. 6.8b), and the mean value for 7 patients was approximately 2 times lower than the corresponding value in Group I.

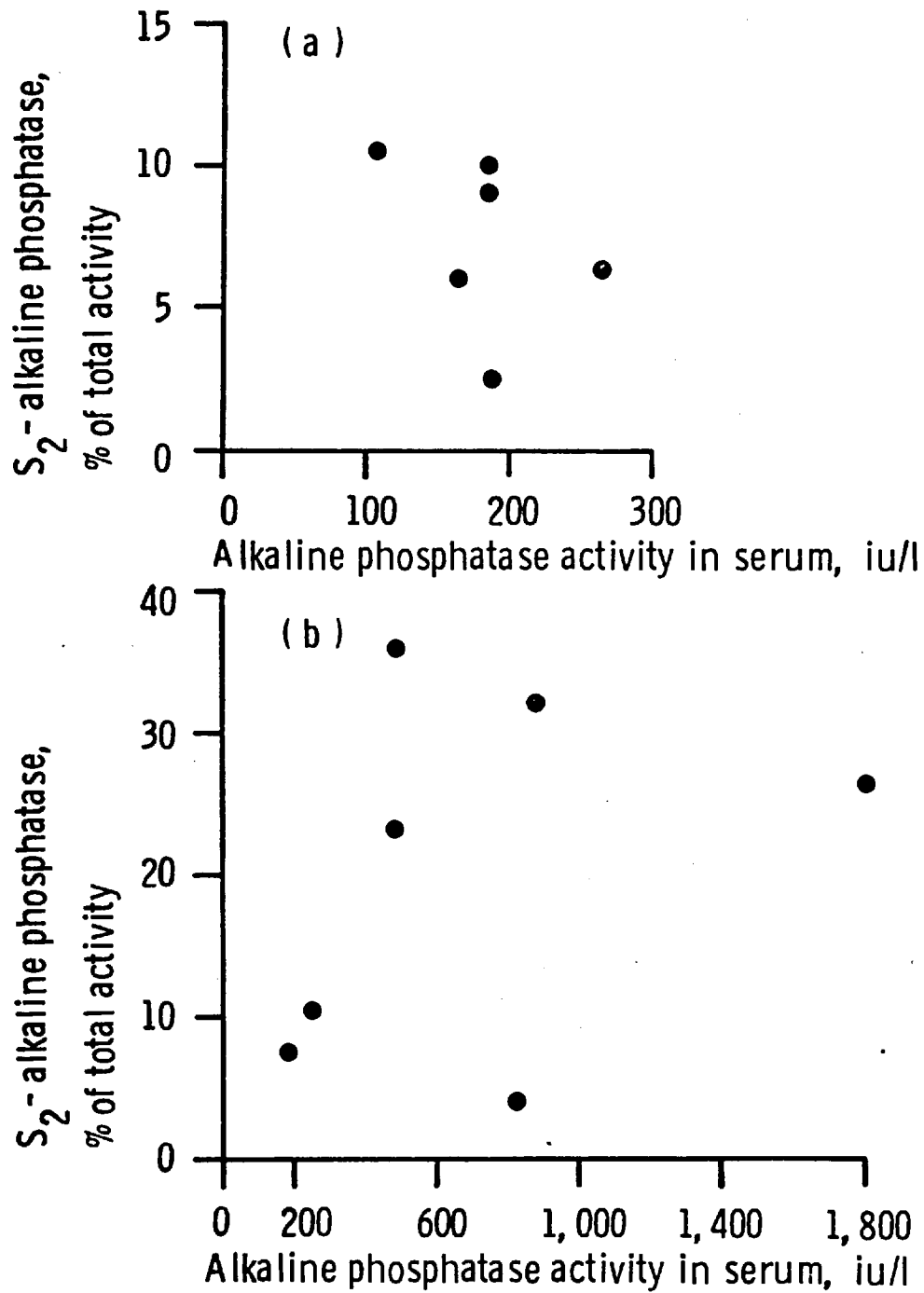


Fig. 6.7

Relationship between the proportion of S_2 -form of alkaline phosphatase and total activity in serum samples of cancer patients with: (a) no liver metastases and (b) either primary or secondary liver cancer.

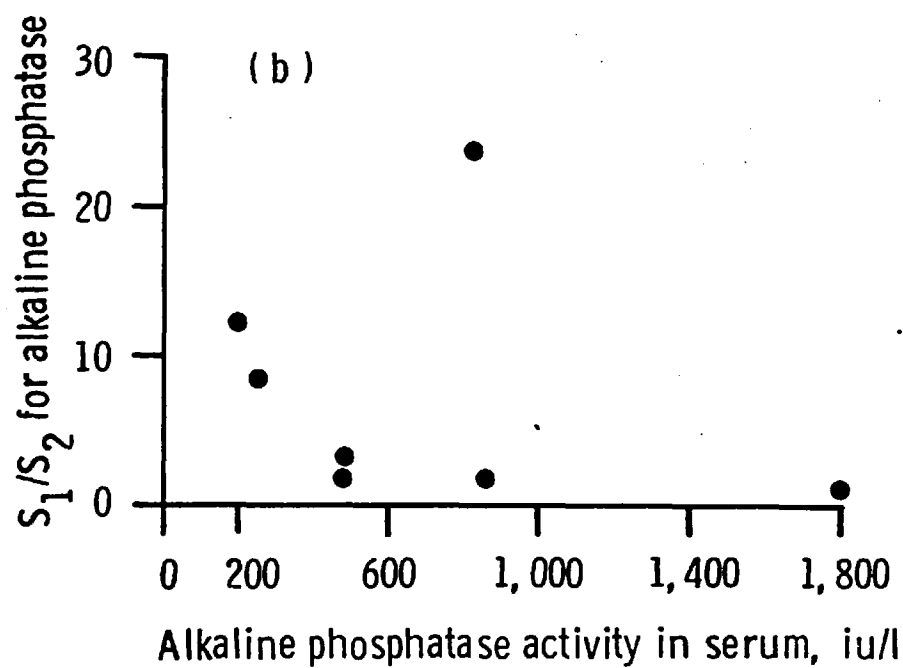
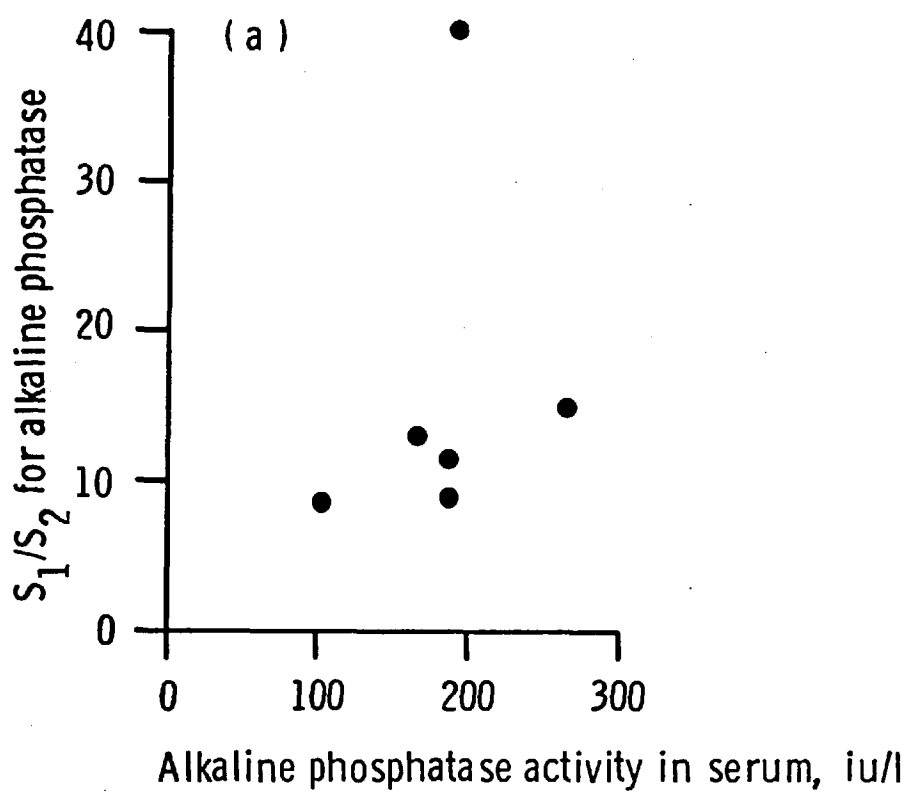


