THE HUMAN GLUTATHIONE S-TRANSFERASES:

A STUDY OF THE TISSUE DISTRIBUTION, GENETIC VARIATION AND DEVELOPMENT OF THE GST1, GST2 AND GST3 ISOENZYMES.

GEORGE CHARLES FAULDER, M.Sc.

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DECLARATION OF ORIGINALITY

I declare that the work presented herein
and the composition of this thesis is my own.

George Charles Faulder.

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ABSTRACT

The glutathione S-transferases are a group of detoxicating enzymes found in all human tissues studied. They are believed to be the products of at least three autosomal gene loci (GST1, GST2 and GST3). The expression of these loci was examined using starch-gel electrophoresis and chromatofocusing and shown to demonstrate tissue-specific patterns.

Genetic variation was observed at the GST1 locus and data from hybridisation studies supported the suggestion that this locus is polymorphic in humans. The expression of the GST2 isoenzymes was more complex and data from starch-gel electrophoresis and chromatofocusing was harder to interpret. The expression of the GST2 isoenzymes, unlike that of the GST1, was not a constant characteristic in different tissues from the same individual and a previous proposal that this locus is also polymorphic was not supported since the population data failed to achieve Hardy-Weinberg equilibrium. Since further hybridisation studies of GST2 isoenzymes demonstrated that some of the forms were interconvertable I believe that the various basic isoenzymes arise by a process of post-translational modification. The GST3 isoenzymes were less intensely investigated and the data revealed no evidence of genetic variability at this locus.

Differential expression of the three loci throughout development was studied in four tissues and compared with

the expression in corresponding adult tissues. Chromatofocusing was used to quantify the products of the three loci at different developmental ages so that the relative expressions of the loci could be determined. At least one of the loci demonstrated developmental changes in expression in each of the tissues studied. Tissues of the same embryological origin showed similar developmental expression of GST whereas tissues of different embryological origins revealed marked differences in the ontogeny of the enzyme. No specifically fetal forms of GST were demonstrated.

In some tissues GST loci were not fully expressed until about one year post-natal age and these observations are believed to provide biochemical evidence for the theory of neoteny in humans.

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INTRODUCTION

Many endogenous and exogenous substances are so hydrophobic that they would remain in the body indefinitely were it not for the activities of a wide variety of detoxicating enzymes (Hodgson & Dauterman, 1980). These enzymes have been divided into two groups; phase 1 and phase 2. The phase 1 enzymes are typified by the mixed function oxidases and much interest has been expressed concerning the possibility that variations in the structure or activities of enzymes such as arylhydrocarbon hydroxylase may have important consequences in terms of individual susceptibility to carcinogens. The phase 2 enzymes usually further modify substrates initially acted on by phase 1 enzymes (Dauterman, 1980). Examples of substrates used by phase 2 enzymes are glucuronides, sulphates, phosphates and glutathione. Addition of any of these substrates to a hydrophobic compound will result in an increase in aqueous solubility and subsequently in the ease with which it can be eliminated from the body (Matthews, 1980).

Conjugation with glutathione is a reaction catalysed by the glutathione S-transferases (GST) and recent work demonstrating that this group of enzymes exhibits both genetic and non-genetic variation has raised the suggestion that these enzymes may be important in the detoxication of carcinogens and other harmful substances.

This thesis initially describes studies concerning the extent of variation of the GST in human tissues.

Subsequent studies investigate the development of the different GST isoenzymes and describe the expression of different GST loci during and after gestation. An attempt has been made to quantify the expression and also to determine the origins of some of the forms found in human tissues.

1.1. THE GLUTATHIONE S-TRANSFERASES.

1.1.1. Reactions Catalysed by GST.

The basic reactions catalysed by this group of enzymes involves the nucleophilic attack of an electrophilic substrate. The sulphur atom of the reduced glutathione (GSH) provides the electrons for this attack. The electrophile can have a varied structure but so far no well-documented alternative to glutathione has been described. The glutathione conjugates formed by the reaction may be excreted without further modification or they may provide the first step in a reaction leading to mercapturic acid.

There are many authoritative reviews on this aspect of the GST (Smith et al., 1977; Chasseaud, 1979; Jakoby and Habig, 1980) and since this thesis has involved the use of only one of the many substrates available (Fig 1a), this area will not be developed further except to give some examples of reactions catalysed by this enzyme (Fig 1b).

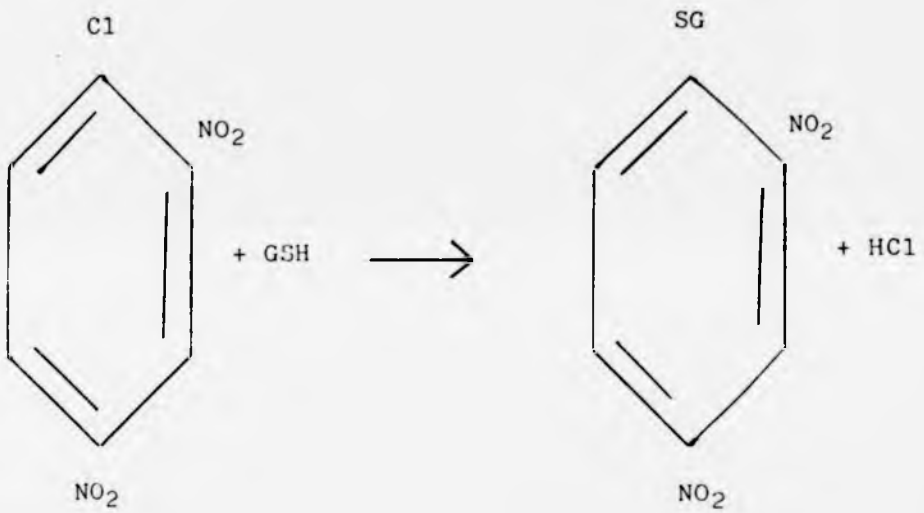


Figure 1 (a).

Figure 1(a) Conjugation (addition-elimination) reaction of 1-chloro-2,4-dinitrobenzene with reduced glutathione. In the assay the non-enzymic (blank) reaction was subtracted from the total rate to give the enzyme-catalysed rate. The conjugate produced absorbs at 340nm.

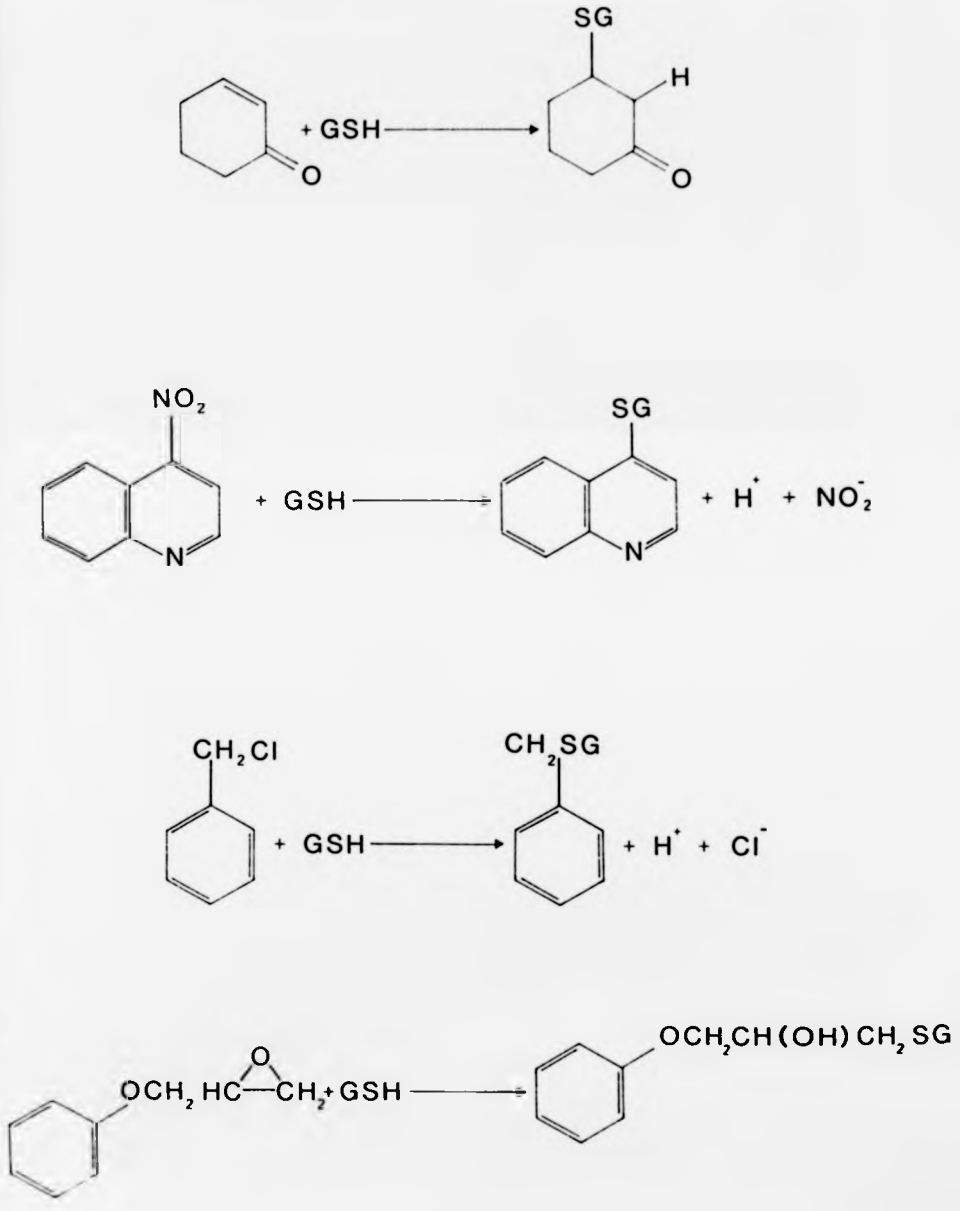

SG
H
+ GSH
O
O
NO2
SG
+ GSH
+ H+ + NO2-
N
N
CH2Cl
CH2SG
+ GSH
+ H+ + Cl-
O
OCH2HC—CH2 + GSH
OCH2CH(OH)CH2SG

Figure 1 (b)

Figure 1 (b) Examples of reactions catalysed by GST.

1.1.2. Distribution of GST.

GST have been identified in all the cell types obtained from many different life forms and it would appear therefore that the enzyme is essential for life. The range of cells studied is impressive and examples from all three kingdoms have been shown to have GST activity. In the Protista Kingdom GST has been identified in the Protozoa, Trypanosoma cruzi (Yawetz and Agosin, 1981) and Euglena sp. (Faulder, Cotton and Strange, unpublished data). GST has also been detected in all tissues of the plant kingdom so far studied; however, only the Angiospermae have been seriously investigated with reports of GST in pea-seedlings, pisum sativum (Diesperger and Sandermann, 1979), Corn, Zea mays (Frear and Swanson, 1970), the rubber tree, Hevea brasiliensis (Balaskaran and Munrandy, 1984). The GST of the animal kingdom have received much more attention with representative animals from many phyla having been studied. GST has been identified in all the following examples (the list is not exhaustive); the Coelenterata, sea anemones, Actinia equina (Cotton, Faulder and Strange, unpublished data); Annelida, earthworms, Lumbricus rubellus (Stenerson et al., 1979; Stenerson and Oien, 1981); Arthropoda, Waxmoth larvae, Galleria mellonella (Clark et al., 1977) and houseflies, Musca domestica,(Motoyama and Dauterman, 1977, 1978, 1979),

Mollusca, snails, Helix sp. (Clark and Smith, 1975; reference to unpublished work by Balaskaran and Smith); Echinodermata, Starfish,Asterias sp. (Faulder, Cotton and Strange, unpublished data); Chordata, rainbow trout, Salmo gairdnerii (Ramage and Nimmo, 1983); Aves, domestic fowl (Yeung and Gidari 1980), Mammalia, cow (Saneto et al., 1980), sheep (Clark et al., 1973), rabbit (Boobis et al., 1979), hamster (Smith et al., 1980), guinea pig (Irwin et al., 1980) and mouse (Ohkawa et al., 1972).

The GST isoenzymes identified all appear to be dimeric and to have molecular weights between 45,000 and 50,000 daltons. The various discrepancies over molecular weight assignments to GST have been ascribed by Motoyama and Dauterman (1979) to variations in buffers and stabilizers used in the gel-filtration techniques in the determination of GST molecular weights. They conducted a carefully controlled series of experiments in which insect and mammalian GST molecular weights were compared. They found no intrinsic difference between the GST from these two widely different sources and concluded that there was very little difference between the molecular weights of GST irrespective of source.

So far there are few detailed structural studies of the GST isoenzymes and there is insufficient amino acid sequence data available to allow comparison of the enzymes from different stages of evolution.

The GST are an interesting group of enzymes to study

for several reasons. Apart from the inherent scientific interest, studies in mammals have shown that the enzyme composition of many tissues is complex because of multiple allelism, multiple loci and post-synthetic modification. These enzymes therefore offer another system that would be suitable for studying the complex factors that regulate isoenzyme expression. The GST enzymes in agriculturally destructive animals are of considerable commercial interest. In this regard these enzymes have attracted the attention of various groups. For example, the failure of some chemical agents to erradicate pests has led a number of investigators to determine whether GST is responsible for detoxicating anthelminthic drugs, antimicrobial drugs, herbicides and insecticides in resistant organisms. Kawalek et al. (1984) demonstrated that GST activity in the sheep tapeworm, Haemonchus sp., was significantly higher in drug-resistant strains than in susceptible strains. GST activity in insects resistant and susceptible to insecticides has also been determined by Motoyama and Dauterman (1977; 1978).Stenersen et al. (1979) have studied GST activity in earthworms in an attempt to understand how these environmentally important animals withstand constant exposure to pesticides and pollutants. These studies were supported by agricultural, forestry and environmental agencies.

Properties of GST have also been investigated in commercially important plants such as the rubber tree,

Heva brasiliensis (Balaskaran and Munrandy, 1984). A significant increase in GST activity was found in corn tolerant to the potent herbicide, substituted s-triazines compared with susceptible plants (Frear and Swanson, 1970).

Another area of interest concerns their putative role in the detoxication of xenobiotics including carcinogens as well as the possibility that the glutathione peroxidase activity demonstrated by some GST isoenzymes (Warholm et al., 1981; Awasthi and Dao, 1981) may be important in the neonatal period.

1.1.3. Nomenclature of GST.

The ubiquitous occurrence of the GST together with the wide range of electrophiles used in the conjugation reaction has resulted in a confused nomenclature particularly in some early publications. Initially the enzymes were described by their assumed substrate specificities as glutathione epoxide transferase, glutathione alkene transferase and many others. This type of nomenclature has largely been discarded but as yet no universally accepted alternative has been established. In mammals the need for a standard system is pressing because in experimental animals such as the rat, many different isoenzymes appear to be present in liver (Jakoby et al. 1984). Since the origins of many of these forms is unknown any reference system must be arbitrary. However,

in an attempt to overcome this problem Mannervik et al. (1985) have developed a classification scheme that relates various cytosolic GST from different mammalian species including humans. Three classes of GST isoenzymes have been identified and these provide divisions into which GST from different mammalian species are incorporated according to shared characteristics such as isoelectric point, substrate specificity, sensitivity to inhibitors and terminal amino acid sequences.

Bass et al. (1977) first described three monomer proteins termed Ya (molecular weight approximately 22,000 daltons), Yb (molecular weight approximately 23,500 daltons) and Yc (molecular weight approximately 25,000 daltons) that comprised the GST in rat liver cytosol. Following this report enzymes were referred to as combinations of Ya, Yb or Yc. However, subsequent reports showing many more than three monomers has led to a confused literature since some enzymes originally believed to be composed of single monomers are now known to be mixtures of enzymes composed of several monomers. In an attempt to establish a universally acceptable system, Jakoby et al. (1984) suggested that each enzyme should be identified by the organism from which it is isolated but not by the tissue from which it is obtained. Since each isoenzyme differs in its subunit composition each subunit received its own number. Identification therefore, of the dimeric enzymes, was based on the numbers given to the two constituent subunits.

Whilst this system has been used by some investigators to classify rat GST, the system has not been used to describe the isoenzymes from other species.

As with other animal GST, the human GST were originally identified by their substrate specificity. As soon as it became clear that some of these had overlapping substrate specificities dichotomous systems of nomenclature developed. Some groups used Greek letters to identify the various isoenzymes, whereas others used a system first suggested by Board (1981a) based on a genetic model; this related the GST isoenzymes to products of a particular locus. Fortunately, as these diverging systems of nomenclature developed, a unifying scheme emerged that conveniently grouped the various isoenzymes according to their isoelectric points. Hence the human GST can be described as basic GST isoenzymes to include α, β, γ, δ and ϵ of one system or GST2 of the alternative system; near-neutral GST isoenzymes to include μ of one system or the GST1 of the other and finally the acidic GST isoenzymes to include ρ, π, ψ and ω of one system or GST3 of the Board (1981a) system (Table 1).