Fig. 6.8

Relationship between the S_1/S_2 ratio and total alkaline phosphatase activity in serum samples of cancer patients with: (a) no liver metastases and (b) either primary or secondary liver cancer.

6.4 Gel-filtration analysis of bile samples

Twenty seven samples of bile obtained from 3 patients undergoing percutaneous intrahepatic decompression for obstructive jaundice were subjected to gel-filtration on the same column as serum samples (see Section 6.2).

Bile samples of the following patients were studied:

Patient I	(M, D.O.B. 1932)	-	cancer of the bile duct
Patient II	(M, D.O.B. 1921)	-	cancer of the pancreas
Patient III	(M, D.O.B. 1945)	-	suspected cancer of the bile duct

Before application to the column, all bile samples were filtered through glasswool. The volume of each sample for gel-filtration was 2 ml. After gel-filtration, the total activity of γ -glutamyl transferase in the bile usually separated into two activity peaks (B_1 and B_3), in some samples, however, intermediary peaks of activity were also observed (see Chapter 3). The distribution of activity between these two main zones (B_1 and B_3) was different between patients as well as for bile samples obtained at different intervals of time from the same patient. The latter case is shown in Fig. 6.9. For gel-filtration of each bile specimen, total γ -glutamyl transferase activity applied to the column and total activity eluted from the column were calculated. The enzyme activity in the B_3 (high) molecular weight peaks was estimated separately. The percentage of activity present in the high molecular weight form of the enzyme was independent of the total activity of the bile (Fig. 6.10).

As can be seen from Fig. 6.10, the proportion of the high

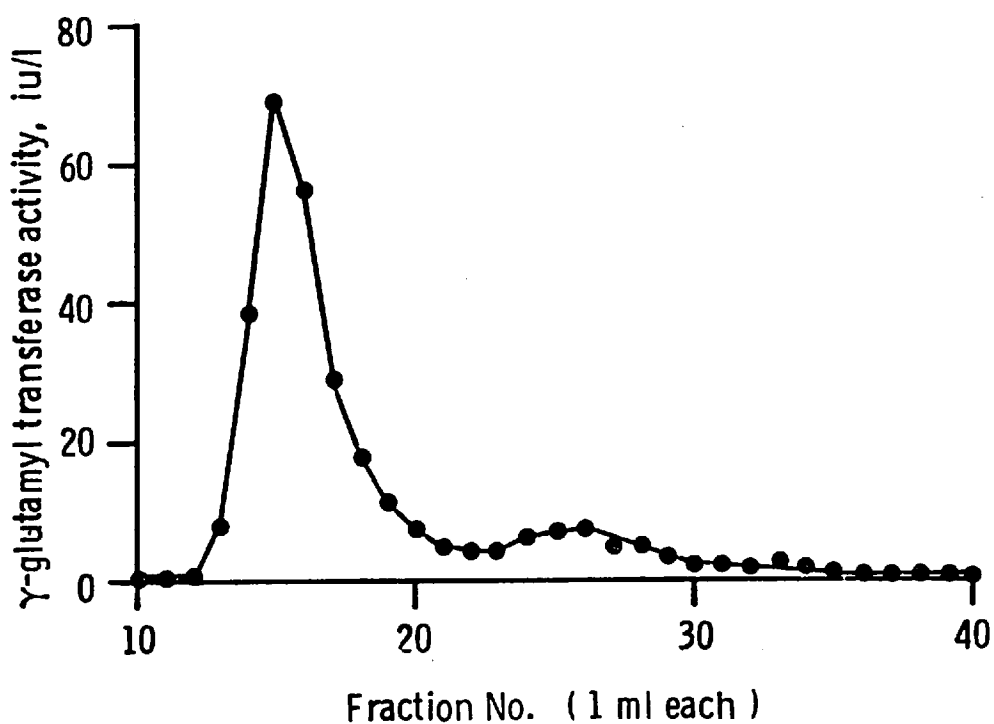
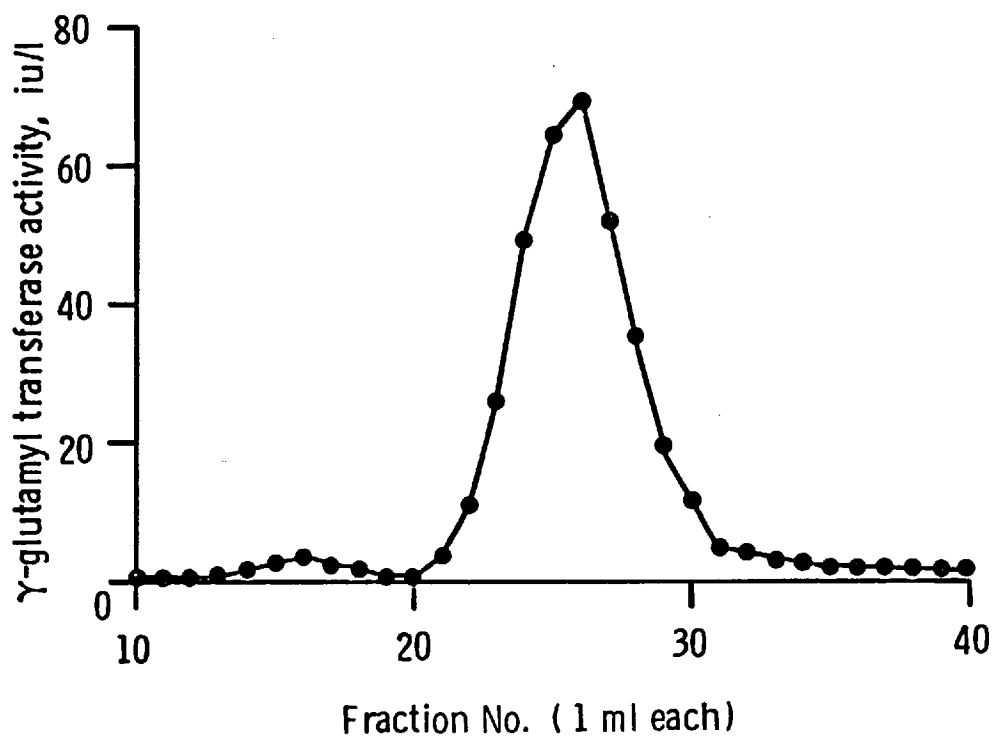