The system of nomenclature proposed by Board (1981a) has been used in this thesis. The main reason is that it is more convenient to discuss some of the human GST isoenzymes in terms of a genetical model. However, because some of the work in this thesis is closely involved with the results of other groups who have used the Greek letter

Nomenclature of the human GST.

Genetic Model	Isoelectric Point	Greek Letters
GST1	Near-neutral	μ
GST1 0 GST1 1 GST1 2		
GST2	Basic	α β γ δ ε
GST3	Acidic	ρ π ψ ω

Table 1.

system, the unifying simple nomenclature of basic, near-neutral and acidic has also been used in an attempt to clarify some points of discussion.

1.1.4. Anion Binding by GST.

A further area of interest concerning the GST is the well-described ability of various enzymes from different sources to bind hydrophobic ligands. Although a good deal of the initial enthusiasm concerning a suggested role for these enzymes in intracellular transport has now waned , this area provided a major impetus for the study of the enzymes and although it is not of primary interest in this thesis, some of the salient work will be briefly reviewed.

In 1964, Brown et al. showed that the bulk of intravenously administered tritiated bilirubin was found in either liver or bile of anaesthetised rats. The impressive features of this study were the speed and efficiency with which the liver was able to remove the radioactive ligand. Subsequent studies have shown that many low molecular weight anions are also removed with great speed and that first-pass clearance often exceeds 90%. Arias and his colleagues investigated the interaction of ligands such as bilirubin with cytosol in a series of studies that resulted in the hypothesis that a soluble protein present in the liver cytosol of many animals was central to efficient transhepatic transport of anions (Levi et al.,

1969a; Levi et al., 1969b; Levi et al., 1970; Levine et al., 1971; Arias et al., 1980). In their initial studies cytosol treated in vitro with radioactive ligand was eluted from columns of Sephadex G-75. Ligand was found to be bound by species of large molecular weight (100,000 daltons; termed X protein), a protein of intermediate molecular weight (45,000 daltons, termed Y protein) and a low molecular weight protein (12,000 daltons, termed Z protein). Little interest was expressed in the nature of the X protein and this has generally been considered to reflect non-specific binding of ligand by lipoproteins. Y and Z proteins however, have been extensively studied and in a series of papers Arias and his colleagues provided further evidence that Y protein was particularly important in intracellular transport.

Two interesting studies were concerned with the efficiency with which a model ligand, bromosulphthalein, was taken across the liver in the presence of Y protein. The protein was detected by the ability of the cytosol to bind bromosulphthalein. Thus it was shown that in a series of animals from the principal vertebrate classes, the appearance of Y protein in liver coincided both with transition from water to land-living habitats and with the development of efficient clearance of bromosulphthalein from plasma.

Some interesting phylogenic studies of the efficiency of hepatic uptake of the model ligand bromosulphthalein

have been reported. As a result of work carried out into the role of organic-anion binding proteins in marine elasmobranchs, Levine et al. (1971) concluded that Y protein first appeared when animal life evolved from water to land. Further studies by Boyer et al. (1975) also found that marine elasmobranchs lacked Y protein. Following these reports Sugiyama et al. (1981) found that GST activity was associated with hepatic anion binding ability but that the ligand specificities and affinities were different in marine elasmobranchs to those of rat GST. The claim that the appearance of ligandin coincided with the transition of animals from water to land first suggested by Levine et al. (1971) was therefore refuted by this latest evidence.

In another interesting study it was shown that neonatal monkeys are deficient in Y protein and that the subsequent increase in hepatic Y protein concentrations coincides with the development of efficient clearance mechanisms for bromosulphthalein from plasma. Whilst these interesting studies support the hypothesis that Y protein is involved in intracellular transport, they do of course provide only circumstantial evidence. An examination of these studies allows a number of alternative conclusions. A criticism of the studies by Levine et al. (1971) and Boyer et al. (1975) is that many of the water living animals have little plasma albumin and intravenous administration of hydrophobic ligands would therefore be

expected to result in their wide dispersion. Furthermore, the detection system used to identify Y protein is at best crude. Perhaps the most surprising and difficult finding to accommodate is the report by Kaplowitz et al. (1973) that Y protein was one of the GST found in rat liver. Subsequent investigators have found that many of these enzymes have ligand binding activities and that Y protein, also referred to as ligandin, is therefore not a single protein.

1.2. GST AND CANCER SUSCEPTIBILITY.

Early studies of the reactions catalysed by the GST revealed their ability to metabolise potentially dangerous hydrophobic electrophiles and led to the suggestion that these enzymes are important in the metabolism of carcinogens. The subsequent finding that the isoenzyme composition of many animal tissues is complex and in some cases reflects genetical variation led to the interesting speculation that differences in the level of GST activity or amino acid sequence of particular GST could have important implications in the determination of individual susceptibility to carcinogens. Smith et al. (1977) were perhaps the first to present a unified hypothesis that related the ability of some GST to bind xenobiotics either covalently or non-covalently with their catalytic activity and show that for some carcinogens the GST could be critical in detoxication.

The suggestion that cancer susceptibility could be quantified by assaying a particular key detoxicating enzyme is neither new nor restricted to the GST (Nebert, 1980). Perhaps the best studied system is that of arylhydrocarbon hydroxylase in lymphocytes. Kellerman et al. (1973) suggested that Caucasian Americans could be divided into three groups on the basis of whether cultured lymphocyte activity of this enzyme was of low inducibility or high inducibility. Thus, whereas only a small minority of healthy subjects demonstrated high inducibility, 30% of patients with bronchogenic carcinoma were of this type. Similarly, whilst 45% of healthy subjects demonstrated low inducibility, only 4% of patients with carcinoma were low inducers. Although subsequent work has questioned these data and the hypothesis that susceptibility to bronchogenic carcinoma can be determined from the inducibility of this enzyme, the fundamental premise that between-individual differences in enzyme activity or structure are key determinants of cancer susceptibility has remained an important area of study. There is evidence supporting the suggestion that the GST are important in this context although at present much of it is circumstantial. A few of the studies that are relevant are cited below although they represent only a small part of the available literature.

The incorporation of benzo(a) pyrene metabolites into isolated rat hepatocyte nuclei has been found to

be inhibited by the presence of GST and GSH (Hesse et al., 1980). In a further report, Hesse et al. (1982) suggested that in rat hepatocytes both GST and GSH are important agents in the protection of DNA from electrophilic attack by benzo(a)pyrene metabolites. Studies carried out by Sparnins et al. (1982a,b) showing that orally administered agents that increase tissue GST activity also protect against the effects of carcinogens, add support to the suggestion that the GST have a role in the metabolism of carcinogens in rodents.

1.2.1. GST and Mutagenicity.

In experiments designed to study the role of mammalian GST in the reduction of mutagenicity in microorganisms, Glatt and Oesch (1977) concluded that GST in mouse cytosol was responsible for decreasing the mutagenic effects of benzo(a)pyrene metabolites on Salmonella typhimurium. The observation by Mukhtar et al. (1978) that rat ovarian and testicular germ cell GST had twice the specific activity of interstitial cell GST was suggested as support for the proposal that GST prevents the mutagenic effects of xenobiotics. Additionally, protection appeared to be afforded from an early age since high GST activities were found in neonatal ovaries.

1.3. CAUSES OF PROTEIN DIVERSITY.

Much of the work to be described in this thesis is

concerned with the identification of the various GST isoenzymes found in human tissues. Studies on many enzymes have shown that multiple molecular forms can arise by different mechanisms. Detailed reviews of this field are given by Harris (1980) and Moss (1979; 1982) in their authoritative texts on isoenzymes. Since some of the basic principles are relevant to the experimental work described in this thesis they will be listed and briefly discussed below.

Multiple molecular forms of proteins can arise by one of at least three mechanisms; multiple allelism at a single gene locus, multiple gene loci and post-synthetic modification. Much of the earliest work on protein variation arose because of the intense clinical interest in sickle cell disease. The finding in 1949 (Neel, 1949) that individuals with the sickle cell trait and with sickle cell anaemia were respectively heterozygous and homozygous for a gene located on an autosomal chromosome together with the findings of Pauling (1949) that the haemoglobin found in erythrocytes of subjects with sickle cell anaemia is different from the protein of normal red cells was perhaps the starting point for the numerous studies that are now beginning to quantify the extent of genetic and non-genetic variation in humans.