Fig. 6.9

Sephacrose 6B gel-filtration of human bile: distribution of γ -glutamyl transferase activity in samples from patient I obtained on 2 consecutive days; 2ml samples with activities of 207 I.U./l (a) and 148 I.U./l (b) were applied to the column.

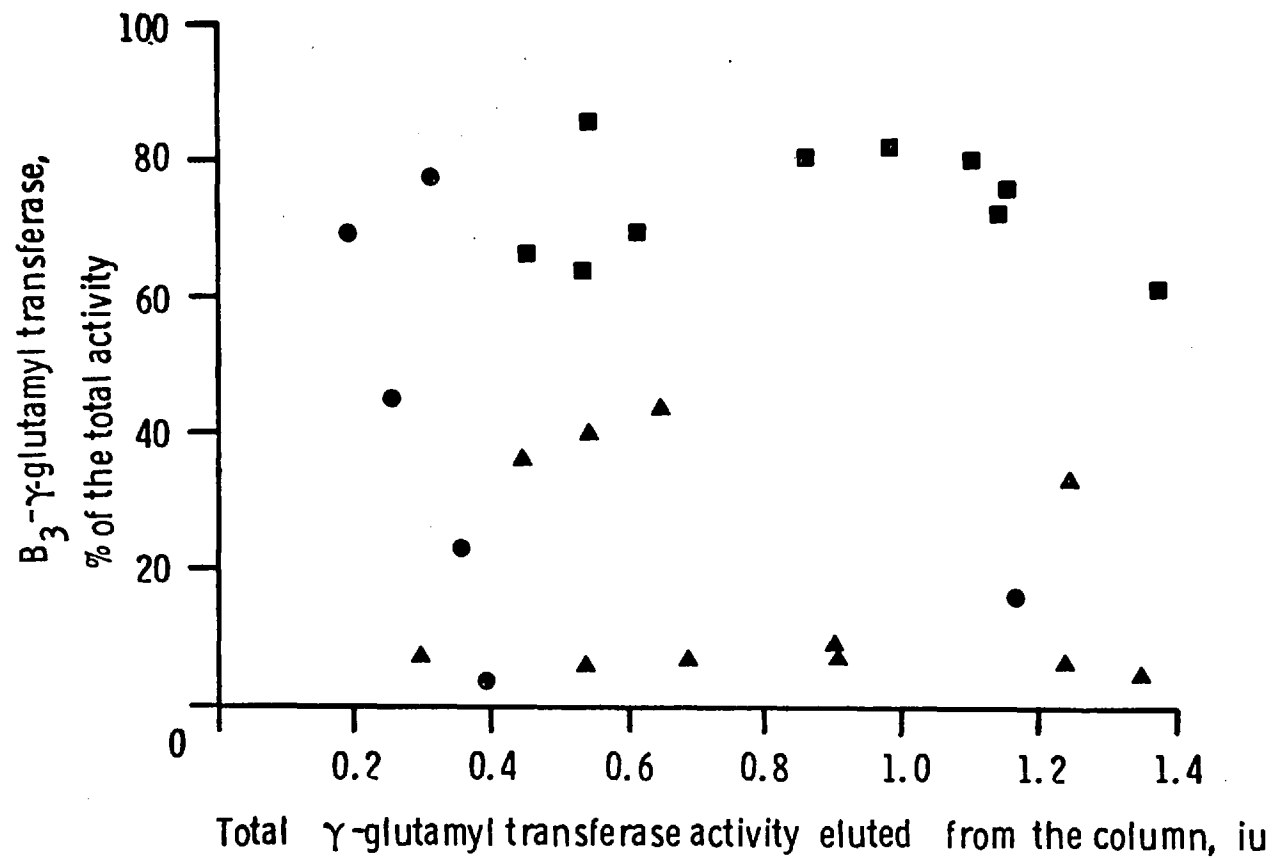


Fig. 6.10

Sephacrose 6B gel-filtration of human bile samples obtained from different patients: patient I (●), patient II (▲), patient III (■).

molecular weight peak of activity (B_3) varied quite a lot for bile specimens from patient I, was lower than 50% in the bile of patient II and represented between 61% and 86% of the total γ -glutamyl transferase activity in the bile specimens of patient III.

6.5 Correlation of high molecular weight form of γ -glutamyl transferase with that of alkaline phosphatase

A pool of serum with raised γ -glutamyl transferase activity, a sample of bile and incubation liquid when human liver tissue was incubated in a pool of serum for 48 h at 37°C (see Chapter 4) were each subjected to gel-filtration on a Sepharose column (1.5 x 95 cm) pre-equilibrated and eluted with 0.1 mol/l Tris-HCl buffer, pH 8.0, containing 0.1 mol/l NaCl. A flow rate of 12 ml/h was used and 3 ml fractions were collected with an LKB 7000 Ultrorac Fraction Collector.

γ -Glutamyl transferase and alkaline phosphatase activities in column fractions were estimated by the appropriate Scandinavian methods (Chapter 2). The elution profiles of both enzymes in "high γ -glutamyl transferase serum pool", in the bile and in the incubation liquid (liver in serum) are shown in Figs. 6.11, 6.12 and 6.13, respectively.

In "high γ -glutamyl transferase serum pool", total γ -glutamyl transferase activity separated after gel-filtration into the two activity peaks, S_1 and S_2 , as was described in Chapter 3. The activity of alkaline phosphatase in this sample also separated into two peaks: one, of a higher molecular weight, eluted from the column in exactly the same position as the S_2 -peak of γ -glutamyl transferase and another one eluted some time after the S_1 - γ -glutamyl transferase (Fig. 6.11).