1.3.1. Multiple Allelism.

The haemoglobin system remains the best understood

example of protein variation particularly with regard to the clinical consequences of this phenomenon and of the many unstable haemoglobins that have been identified, in most cases the nature of the mutation is known. The recognition that various forms of a protein could be found in naturally occurring populations led to the development of electrophoretic techniques that would allow the screening of large numbers of subjects (Smithies, 1955). Using this approach it has been possible to detect the presence of more than one allele at many loci and it is now known that there are many examples of enzymes that demonstrate allelic variance (Harris et al., 1968). It has also been possible to identify the hybrid enzymes that occur in heterozygotes.

An example of this phenomenon is given by the dimeric enzyme peptidase A (Lewis and Harris, 1967; Lewis et al., 1968; Lewis, 1973). In early studies in which erythrocytes from large numbers of individuals were screened, it was found that three electrophoretic patterns could be identified. In the first case a single cathodally migrating band of relatively slow mobility was seen (PEP A1). In the second case a single band of faster mobility was identified (PEP A2) and in the third case a pattern of a fast and slow band as well as one intermediate between these two: (PEP A1-2). Family studies have shown that these three electro-

phoretic patterns arise from the presence of two autosomal alleles PEP A^1 and PEP A^2 so that patterns PEP A1 and PEP A2 represent the homozygotes and PEP A2-1 the heterozygote.

Generally any enzyme that contains more than one monomer and that demonstrates multiple allelism would be expected to demonstrate hybrid enzymes in heterozygotes. The simplest case is that of a dimeric enzyme coded by a locus at which only two alleles occur. Where an enzyme is, for example, a tetramer comprising either of α or β monomers, the number of potential isoenzymes becomes larger; α_4, $\alpha_3\beta$, $\alpha_2\beta_2$ $\alpha\beta_3$ and β_4. An example is the tetrameric enzyme lactate dehydrogenase (Markert, 1968).

The relative intensity of these isoenzymes in a sample from a heterozygote such as those shown in figure 2 will depend on the rate of synthesis of the monomers and on the relative ease with which the two monomers are able to combine. In the simplest case the two polypeptides would be synthesised at the same rate and $\alpha\alpha$ combinations would be as readily formed as $\alpha\beta$ and $\beta\beta$ combinations. In such a case the relative activities of the three isoenzymes separated by electrophoresis or chromatography would be in the ratio 1:2:1. In the case of a tetrameric enzyme the ratio of activities of the five isoenzymes would be 1:4:6:4:1. Many examples of dimeric enzymes in which a 1:2:1 pattern occurs have been

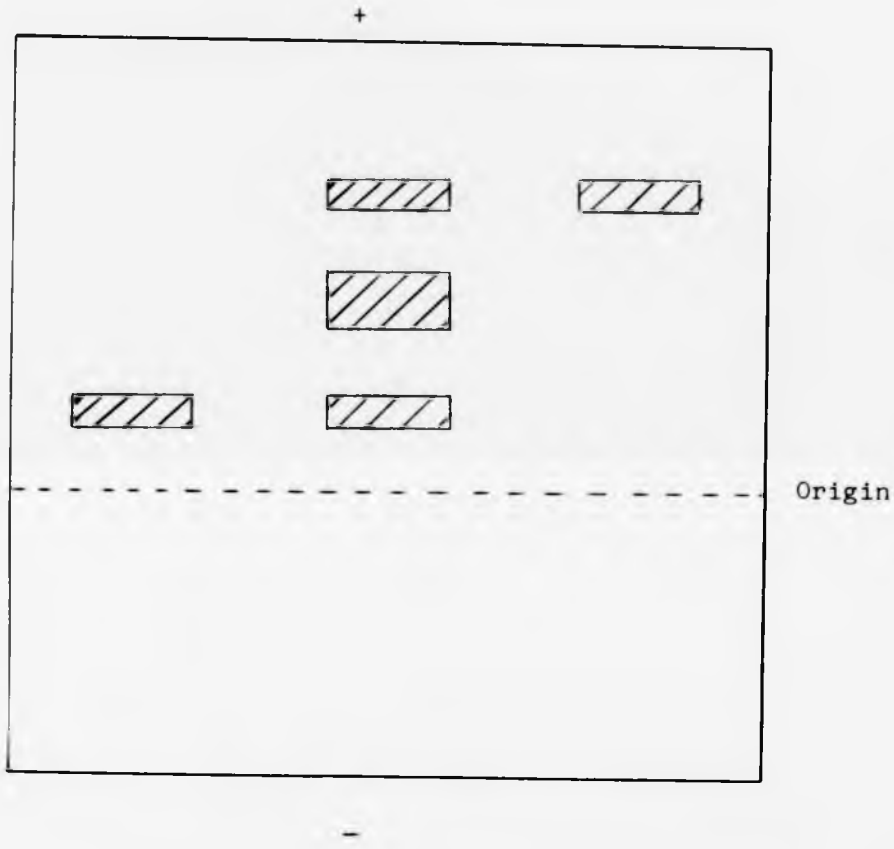


Figure 2.

Figure 2. Diagrammatic representation of the mobilities of GST1 isoenzyme.

(i) GST1 2 Homozygote

(ii) GST1 2-1 Heterozygote

(iii) GST1 1 Homozygote

described and such a pattern will be presented for one set of GST isoenzymes. However, examples of asymmetric patterns have also been reported and are interpreted as indicating that one of the polypeptides contributes less to total activity that the other (Fritz et al., 1971). The reason for this difference in contribution could be a result of differences in synthesis rate, degradation rate or stability.

1.3.2. Multiple Gene Loci.

Further complexity in the isoenzyme patterns of tissues will result when two or more separate gene loci are involved in the synthesis of the protein set. Again the best studied example of this phenomenon is that of haemoglobin. Normally, an adult synthesises haemoglobin A whereas the fetus synthesises haemoglobin F. A further molecular form of haemoglobin, haemoglobin A_2 is also found in small amounts in healthy adults. The composition of these three haemoglobin molecules is:

Hb A : $\alpha_2 \beta_2$

Hb A_2 : $\alpha_2 \delta_2$

Hb F : $\alpha_2 \gamma_2$

The α, β, γ and δ proteins differ in their amino acid sequences and must therefore be determined at separate gene loci. At least four genes will be involved in the determination of the structure of the three types

of haemoglobin. Interestingly, in the context of this thesis, the haemoglobin genes demonstrate different developmental patterns. Thus, in the newborn human about 70 - 80% of the haemoglobin is haemoglobin F. Progressively after birth haemoglobin A contributes increasingly more, so that at six to twelve months after birth haemoglobin F is present in only trace amounts (Wood and Weatherall, 1983).

The ontogeny of haemoglobin is complicated by the presence of at least two other haemoglobin chains during early embryonic and fetal life; ε and ζ are also synthesised but their structure is much less well-defined than the other monomers.

There are other well documented examples of proteins resulting from more than one gene locus; lactate dehydrogenase is perhaps one of the best studied. In this case three separate gene loci are responsible for the various isoenzyme patterns seen.

It is worth emphasising that apart from the variation resulting from multiple allelism at a particular locus or from the presence of more than one locus, a further level of variation can result when both these phenomena occur. Thus, apart from interallelic hybrids, interlocus hybrids can often also be identified.

1.3.3. Post-Synthetic Modifications.

Multiple allelism and multiple loci provide the

genetic framework for the formation of multiple molecular forms. Further variation can also arise when synthesised proteins are reacted with other cellular constituents and modified. Many examples of such secondary isoenzymes have been demonstrated and this phenomenon would appear to be quite common. Usually the modifications are simple examples of chemical modifications. For example, erythrocyte enzymes containing a sulphydryl group may be reacted with oxidised glutathione to form a mixed disulphide. Such a reaction has been documented in the case of adenosine deaminase. A further example is the deamidation of glutamine and asparagine residues. Usually the reactions that give rise to secondary isoenzymes result in products that are more negatively charged than their parents and they can therefore be identified by their anodal mobility in electrophoretic systems or their lower isoelectric points.

Whilst the extent of modification to the protein is generally small and often reversible, it is sometimes difficult to determine which modification has occurred.

1.3.4. Other Possible Causes of Protein Variation.

It has been suggested that in some cases isoenzymes with the same primary amino acid sequence can differ in their three dimensional shape giving rise to two or more stable configurations. These are referred to as conformational isomers.

The difficulty in obtaining suitable tissue can often lead to the need to accept old specimens or ones that have been kept in less than ideal circumstances. Obtaining fresh human tissue is difficult in some cases. It is therefore necessary to be alert for the possibility that the enzyme variation identified is a result of *in vitro* storage artefacts or aggregation of the enzyme molecules. Warholm *et al*. (1980) refer to a useful guide (von Bahr *et al*., 1980) to determine whether post-mortem changes to GST were likely to have occurred. Analysis of various microsomal enzymes is used to determine whether degradation or inactivation has occurred. These data give an assessment of the likelihood of post-mortem changes in cytosolic enzymes such as GST.

The term 'isoenzyme' has been used in this thesis to refer to members of an enzyme set. The term was first introduced by Markert and Møller (1959) and is generally used without any implication of the relationship between different protein species.

1.3.5. Estimates of Protein Diversity.

It is recognised that even the simplest life forms can synthesise a large number of enzymes and other proteins. Mutations in the structural genes that code for these proteins can result in different alleles being present at a given locus. Since the different alleles may give rise to a structurally altered protein, natural

populations frequently demonstrate a variety of forms of a particular protein. These forms may differ by only one amino acid in the total sequence but frequently this small alteration is sufficient to allow differentiation of the various forms. Once this concept had become established, many investigators began to formally estimate the extent of variation in different populations and it is now clear that all life forms studied so far demonstrate extensive genetic diversity.

1.3.6. Estimation of the Number of Alleles.

Any estimate of the extent of genetic variation requires knowledge of the number of alleles at a given locus. Theoretically the number of structural alleles that could be generated at any locus by mutation is very large. Harris (1979) has calculated that for a typical protein containing 300 amino acids and a corresponding DNA sequence of 900 bases, as many as 2,700 different alleles could be generated. This takes account of substituting one base for one of the three others and therefore does not include other recognised mutational events such as small deletions, duplications and frame shifts.

Haemoglobin has been intensively investigated and a very large number of variants identified. The α-chain of this protein contains 146 amino acids and the DNA sequence must therefore contain 438 bases. Consequently,

mutations resulting in a single base change could generate 1314 different alleles. This number of alleles would not be expected since the genetic code is degenerate and about 23% of mutations would result in no change in the α-chain. Further, some mutations (about 4%) will result in chain termination. It would be expected therefore that about 70% of the expected number of alleles (958) could be identified in human populations.

The most convenient method for screening populations is electrophoresis. The various media usually offer good separation of proteins and the techniques are usually simple and reliable. However, since electrophoresis relies on charge differences between proteins, only variants that result from a mutation that leads to a change in charge of the original protein will be detected. Harris (1979) has estimated that about two thirds of mutations would not be detectable if only an electrophoretic approach was used. Taking into account all of the difficulties in estimating the number of possible allelic variants Harris (1979) has estimated that 287 variants should be detected and his survey of the literature resulted in 61 (21.3%) of these as having already been identified. This figure is surprisingly high since many of the already identified variants are extremely rare and the percentage of the human population that has been screened is extremely small.

It seems, therefore, reasonable to assume that a very high proportion of the possible mutations occur somewhere in the human population.

Many other proteins have been studied and, for example, more than 80 variants of glucose-6-phosphate dehydrogenase have been identified. Apart from the differences in their charges, some of the variants demonstrate altered catalytic properties, thermostabilites and pH optima.

1.3.7. Polymorphism.

It is well established that substantial allelic variation occurs in natural populations. In some cases the products of one allele are usually present and that protein can be regarded as the normal form for that particular population. In other cases, various forms are common in the population and none of the forms could be regarded as standard.

A further consideration concerns how many of the numerous gene loci in a particular life form demonstrate allelic diversity.

The concept of polymorphism is central to these considerations. The polymorphic locus has been defined by Harris (1980) as one in which the most commonly occurring allele in the population has a frequency that is less than 0.99. Thus at least 2% of the individuals will be heterozygous at that locus. In many populations

and for many proteins, the proportion of heterozygotes will be much greater. Harris and Hopkinson (1972) estimated the extent of polymorphism at a variety of loci coding for enzymes in humans. They found that about 30% of the loci were polymorphic. As has already been stated this estimate may be too low since electrophoresis, the technique used in the survey, can only detect charge differences.

Studies have also been described that allow comparison of the extent of genetic diversity in different life forms. Such studies often compare the average heterozygosity per locus. This refers to the proportion of gene loci in an individual at which different alleles occur.

$$\text{Average Heterozygosity} = \frac{\text{Total number of Heterozygotes per locus}}{\text{Total number of individuals}}$$

In general the average heterozygosity per locus is much greater in invertebrates than in vertebrates. This reflects the greater proportion of polymorphic loci and the greater number of common alleles per locus in invertebrates.

An important consequence of the realisation of the extent of genetic variation in humans and other animals is the concept of the uniqueness of the individual. It now seems clear that if sufficient loci are sampled

and consequently a large number of polymorphisms identified, any individual could be identified as being unique in his enzyme and protein make-up. Furthermore, the concept of individual susceptibility to disease, already commented on in the context of cancer is of importance although at present data are extremely limited.

1.3.8. Rare Alleles.

Apart from the genetical variants covered by the term polymorphism, many loci also demonstrate rare alleles. Polymorphism can usually be demonstrated in a relatively small population sample of between 50 - 100 individuals. Rare alleles however have extremely low incidences and many thousands of subjects need to be studied before they can be detected. For example, phosphoglucomutase demonstrates three common phenotypes that result from two common alleles (frequency 0.76 and 0.24). Harris et al. (1974) screened 10,333 Europeans (20,666 alleles) and detected five different rare alleles. The individual frequencies of these alleles were less than 0.0002. Other enzymes also demonstrate rare alleles and it would appear that the incidence of these is about the same in loci that are polymorphic as loci that are not polymorphic.

1.4. METHODS USED TO DETECT PROTEIN VARIATION.

Much of the protein diversity already identified has been demonstrated by electrophoresis. The initial impetus for this work came from the development of the zymogram technique using starch-gel electrophoresis (Smithies, 1955). Use of this medium is extensive and Harris and Hopkinson in their manual (Harris and Hopkinson, 1976) list methods for many enzymes. The advantages of starch are cheapness and relative simplicity in the preparation and staining of gels. The extent of resolution of proteins is generally good and although some variants will be missed, the technique offers a practical way of assessing the extent of variation in an enzyme (or protein) not previously investigated in a population.

More recently other electrophoretic techniques have been introduced and the use of starch-gels has somewhat declined. Gel iso-electricfocusing offers a particularly sensitive method since narrow pH ranges can be applied to obtain maximum resolution. Gel-affinity chromatography (Swallow, 1977) has also been advocated and recently Whitney *et al*. (1985) have used immobilised gradient iso-electricfocusing as a means of detecting neutral amino acid substitutions in proteins.

The choice of an electrophoretic medium can often

be made by subjective reasoning since on occasions unexpected resolution of proteins can be obtained using one medium but not a similar alternative. The initial survey of GST described in this thesis was made using starch-gels. This medium was chosen for the reasons given above as well as the then recent publication by Board (1981a) describing a zymogram method for separating GST in human liver.

Since electrophoretic methods are unlikely to result in the identification of more than one third of the variation present, exhaustive surveys demand that techniques that study other aspects of protein structure be used. A simple approach would be to alter the electrophoretic conditions and using this approach Singh et al. (1976) were able to distinguish 37 alleles for xanthine dehydrogenase in Drosophilia sp.rather than the previously identified six alleles.

Other investigators have used heat denaturation to resolve isoenzymes. Perhaps the best studied example using this method is that of alkaline phosphatase. Since separation of the bone and liver isoenzymes of alkaline phosphatase is difficult using electrophoresis, differential heat stability of these two isoenzymes can be used to good effect (Moss and King, 1962).

A further well recognised example of protein variation that is not easily detected by electrophoresis is that of cholinesterase in human serum. The usual

enzyme is particularly sensitive to the inhibitor dibucaine whereas one of the atypical forms that causes scoline sensitivity is relatively insensitive. Affected individuals therefore can be detected by studying the extent of inhibition by dibucaine (Kalow and Genest, 1957). A further genetic variation in cholinesterase can be detected by using fluoride and it is usual to study individuals who are sensitive to suxamethonium or their families by using both dibucaine and fluoride.