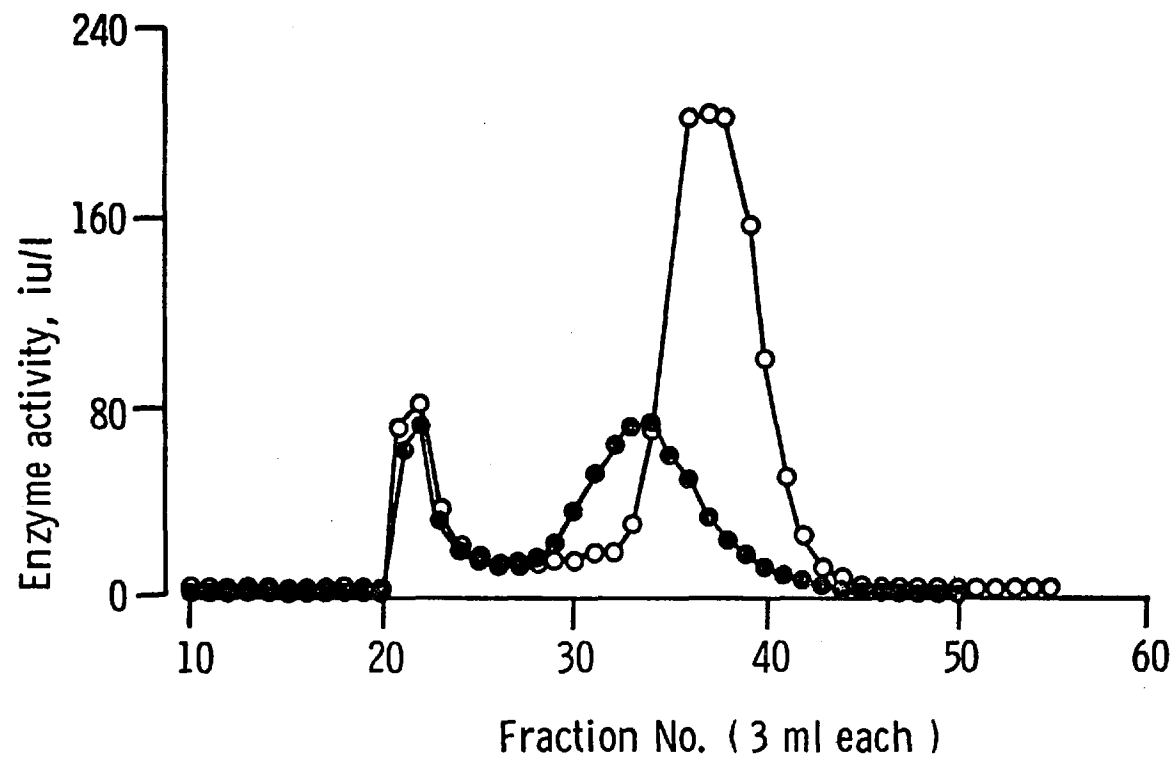


Fig. 6.11 Sepharose 6B gel-filtration of a sample of serum: γ -glutamyl transferase ($\bullet$ — $\bullet$) and alkaline phosphatase activity ($\circ$ — $\circ$); 3 ml sample with activities of 658 I.U./l and 1,083 I.U./l, respectively, was applied to the column.

In the bile, alkaline phosphatase activity separated into two molecular weight forms, and the elution position of the higher molecular weight peak of alkaline phosphatase coincided with that of the B_3 -peak of γ -glutamyl transferase (Fig. 6.12).

After gel-filtration of the incubation liquid (liver in serum), the elution position of the exclusion-volume peak of γ -glutamyl transferase (LS_2) was the same as that of a peak of alkaline phosphatase activity; the latter represented only a small part of the total alkaline phosphatase activity in the sample (Fig. 6.13).

The exclusion-volume peak of activity of "high γ -glutamyl transferase serum pool" contained about the same amounts of activity of both enzymes, whereas in the bile, the activity of alkaline phosphatase in it was higher than that of B_3 - γ -glutamyl transferase, and in the incubation liquid (liver in serum) the activity of alkaline phosphatase in the LS_2 -peak was rather low.

6.6 Summary

Sephacrose 6B gel-filtration of human bile samples demonstrated γ -glutamyl transferase activity in the bile to be present mainly in the high molecular weight form, B_3 (M.W. $>1 \times 10^6$) and in the B_1 -form (M.W. approx. 82,000), which may probably be the proteolyzed form of the enzyme. The distribution of activity between these two peaks varied from patient to patient, as well as in day-to-day samples of bile obtained from the same patient. It is therefore difficult to relate molecular-size distribution of activity to an underlying disease. Since in old bile

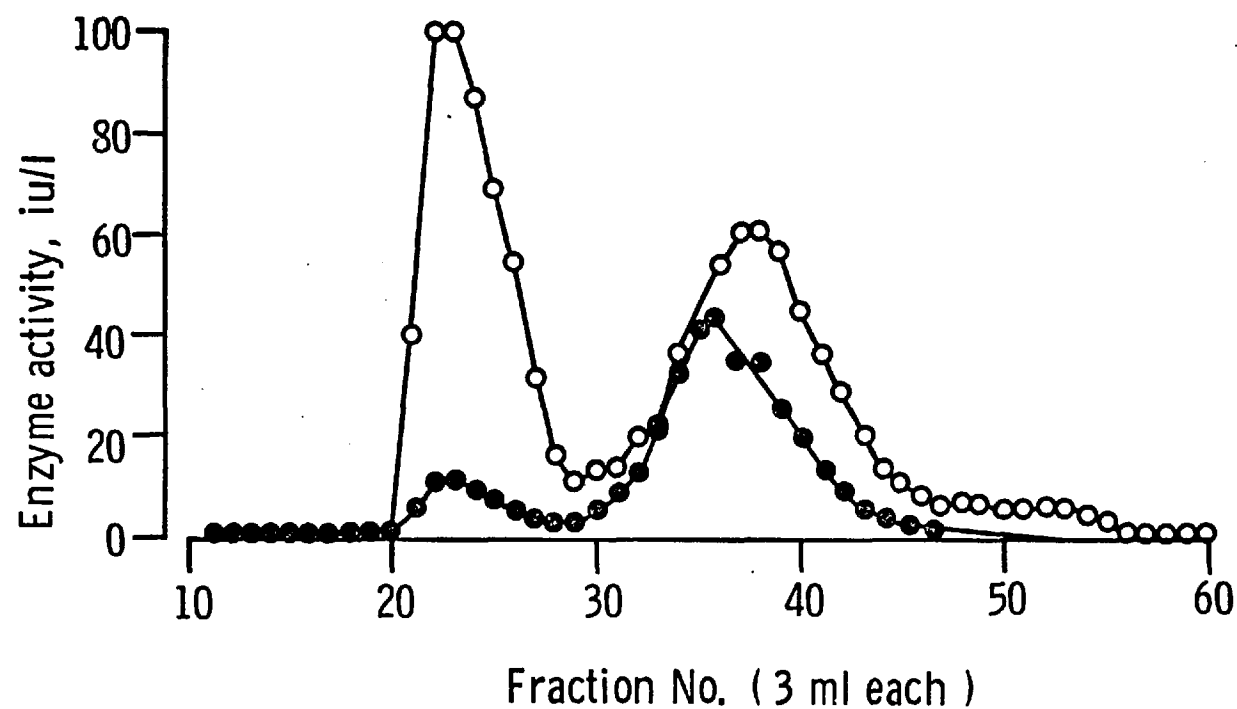


Fig. 6.12 Sepharose 6B gel-filtration of a sample of bile: γ -glutamyl transferase ($\bullet$ — $\bullet$) and alkaline phosphatase activity ($\circ$ — $\circ$); 7.3 ml sample with activities of 167 I.U./l and 365 I.U./l, respectively, was applied to the column.

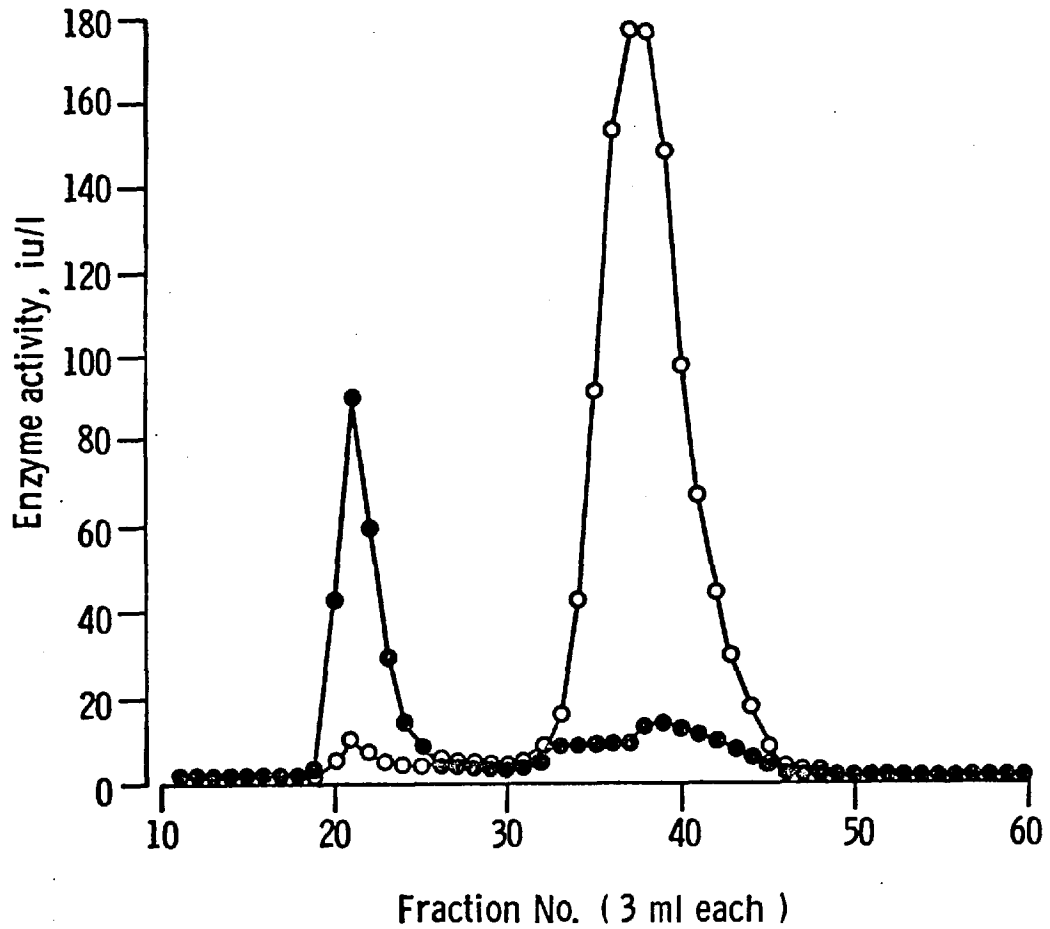


Fig. 6.13

Sepharose 6B gel-filtration of the incubation liquid (liver tissue in serum): γ -glutamyl transferase (●—●) and alkaline phosphatase activity (○—○); 6 ml sample with activities of 206 I.U./l and 4,542 I.U./l, respectively, was applied to the column.

specimens B_3 -form may get converted into B_1 -form of γ -glutamyl transferase (Chapter 3), it may be advisable to perform gel-filtration analysis of bile on freshly-obtained samples.

The high molecular weight form of γ -glutamyl transferase which occurs naturally in human serum (S_2) and bile (B_3) and is released by liver into serum in vitro (LS_2) may represent the enzyme in a bound or complexed form. Such a view is supported to some extent by experiments described in section 6.5 of this chapter, since alkaline phosphatase is another enzyme which is known to be membrane-bound.

Sephacrose 6B gel-filtration of individual patients' serum samples caused the activity of γ -glutamyl transferase to separate into two main peaks, S_1 and S_2 . Sometimes, however, a shoulder of activity on the S_1 -peak was also observed, which probably corresponds to the proteolyzed form of γ -glutamyl transferase; such proteolyzed fraction of the enzyme activity was observed by Huseby (1978) after gel-filtration of normal human serum pool.

The distribution of activity between the S_1 - and S_2 -forms of γ -glutamyl transferase varied from patient to patient, and the percentage of activity present in the high molecular weight form (S_2) was found to be generally independent of the total activity of the serum (Section 6.2). Patients with alcoholism and cirrhosis seem to have a much higher proportion of the S_1 -fraction of the enzyme than patients with malignant diseases. The mean value of the S_1/S_2 ratio for serum samples in the group of alcoholism and cirrhosis was about 8.1, whereas such value in malignant diseases was found to be about 2.0. On the whole, sera of

patients with various malignant diseases seem to have a bigger proportion of the peak of γ -glutamyl transferase activity with molecular weight of over 1,000,000, than sera of patients with other types of diseases. The S_2 -fraction of activity was predominant in serum of one kidney transplant and three bone marrow transplant patients. Their S_1/S_2 ratios were below unity, which probably resulted either from the rejection of transplants or from the effect of drugs such patients had been receiving.

In hepatic metastases study, sera of cancer patients without liver secondaries had a lower proportion of the S_2 -form of γ -glutamyl transferase than sera of patients with either primary or secondary liver neoplasms. Their respective S_1/S_2 ratios for γ -glutamyl transferase were 4.03 and 1.94. The proportion of the S_2 -form of alkaline phosphatase, which may probably be the same as the corresponding form of γ -glutamyl transferase, was also higher in the cases with hepatic malignancy. The mean S_1/S_2 ratio for alkaline phosphatase was 16.2 when liver metastases were absent, and about 7.8 when they were present. Two patients with primary liver cancer had the values of the S_1/S_2 ratio for γ -glutamyl transferase well below one. A patient who had cancer of the gall-bladder with liver secondaries and another one with breast cancer and metastases to the liver had the S_1/S_2 ratio for γ -glutamyl transferase of 0.41 and 0.62, respectively, and both died soon after the test had been carried out. It may be, that the lowering of the S_1/S_2 ratio for both γ -glutamyl transferase and alkaline phosphatase in serum of cancer patients indicates a rather poor prognosis. If the S_2 -fraction represents

the activities of these two enzymes in a membrane-bound form, the increase in the proportion of this type of activity may indicate the destruction of liver cells in cancer. It is then possible that, as the liver involvement in patients with cancer increases, the high molecular weight fraction of γ -glutamyl transferase and alkaline phosphatase activity after gel-filtration becomes more and more prominent.