1.4.1. More Recent Assessments of the Extent of Genetic Variation.

The study of isoenzymes has, in recent years, benefitted from advances in the technology of protein separation as well as in the ability to handle DNA and some re-thinking has been necessary. In 1975, O'Farrell published details of his two dimensional electrophoretic method. This approach gives different information to the traditional one dimensional zymogram techniques used to assess protein variation since proteins are first denatured. It is usual therefore for protein detection to be performed using a non-specific protein stain or autoradiography. Samples are run in the first direction using isoelectricfocusing and in the second direction by SDS gel electrophoresis. The technique allows the protein variation in whole cells to be assessed and the data obtained are

generally extremely complex. Several groups have used two dimensional electrophoresis to study protein variation in fibroblasts and surprisingly estimates of the average heterozygosity were found to be very much lower (about 1%) than previous estimates that were obtained for cytosolic enzymes. This result could be a consequence of less genetic variation in proteins that are present in cells in high concentrations. This is the group of proteins most easily detected by two dimensional electrophoresis. This hypothesis seems untenable since haemoglobin, as well as other abundant proteins, exhibit well-documented genetical variation. A further possibility is that the two dimensional approach is less able to detect variation. This also seems untenable and indeed surveys comparing genetic variation of enzymes in HeLa cell extracts (Milman et al., 1976) and polypeptides in brain (Comings, 1979) have shown that this technique is sufficiently sensitive to detect all previously recognised polymorphisms. Edwards and Hopkinson (1980) have commented on these data and suggested that since one dimensional and two dimensional approaches sample proteins that differ in their subcellular origin, the degree of genetic variation amongst structural proteins may be very much lower than amongst soluble proteins. This conclusion is in keeping with previous work from this group that multimeric proteins that require

protein-protein interaction between their constituent monomers exhibit less genetical variation than do monomeric proteins. The argument is that since protein-protein interactions depend on particular amino acid sequences, mutations leading to substitutions in these domains, will generally be unacceptable because they will prevent association. Thus in a dimeric enzyme, a part of the protein is not available for genetical variation and subsequently the expected level of polymorphism is not achieved. Harris et al. (1977) confirmed this hypothesis by comparing monomeric enzymes with those that demonstrated various levels of quaternary structure. A similar limitation in the freedom of structural proteins to undergo amino acid substitution might also be expected since they are involved in vital protein-protein interactions as well as associations with lipid and carbohydrate moieties in membranes. It seems likely therefore that the current high level of genetical variation is in part a reflection of the protein population sampled and that as other parts of the cell are investigated lower levels of variation will be identified.

A further consideration is that almost all estimates of genetical variation have been made by studying proteins. It is well recognised that some mutations are almost impossible to detect because the overall change to the protein is so small. However,

it now seems clear from studies of DNA that the extent of polymorphism is considerably greater than that identified by protein studies even if one makes allowance for the occult changes in protein structure. Ayala (1983) has argued that whilst a typical sexually reproducing organism may be found to be heterozygous at 20% of its loci by studying proteins, at the level of the DNA nucleotide sequence, every individual is heterozygous at every locus if variation in introns as well as exons is taken into account. This suggestion is important in the current debate concerning the evolutionary significance of protein variation. Since this complex subject is not relevant to this thesis it will not be further discussed.

1.5. VARIATION IN THE GST.

As already discussed the GST in many cells present a complex mixture. In humans, Kamisaka *et al.* (1975) first resolved the basic isoenzymes present in liver into five isoenzymes using CM-cellulose chromatography. These isoenzymes were named in increasing order of isoelectric point value as α, β, γ, δ and ϵ. Each was purified to homogeneity and the amino acid composition of each determined. Further, antiserum was prepared to human ligandin and tested against each of these preparations. The results of these studies were interpreted as the five separated isoenzymes being charge

isomers of a parent protein that had arisen by a process of mixed disulphide formation or by deamidation of glutamine or asparagine residues. At this time near-neutral or acidic isoenzymes were not identified. Subsequently Marcus et al. (1978) showed that human erythrocytes contained an acidic isoenzyme termed ρ that was structurally different to the α - ε set. Further investigations by Warholm et al. (1980) resulted in the identification of another GST in humans. A near-neutral form termed μ was found in human liver although interestingly it was detected in only 60% of the small number of subjects studied. Other isoenzymes have also been identified in human tissues by Laisney et al. (1984) and Vander Jagt et al. (1985). It was Mannervik (Mannervik, 1985; Mannervik et al. 1985) who proposed a useful and simple classification for the human enzymes based on isoelectric points. Thus the human GST isoenzymes are described as basic, near-neutral or acidic and it would appear that the isoenzymes in the mouse and rat can be similarly classified (Mannervik et al., 1985). This system suggests the possibility of earlier gene duplication that has given rise to three loci that code for the different isoenzyme sets and further studies to investigate the phylogeny of these enzymes would be interesting.

As reports of the different isoenzymes of GST in human tissues were being published, Board (1981a)

presented a genetical model for the isoenzymes in human liver. He suggested that most of the activity in this tissue results from the presence of the products of two autosomal gene loci termed GST1 and GST2. GST2 codes for the positively charged enzymes that migrate towards the cathode. Between sample variation in this isoenzyme set was identified and since the starch-gel patterns appeared to be similar to those expected for two homozygote and one heterozygote phenotypes he proposed that the GST2 isoenzymes were coded by a locus that could contain two alleles. The work described in this thesis has reinvestigated the possibility that the GST2 locus is polymorphic and as described below a different interpretation is made to that proposed by Board. Study of the GST2 isoenzyme set is further complicated by the recent report by Stockman et al. (1985) that more than one monomer is to be found in the basic isoenzyme set. These data however are not easy to fit into a genetic model and will be discussed in detail below.

Another locus in human liver, GST1 codes for the enzymes with intermediate anodal mobility and this isoenzyme set is believed to correspond to the μ isoenzyme described by Warholm et al. (1983). The incidence of GST1 isoenzymes in various racial groups approximates to that expected for the μ isoenzyme. However, whereas μ appears to be a single homogenous enzyme, Board described up to three isoenzymes that are apparently coded by GST1.

These data agree well with the hypothesis that three alleles (GST1*0, GST1*1 and GST1*2) can be found at the GST1 locus. Four phenotypes can be identified by starch-gel electrophoresis; these result from allele combinations, GST1*0/GST1*0 (null allele), GST1*1/GST1*1, GST1*2/GST1*2 and GST1*1/GST1*2 (heterozygote). Individuals who are heterozygous for the GST1*1 and GST1*0 or GST1*2 and GST1*0 alleles (GST1*1/GST1*0; GST1*2/GST1*0) cannot be differentiated from the corresponding homozygote subjects (GST1*1/GST1*1; GST1*2/GST1*2 respectively). Board's findings have been re-examined and extended in this thesis and the data have been found to support those of Board in that GST1 is a polymorphic locus.

A further point of interest concerning the GST1 locus is the high incidence of individuals who demonstrate no GST1 activity. About 40% of individuals so far studied appear to be null at this locus and in view of previous work suggesting that the GST are implicated in hepatic anion transport as well as the detoxication of carcinogens, it has been suggested that individuals with the GST1 0 phenotype may demonstrate impaired hepatic anion transport or be at a greater risk of developing cancer (Board, 1981b; Silberstein and Shows, 1982; Scriven et al., 1986).

Board also detected a small amount of GST activity that demonstrated a marked anodal mobility. This acidic isoenzyme, classified as a product of the GST3 locus,

appears to correspond to the ρ form originally found in erythrocytes. Studies in our laboratory have attempted to identify genetical variation at this locus but, although extensive variation in between-individual activity has been detected in erythrocytes and platelets (Strange et al., 1982, Rogerson et al., 1984) no genetical variation has so far been observed for GST3 isoenzymes. Acidic isoenzymes are present in all human tissues (Laisney et al., 1984) although there remains considerable controversy as to whether the forms found in erythrocytes, placenta, liver, skeletal muscle and other tissues are products of the same locus.

In 1981, Koskelo et al. suggested that there was a non-identity between acidic lung GST and acidic erythrocyte GST. Immunological studies by Dao et al. (1982) supported this work and additionally they found acid lung GST to be immunologically identical to acidic placental GST. In contrast, however, Mannervik and Guthenberg (1981) found that acidic placental GST was very similar, if not identical, to acidic erythrocyte GST. Conflicting data have also been reported with respect to the acidic liver and erythrocyte forms of GST. Koskelo and Valmet (1980) and later Board (1981a) found the acidic liver GST to resemble the acidic erythrocyte GST. However, also in 1981, Awasthi and Dao found all acidic liver GST to be immunologically distinct from the acidic erythrocyte GST.

In some of the studies described in this thesis it has been possible to compare enzymes from several human tissues and comment will be made on this controversy in the discussion.