With the limited number of samples analyzed, it is difficult to say at the moment whether the increase in the proportion of the high molecular weight form of both these enzymes is specific for cancer involving liver. One cannot rule out the possibility that such an increase could be caused by a prolonged chemo- or radiotherapy.

However, since there is a considerable overlapping of S_1/S_2 values in both groups of cancer patients (Tables 6.5 and 6.6), it may not be possible to distinguish between the absence or presence of hepatic secondaries on the basis of one such test alone. One would therefore propose to use a combination of this test with some other tests for metastases, such as CEA measurements (see Chapter 1). It may be of value to repeat the estimation of the S_1/S_2 ratio for γ -glutamyl transferase in serum of some cancer patients over a period of time. Tentatively, the progressive lowering of the ratio in the same patient may indicate the beginning of metastatic spread of cancer from other sites in the body to the liver. However, before any definite conclusions are made, much more work needs to be done with many more patients and different types of malignant diseases. It still remains to be established whether the measurement of the S_2 -form of γ -glutamyl transferase (or the S_1/S_2 ratio) is a more sensitive indicator for hepatic malignancy than the measurement of total activity of this enzyme in serum.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

7.1 Summary

The results of the experimental work reported in this thesis can be summarized as follows:

- 1) γ -Glutamyl transferase solubilized from human liver with sodium deoxycholate and Lubrol WX has been purified to a specific activity of 11.1 I.U./mg protein. The molecular weight of the enzyme-detergent complex was estimated to be approximately 160,000. In the absence of detergent, the molecular weight of the enzyme increases, presumably as a result of aggregation.
- 2) Multiple forms of the enzyme have been identified in human bile and serum and some of the physical and kinetic properties of these forms and the purified liver enzyme were compared. The main differences between them appear to be mainly in their size and net charge. No significant differences in catalytic properties were observed.
- 3) An attempt was made to reproduce in vitro the various forms of γ -glutamyl transferase naturally occurring in human serum by incubating human liver tissue in serum and protein-free solutions. These experiments demonstrated that forms of the enzyme released from liver tissue into serum in vitro resembled to some extent those present in native serum with respect to their electrophoretic mobility. Physical and catalytic properties of such artificially-prepared forms of γ -glutamyl transferase were studied.

- 4) The relationships between the multiple forms of γ -glutamyl transferase obtained from human liver, bile and serum were investigated further by means of interconversion experiments. Components common to all enzymic forms have been identified.
- 5) The occurrence of fractions of γ -glutamyl transferase heterogeneous with respect to size in individual samples of human serum and bile was studied. A tentative hypothesis relating the distribution of the enzyme activity between two different molecular weight fractions in serum to the nature of the underlying disease was made.

Some generalizations about the nature and properties of human γ -glutamyl transferase arising from these observations, and their relationship to current knowledge of the enzyme, are discussed in the following pages.

7.2 Nature of γ -glutamyl transferase molecule

γ -Glutamyl transferase purified from human liver using Na-deoxycholate and Lubrol WX had a molecular weight (estimated by gel-filtration through Sephadex G-200 in the presence of 0.1% Na-deoxycholate) of about 160,000. This value most probably represents the molecular weight of an enzyme-detergent complex. Huseby (1978) reported the molecular weight of the γ -glutamyl transferase-detergent complex to be approximately 175,000. Our value is in agreement with this estimate. However, it can be concluded that the detergent-purified form of the enzyme is not very stable from the fact that, after prolonged storage at -25°C , the molecular weight of the purified liver enzyme preparation increased to an average value of 550,000.

This apparent increase in molecular weight of the initial preparation of γ -glutamyl transferase occurred after the removal of Na-deoxycholate in the final step of the purification procedure (Chapter 3), and is therefore probably due to the aggregation of the enzyme molecules. The formation of complexes of varying molecular weights of the detergent-purified γ -glutamyl transferase from human liver (after the removal of detergent) is also implied by a very spread-out elution profile of the enzyme on gel-filtration (Chapters 3 and 5).

This tendency is not surprising, since γ -glutamyl transferase is a glycoprotein with the carbohydrate content as high as 35% (Goldberg, 1979), and a well-known property of glycoproteins in general is their tendency to aggregate and to form complexes with other proteins, lipoproteins, or lipids. Several reports have appeared on the tendency of the detergent-purified form of γ -glutamyl transferase from different sources to aggregate upon the removal of detergent, but to become quite soluble and stable when treated with proteolytic enzymes, such as bromelain, papain, trypsin, or ficin (Leibach and Binkley, 1968; Curthoys and Kuhlenschmidt, 1975; DeLap, Tate and Meister, 1975; Tate and Meister, 1975; Hughey and Curthoys, 1976; Miller, Awasthi and Srivastava, 1976; Zelazo and Orłowski, 1976; Huseby, 1977; Hada et al., 1978; Horiuchi, Inoue and Morino, 1978; Yasumoto et al., 1978; Jaken and Mason, 1979; Elce, 1980).

When the purified liver enzyme obtained in the present work was treated with trypsin, proteolysis resulted in the appearance of the lower molecular-weight form (M.W. 82,000). This form of γ -glutamyl transferase was soluble and quite stable, since its molecular weight did not change on

storage or during concentration of the enzyme solution. Some investigations have suggested that proteolytic treatment of the detergent-purified γ -glutamyl transferase splits off a hydrophobic polypeptide chain which is thought to be responsible for the binding to other proteins, lipoproteins and lipids, and that this accounts for the failure of proteolyzed form of the enzyme to aggregate or to form complexes (Hughey and Curthoys, 1976; Horiuchi, Inoue and Morino, 1978; Shaw, London and Petersen, 1978; Curthoys and Hughey, 1979; Hughey, Coyle and Curthoys, 1979). The properties of the human liver enzyme reported here are consistent with this view.

When properties of γ -glutamyl transferase purified from different sources are compared (Table 7.1), it can be seen that, irrespective of the source of the enzyme, the detergent-purified preparations all exhibit a much higher molecular weight than the proteolyzed ones. The molecular weight estimations of the proteolyzed form of the enzyme obtained from different sources all gave rather similar values of 64,000 to 90,000 (Table 7.1). As can be seen from this table, the γ -glutamyl transferase molecule consists of two subunits, which are different in size. The "heavy" form of the enzyme and the "light" (proteolyzed) form from a particular tissue in most cases are reported to contain the same two hetero-subunits with molecular weights of 22,000 - 27,000 and 45,000 - 66,000. However, one report on γ -glutamyl transferase purified from rat renal brush-border membranes showed the molecular weight of the bigger subunit of the detergent-purified form ($66,000 \pm 8,000$) to be slightly greater than that of the corresponding subunit of the papain-treated enzyme ($45,000 \pm 5,000$) (Horiuchi, Inoue and Morino, 1978). Another report

Table 7.1 - Isolation and some properties of γ -glutamyl transferase from different sources

No.	Enzyme source	Method of extraction	Approximate M.W.	Subunit M.W.
1	Rat kidney (Tate & Meister, 1975)	Lubrol Lubrol, bromelain	>250,000 68,000-70,000	1) 22,000 2) 46,000 (Tate & Meister, 1977)
2	Rat kidney (Hughey & Curthoys, 1976)	Triton X-100 Papain	169,000 (87,000* = γ GT 82,000 = Triton X-100) 68,000	1) 27,000 2) 54,000 1) 27,000 2) 51,000
3	Rat hepatoma (Taniguchi, 1974)	Na-DCC	113,000	N.D.
4	Rat Morris hepatoma (Szewczuk, Milnerowicz, & Sobiech, 1978)	Na-DCC	108,000 (estimated with Triton X-100 in sucrose gradient)	N.D.
5	Rat renal brush-border membrane (Horiuchi, Inoue & Morino, 1978)	Detergent Papain	102,000 64,000	1) 25,000 $\pm$ 4,000 2) 66,000 $\pm$ 8,000 1) 23,000 $\pm$ 3,000 2) 45,000 $\pm$ 5,000
6	Rat normal mammary tissue	Triton X-100, papain	80,000	N.D.

continued . . .

7	Rat mammary adenocarcinoma (Jaken & Mason, 1979)	Triton X-100, papain	80,000	N.D.
8	Rat seminal vesicles (DeLap, Tate & Meister, 1975)	Lubrol, bromelain Na-DOC	70,000 >200,000	N.D. N.D.
9	Hog kidney (Leibach & Binkley, 1968)	Ficin	80,000	N.D.
10	Hog kidney (Orlowski & Meister, 1965)	Na-DOC		N.D.
11	Bovine colostrum (Yasumoto et al., 1978)	Papain	80,000	1) 25,000 2) 55,000
12	Human kidney (Miller, Awasthi & Srivastava, 1976)	Lubrol, bromelain	90,000	4 s/u, M.W. 21,000 each
13	Human kidney (Tate & Ross, 1977)	Lubrol, bromelain	84,000	1) 22,000 2) 62,000
14	Human kidney (Hada et al., 1978)	Bromelain	90,000	N.D.
15	Human renal carcinoma (Hada et al., 1978)	Bromelain	130,000	N.D.

continued . . .

16	Human liver (Huseby, 1977)	Na-DOC, Lubrol, trypsin	90,000	1) 22,000 2) 47,000
17	Human liver (this work)	Na-DOC, Lubrol Trypsin	160,000 82,000	N.D.
18	Human bile (Indirani & Hill, 1977)	Protamine sulphate	>250,000	N.D.
19	Human seminal fluid plasma (Krisnaswamy, Tate & Meister, 1977)	Bromelain	70,000	N.D.

* calculated M.W.

indicated that human kidney γ -glutamyl transferase is a tetramer with subunits of molecular weight of about 21,000 each (Miller, Awasthi and Srivastava, 1976). These authors found that their preparations of the human kidney enzyme (M.W. 90,000) dissociated on sodium dodecyl sulfate-urea- β -mercaptoethanol polyacrylamide disc electrophoresis into two protein bands with molecular weights of 21,000 and 57,000, from which they concluded that subunits may be heterogeneous, but did not rule out the possibility that the protein band corresponding to a molecular weight of 57,000 represented a trimer. Later work of Tate and Ross (1977) showed the human kidney γ -glutamyl transferase to be a glycoprotein which is composed of two glycoproteins of molecular weights of 62,000 and 22,000, respectively.

The active site of the enzyme is believed to be on the outside surface of mammalian cells and the light subunit is known to contain the γ -glutamyl binding site (Tate and Meister, 1976; Tate and Meister, 1977; Tate and Ross, 1977; Novogrodsky, Tate and Meister, 1976; Horiuchi, Inoue and Morino, 1978; Shaw and Petersen-Archer, 1979). It has been suggested that the heavy subunit contains a polypeptide chain which anchors the enzyme to cell membranes and that this anchoring polypeptide chain can be removed by proteolytic digestion (Hughey and Curthoys, 1976).

In this work, a component with molecular weight in the region of about 20,000 was separated by extracting the various fractions of the enzyme from human liver, bile and serum with 0.5% Na-deoxycholate and subjecting such extracts to gel-filtration on Sepharose 6B column. This component retained its transferase activity. It is thus tempting to identify it with the light subunit prepared by other workers, and to

regard its properties as evidence that the binding sites for both donor and acceptor substrates are located on this component of the molecule. Since the presence of no intermediate enzymically active molecular weight form has been demonstrated between the light subunit and the proteolyzed form of γ -glutamyl transferase (containing both subunits), it may be concluded that the heavy subunit of γ -glutamyl transferase from human liver, bile and serum does not contain an active site.

7.3 Nature of multiple forms of γ -glutamyl transferase

In this work, electrophoresis on agarose gel and gel-filtration through Sepharose 6B have demonstrated the presence of heterogeneous forms of γ -glutamyl transferase in human liver, bile and serum. The naturally-occurring multiple forms of the enzyme have nearly identical catalytic properties, affinity to Concanavalin A and rather similar stability to inactivation by heat. They were different mainly with respect to size and charge. The purified liver enzyme had an average molecular weight of approximately 550,000 and an electrophoretic mobility of 0.73 that of albumin. γ -Glutamyl transferase in the bile was represented mainly by two peaks of activity on gel-filtration: one with a molecular weight of over 1,000,000, and another with a molecular weight of 82,000, but in some bile samples the presence of intermediate-size peaks of activity has been observed. The largest molecular weight fraction in the bile was heterogeneous on agarose-gel electrophoresis (electrophoretic mobilities of 0 and 0.6), while the smaller-sized fractions had mobilities of 0.8 that of albumin. In serum, γ -glutamyl transferase activity has also been found to be heterogeneous with respect to both size and charge. On gel-filtration

two main fractions were observed: a larger one with molecular weight of over 1,000,000 and an average electrophoretic mobility of 0.6 - 0.7 relative to albumin, and another one of about 300,000 and an electrophoretic mobility ranging between 0.6 and 0.95 that of albumin.