1.6. ENZYME DEVELOPMENT.

Apart from their importance to geneticists, isoenzymes are also of great interest to developmental biologists. The study of gene expression during normal development can be advanced by determining the expression of the different alleles in heterozygotes within a species. Understanding the mechanism whereby genes are regulated during development has become a central issue in biology and because multi-locus enzymes are often differently expressed, analysis of such changes can help in determining how closely related genes are regulated. Multi-locus enzymes are interesting in this context because they are apparently partially functionally redundant since the different isoenzymes catalyse the same basic reaction. It seems reasonable therefore,to expect that these loci can be more finely regulated than enzymes encoded by only a single locus since an enzyme carrying out an important function and existing in only one form must always be expressed. Thus the differences that have already been documented in the expression of isoenzymes suggest that the multi-locus condition has resulted in considerable regulatory flexibility.

Some generalisations concerning gene expression during embryogenesis have been put forward by Whitt (1983):

1. Isoenzyme loci that are most tissue restricted in their expression are not usually expressed until later in development.
2. Enzyme loci that are widespread tend to be expressed continuously.

These guidelines are by no means proven and have in part resulted from studies in fish. To determine whether these generalisations are applicable to humans, the expression of the GST loci has been studied in various tissues.

Edwards and Hopkinson (1977) have also reviewed enzyme development but in their case specifically in humans. They were also able to offer some generalisations concerning enzyme ontogeny, for example:

1. There is least developmental change among enzymes determined by a single structural gene locus.
2. Changes in expression generally occur during the second half of gestation and early infancy.
3. Relatively few examples of foetal specific enzymes are found.
4. Changes in expression of loci generally occur gradually.
5. Some specialised enzymes with limited tissue distribution demonstrate a sudden increase in expression during the latter part of gestation.

6. Generally simpler isoenzyme patterns are seen in fetal material compared with expression in adults.
7. Isoenzyme patterns in the brain tend to be established early whereas those of muscle are late.
8. Acidic isoenzymes tend to be expressed early in gestation and as development progresses more basic isoenzymes appear.

An important comment made by Edwards and Hopkinson (1977) concerned the difficulty in studying humans and because of the ethical problems, enzyme development in humans has usually not been as exhaustively documented as that in experimental animals.

1.7. AIMS OF THIS THESIS.

The overall aim of this thesis was to study the extent of genetic variation in the human GST with special reference to the basic isoenzymes. In his original study Board (1981a) proposed that this isoenzyme set exhibited genetical variation. He studied the isoenzymes in only liver tissue and his basis for believing that genetical variation occurred at GST2 was based on the good agreement between observed and expected phenotypes using the Hardy-Weinberg equilibrium. I have extended this study in liver as well as other tissues from the same individuals. In this regard I was

fortunate in obtaining the permission of Her Majesty's Coroner as well as the generous support of several pathologists who obtained tissue sets from suitable adult individuals at post-mortem. I then prepared cytosols and determined their isoenzyme composition using starch-gel electrophoresis and other techniques These studies form the first part of the thesis.

The second part of the project was to study the ontogeny of the isoenzymes of GST. Over the past few years a number of reports of GST activity in foetal tissues have appeared in the literature (Mukhtar et al., 1981, Warholm et al., 1981 and Polidoro et al., 1982). These have generally been limited to measurements of total activity in cytosol during the first trimester and little is known of the expression of the various isoenzymes during human development. I was particularly fortunate to be able to collaborate with Dr. Robert Hume in Edinburgh. He obtained tissue sets during development and these have allowed a formal study of the expression of the various GST isoenzymes. Initially expression was determined qualitatively using starch-gel electrophoresis since this technique was our reference method for assigning isoenzymes to particular loci. But since it was clear that marked changes in expression were occurring, I decided that it was necessary to quantify the activities of the different isoenzymes. I therefore chromatofocused cytosols and quantitively assayed the contribution of the different loci.

MATERIALS AND METHODS.

2.1. CHEMICALS.

Agarose, ammonium sulphate (Analar grade), butan -1-ol (Chromatographic grade), 1-chloro-2,4-dinitrobenzene (Analar grade) citric acid (Analar grade), cumene hydroperoxide, disodium hydrogen phosphate (Analar grade), ethanediol (Analar grade), ethanolamine (Analar grade), guanidinium chloride (Aristar grade), hydrochloric acid (Analar grade), imidazole (GPR extra pure grade), iodine (Analar grade), potassium dihydrogen phosphate (Analar grade), potassium hydroxide (Analar grade), potassium iodide (Analar grade), sodium dihydrogen phosphate (Analar grade) and sucrose (Analar grade) were obtained from B.D.H. Chemicals Ltd, Poole, Dorset, U.K.

Nitrogen was obtained from British Oxygen Company Ltd, Worsley, Manchester, U.K.

Absolute ethanol was purchased from J. Burrough Ltd, 60 Montford Place, London, S.E.11.

Starch was purchased from Connaught Laboratories, Willowdale, Ontario, Canada.

Sodium Chloride (S.L.R. grade) was bought from Fisons Ltd, Loughborough. Leicestershire.

DEAE-Sephadex, Phenyl Sepharose CL4B, Polybuffer 74, Polybuffer 96 and Polybuffer Exchanger were purchased from Pharmacia Fine Chemicals (Great Britain) Ltd, Prince Regent Road, Hounslow, Middlesex, U.K.

Epoxy-activated Sepharose 6B, glutathione reductase, β-nicotinamide adenine dinucleotide phosphate (reduced form),

reduced glutathione and Tris-HCl were obtained from the Sigma Chemical Company.

Gigasept was purchased from Sterling Hospital Products, Chapeltown, Sheffield, U.K.

During the first half of this project water was doubled distilled from an all-glass still supplied by Quickfit and Quartz Ltd, Stone,Staffordshire, U.K. For the second half of the project water was obtained from a nanopure II system supplied by Sybron Barstead, Boston, U.S.A.

Biuret reagent was obtained from Technicon Instruments Ltd, Houndmills, Basingstoke, Hampshire, U.K.

DEAE-cellulose (Whatman DE52) and CM-cellulose (Whatman CM52) were purchased from Whatman Ltd, Maidstone, Kent, U.K.

2.2. SOURCE OF TISSUE SAMPLES.

Samples of adrenal, kidney, liver and spleen were obtained within 4h of death from aborted foetuses (10-24 weeks gestation) following termination of pregnancy, premature and term infants (26-42 weeks of gestation) who died in the neonatal period and infants who suffered sudden infant death syndrome (2-84 weeks postnatal age). Approval was obtained from the Reproductive Medicine Ethics Committee of the Simpson Memorial Maternity Pavilion, Royal Infirmary, Edinburgh. Corresponding tissue samples were also obtained from adults who had died following coronary artery disease

and who were without clinical evidence, and where possible, histological evidence of liver disease. These samples were obtained at autopsy, usually within 12h of death. Samples were not taken after 20h of death. Samples were stored at -70°C before use. Subsequent experiments showed no loss of GST activity or alteration in column elution profiles or zymogram patterns after 12 months of storage at -70°C.

Although the tissue samples were obtained from subjects who were selected because they had apparently died from non-infectious conditions, it was necessary to develop a safety protocol that would afford protection against possible infective agents such as Hepatitis B virus and more recently HTLV III virus. The salient safety points are included in the description of the preparation of cytosol.

2.3. PREPARATION OF CYTOSOL.

Disposable rubber gloves, plastic apron and white laboratory coat as recommended by the Howie Commission (H.M.S.O., 1978) were worn throughout the preparation of tissue homogenates and cytosols. Tissue samples stored in screw-cap plastic containers at -70°C were removed from the freezer, placed in a small plastic bag and taken to a safety cabinet sited in a Category B room (H.M.S.O., 1978). The tissue was allowed to thaw slightly and a sample was removed using sharp scissors. The size of

the sample varied considerably depending on the tissue type and gestational age. It was placed in a glass blender, approximate volume 25 ml, that was surrounded by crushed ice. To one volume of tissue was added approximately three volumes of Tris-HCl buffer (20m$\underline{M}$; pH 7.20; 4°C) containing sucrose (250m$\underline{M}$), disodium EDTA (0.1m$\underline{M}$) and reduced glutathione (1m$\underline{M}$). The tissue was carefully chopped into small pieces using scissors and then homogenised in the blender for 30 seconds at 5000 r.p.m. followed by a 30 second pause. This step was repeated eight times. Light microscopy of the samples of homogenised tissues confirmed that more than 80% of the cells were disrupted by the homogenisation process. The homogenate was transferred, using a disposable plastic Pasteur pipette, into 4.2 ml polycarbonate ultracentrifuge tubes (M.S.E.) and centrifuged (MSE Prepspin 65) (20 min; 20,000g; 4°C). The supernatant was removed, carefully avoiding any lipid layer at the surface. The supernatant was then transferred to a clean 4.2 ml polycarbonate ultracentrifuge tube. The small volume lost by removal of the cell debris at the first centrifugation was replaced by using the Tris-HCL buffer. The supernatant was recentrifuged (60 min; 150,000g; 4°C) and the resulting supernatant was removed by a single action using a disposable Pasteur pipette. The supernatant was dispensed into 1 ml screw-cap plastic containers and stored at -70°C. This supernatant was termed cytosol.