To generalize broadly about the occurrence of some of these fractions of γ -glutamyl transferase, it can be said that a fraction with molecular weight of over 1,000,000 occurs very often and in large proportions in human bile, but that a fraction of this size represents usually a smaller proportion of the γ -glutamyl transferase activity of serum. A similar fraction can be obtained from liver tissue in vitro, and presumably consists of the enzyme in a complexed or particulate form. These fractions of activity in native human bile and serum, as well as such fraction eluted from human liver by serum, all eluted with the void volume on Sepharose 6B chromatography and are all associated with alkaline phosphatase, which is known to be a membrane-associated enzyme. The largest-size fraction of γ -glutamyl transferase which occurs naturally in human bile and serum may therefore represent the enzyme eluted from human liver or biliary tract cells in the form of membrane fragments.

The molecular weight of this void-volume fraction can be reduced by various treatments, such as treatment with organic solvents or detergents, and proteolytic treatment with trypsin. The nature of such treatments leads to a speculation that the larger molecular weight forms of γ -glutamyl transferase contain lipids or lipoproteins. A similar proposition has been put forward by several other workers (Freise, Schmidt and Magerstedt, 1976; Beck and King, 1978; Huseby, 1978).

Huseby (1978) suggested that the largest and intermediate molecular

weight peaks after gel-filtration of human liver homogenate, bile and serum contain a hydrophobic domain which can complex with lipids and amphiphilic proteins and that these complexes produce the heterogeneous patterns found on gel-filtration and electrophoresis. Results presented in this thesis seem to support such a hypothesis.

As with the enzyme prepared from liver, proteolysis of other naturally-occurring and experimentally-produced large-molecular weight fractions of γ -glutamyl transferase, produced a fraction with molecular weight of approximately 82,000. This is also the molecular size of the B_1 -fraction which occurs naturally in human bile and of LS_1 and LN_1 forms of the enzyme obtained by incubating human liver tissue in serum and saline solution, respectively. However, differences in charge exist between the proteolyzed forms of the enzyme obtained from various original fractions. These differences may reflect the fact that the molecules may still be associated with different small molecular weight components which change the mobility of γ -glutamyl transferase in electrophoresis, but do not change the molecular weights of the complexes sufficiently to be detectable by gel-filtration on Sepharose 6B or Sephadex G-200. The proteolyzed fraction of γ -glutamyl transferase was not observed in pooled human serum, which contrasts with the findings of Huseby (1978). However, the presence of a smaller molecular weight "shoulder" of activity on the S_1 -peak after Sepharose 6B gel-filtration of some individual serum samples (Chapter 6) may perhaps represent the proteolyzed form of the enzyme. It still remains to be established whether the appearance of this form of the enzyme in human serum has any diagnostic significance. The proteolyzed form of γ -glutamyl transferase is probably the same as the "soluble" form described by Szewczuk (1966).

In summary, one may say that there are two "main" fractions of γ -glutamyl transferase: the largest, probably associated with membrane fragments, of molecular weight of over 1,000,000 (which on treatment with detergent produces an enzyme-detergent complex of approximately 160,000 molecular weight), and a proteolyzed fraction of about 82,000 molecular weight. The occasional presence of intermediate-sized fractions of activity in human bile and serum may result from aggregation of the enzyme with other proteins, lipoproteins or lipids. Different electrophoretic patterns in serum and bile may result from changes in serum or bile lipids or proteins which may form complexes with γ -glutamyl transferase. This has been suggested for serum γ -glutamyl transferase by Freise, Magerstedt and Schmidt (1976) who demonstrated that chylomicrons could change mobility of the enzyme zones on electrophoresis.

All fractions of γ -glutamyl transferase in human liver, bile and serum identified in the present work have virtually the same catalytic properties. Thus, the evidence presented in this work and that of others does not support the idea that multiple forms of γ -glutamyl transferase in human liver, bile and serum are true isoenzymes.

Now, the question of the origin of biliary and serum γ -glutamyl transferase arises. Several possibilities have been discussed.

The present experiments do not provide the evidence of complete identity between γ -glutamyl transferase derived from liver in vitro and the forms occurring in bile and serum which had been hoped for, and which might thus have further supported the presumed hepatic origin of the

γ -glutamyl transferase activity in these fluids. Lack of complete identity of properties between the naturally-occurring and artificially-prepared forms is most apparent with respect to electrophoretic mobility, and, for the LS₂ fraction, in its apparent absence of terminal sialic acid residues. However, against these differences must be set the many similarities between liver-derived γ -glutamyl transferase and the forms occurring in serum and bile, e.g., in kinetic properties and in molecular size. Furthermore, the ease with which the properties of the various γ -glutamyl transferase fractions can be modified, and the apparent presence in them of common components, argues against the acceptance of differences in such properties as net molecular charge as evidence of fundamental differences in structure. The simplest explanation of the increased activity of γ -glutamyl transferase in the circulation in hepatobiliary disease therefore still seems to be that the enzyme originates in the liver and/or biliary tract, and during or after its release undergoes various modifications.

7.4 Value of the existence of multiple forms of γ -glutamyl transferase in serum for diagnostic enzymology

As has been pointed out in Chapter 1, many reports have appeared on the electrophoretic heterogeneity of γ -glutamyl transferase in human serum, but no consistent change in electrophoretic patterns, which is specific to any disease, has been observed. This was also true for our own results on agarose-gel electrophoresis of γ -glutamyl transferase in 89 serum samples. Separations based on charge, therefore, do not seem to have much clinical value.

On the other hand, the separations based on size, such as serum gel-filtration experiments reported in Chapter 6, may be of more use diagnostically. Thus, the increase in the proportion of the largest molecular weight peak of activity (S_2) may mean a rather poor prognosis, possibly indicating the destruction of liver cells in patients with either primary liver cancer or with cancers originating from other organs in the body and metastases to the liver. It is interesting to note, that most cancer patients with values of S_1/S_2 ratio below unity, died soon after the test was carried out. Thus, one may say that, as the liver involvement in patients with cancer increases, the S_2 -fraction of γ -glutamyl transferase activity after gel-filtration becomes more and more prominent.

Increase in the proportion of the S_2 -peak relative to the S_1 -peak was also observed for serum of one kidney transplant and three bone marrow transplant patients. Their S_1/S_2 ratios were less than one and probably indicated the rejection of transplants.

Patients with alcoholism and cirrhosis, however, seem to have a rather different pattern of distribution of the two peaks of γ -glutamyl transferase activity in serum. In this group, S_1 -fraction becomes more prominent than S_2 , and the S_1/S_2 ratio is indeed very high in some patients, especially in chronic alcoholics.

Gel-filtration of serum of patients with raised total γ -glutamyl transferase activity may, therefore, have some value in indicating the nature of the disease and the degree of involvement of the liver. In this respect, however, as in other diagnostic applications of measurement

and characterization of γ -glutamyl transferase in serum, greater practical value will result from a clearer understanding of the pathophysiological processes which lead to the entry of the enzyme into the circulation.

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Published work

Part of the work presented in this thesis has already been published:

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