All equipment used in the preparation of homogenates

and cytosols, with the exception of the homogenisation motor, was autoclaved. Bench surfaces were wiped with a solution of 2% Gigasept (Sterling Hospital Products).

2.4. STARCH-GEL ELECTROPHORESIS.

The first part of this project was to investigate the extent of variation in the basic, GST2 set of isoenzymes. Since the original observations of Markert (1977) it has become increasingly clear that a large number of isoenzymes from all life forms exhibit variation. In some cases this variation is genetical whilst in others it results from secondary modifications of the primary product. Determining the source of variation often requires a large number of samples and traditional biochemical separation techniques involving column chromatography are usually too slow to allow such studies to be realistically carried out. Electrophoresis, on the other hand, allows a relatively large number of samples to be processed and most studies of enzyme variation have used, at least initially, an electrophoretic method. Starch-gels are perhaps the most widely used and although often superceded by more recently introduced techniques such as gel-isoelectric focusing and affinity electrophoresis, still provide a reasonably simple and robust method. Since Board (1981a) had already presented a genetic model for the GST in human liver by using a starch-gel zymogram technique this method was used in the initial studies into the variation of human

GST with particular reference to the basic isoenzymes. As experiments were subsequently carried out using other methods, it became clear that data obtained using the zymogram approach was comparable with that obtained using chromatofocusing and ion-exchange chromatography.

Horizontal starch-gel electrophoresis was performed using a continuous Tris-citrate buffer system at pH 7.50 (Scott and Wright; 1980). The electrode buffer was Tris-citrate buffer (100mM; pH 7.50) and the gels (length 19cm, width 11cm, depth 1cm), containing 13% starch (Connaught Laboratories) were prepared in a 1 in 10 dilution of this buffer. Usually three gels were prepared and run in a day. Starch powder (35g) was added to 270ml of Tris-citrate buffer and mixed with gentle swirling in a glass, round bottomed flask (resistant to implosion) over a Bunsen flame. The heat was maintained as the mixture went through phases, initially being opaque and viscous and then clear and less viscous. The bubbles were removed by de-gassing with a vacuum line for 20 sec. The hot, clear agar was poured into a flat, open-mould tray (19cm x 11cm x 1cm) with care to produce an even, uniform gel that was allowed to solidify at room temperature for 4h. To prevent surface evaporation, the gel was covered with polythene after 1h. Up to five samples (50 µl each) per gel were applied by soaking cytosols onto pieces of Whatman 3MM paper (0.6cm x 0.9cm). The papers were inserted into slits made in the gel with a broad blade. Greater volumes of cytosol could

be applied (in cases where cytosols demonstrated low GST activity) by inserting additional pieces of Whatman 3MM paper soaked with the sample. The origin was sometimes off-set from the centre to give better results for the fast migrating forms of the enzyme. Electrophoresis was carried out for 16h (overnight) at 4°C in a Q11 system (Shandon, U.K.) at a constant current of either 23 mA or 7 mA per gel (approx. voltage 7.0 or 2.5 V/cm respectively). The gels were removed from the mould-trays, sliced into halves horizontally (using a stainless steel wire-cutter), placed into flat trays and stained in two stages, using overlays of agarose (0.75% w/v).

The first overlay was prepared in sodium phosphate buffer (100m<u>M</u>; pH 7.00) and contained the substrates 1-chloro-2,4-dinitrobenzene (2m<u>M</u>) and reduced glutathione (0.8m<u>M</u>). Approximately 200ml of this overlay, at a temperature of about 50°C, was poured onto the cut surface of each half-gel and when set, was incubated at 37°C for 1.75h. To facilitate removal of the overlay the gels were kept at 4°C for 0.25h. After careful removal of the first overlay, a second overlay was prepared in Tris-HCl buffer (100m<u>M</u>; pH 8.00) containing an appropriate amount of iodine in potassium iodide solution (80m<u>M</u> iodine in 120m<u>M</u> potassium iodide) to identify the GST isoenzymes as blue bands on a paler blue, or white background (Scott and Wright, 1980).

The zymogram usually faded after about one hour

and a record of each experiment was made using a Polaroid C.U.5 land camera.

2.5. RESOLUTION OF GST2 ISOENZYMES.

Although the starch-gel electrophoresis studies revealed some variation between adult individuals, the patterns seen were similar in that samples demonstrated a strongly stained cathodal band that migrated ahead of other bands. This prominent band exhibited similar mobility in almost all samples studied. The cathodal band with intermediate mobility also appeared to be a constant feature of the samples that were studied. The species with the slowest cathodal mobility however were more variable. As discussed in the Introduction, electrophoretic techniques cannot resolve all enzyme variants and it was decided to further examine the two main cathodal bands before using chromatofocusing to determine the number of enzyme species present.

These two bands were separated from other GST in an initial DEAE-Sephadex step. Some resolution of the basic isoenzymes was achieved and fractions from the two peaks obtained were separately pooled and subjected to ammonium sulphate fractionation. This step was used since it is rapid, relatively gentle to protein species and allowed easy use of the third stage of the procedure, hydrophobic interaction chromatography. This latter stage was used

since GST are known to readily interact with a wide variety of hydrophobic ligands. Since different GST isoenzymes have different affinities for hydrophobic ligands it was thought that useful resolution of the GST could be achieved. The different peaks that eluted from the hydrophobic interaction column were separately pooled and eluted from a GSH-affinity column.

2.5.1. DEAE-Sephadex Chromatography.

DEAE-Sephadex anion exchange chromatography was used as the first step in the purification of GST2 (basic) isoenzymes since, at the pH chosen, the GST2 isoenzymes were not retained on the column and were easily separated from the retained GST1 (near-neutral) and GST3 (acidic) isoenzymes.

DEAE-Sephadex (Pharmacia Fine Chemicals) was prepared in Tris-HCl buffer (20mM; pH 7.25) and packed into a column (30.0 x 2.6 cm). The column was then equilibrated with the Tris-HCl buffer. Cytosol was pumped on to the column at a flow-rate of 24 ml/h and flow-through fractions (6ml) were collected. GST activity was assayed in each of these. Two major peaks of GST activity were identified and appropriate fractions of each were pooled.

2.5.2. Ammonium Sulphate Precipitation.

This is a simple and efficient method of removing large quantities of unwanted protein. Pilot experiments

were used to determine the percentage saturation of ammonium sulphate required to remove considerable amounts of unwanted protein without loss of GST. It was found that when the ammonium sulphate saturation of the pooled fractions from DEAE-Sephadex was 50%, more than 95% of the GST activity was retained in the supernatant with considerable loss of other proteins. The amount of ammonium sulphate required to produce a 50% saturated solution was determined from the nomogram described by Dixon and Webb (1965). The pooled fractions from DEAE-Sephadex were placed in a small glass beaker and allowed to equilibrate to room temperature. Ammonium sulphate was then slowly added with stirring. When 50% saturation was achieved, the solution was allowed to remain at room temperature for 30 min unstirred. The solution was centrifuged and the supernatant separated from the pellet of unwanted protein.

2.5.3. Phenyl Sepharose Hydrophobic Interaction Chromatography.

Hydrophobic interaction chromatography is a highly versatile technique that selectively binds molecules with hydrophobic sites. Separation is achieved by the interaction of the hydrophobic matrix, water and the hydrophobic solute. Once the interaction is established, any perturbation of one or more of the three components may be used to effect separation.

Since GST2 isoenzymes have the ability to bind hydro-

phobic ligands such as bilirubin (Kamisaka *et al*. 1975) hydrophobic interaction chromatography was selected as one of the procedures in the resolution of the GST2 isoenzymes. An additional reason for choosing hydrophobic interaction chromatography was the use of ammonium sulphate in the preceding step; high concentrations of ammonium sulphate promote hydrophobic interactions (Pahlman *et al*. 1977).

Phenyl Sepharose CL4B (Pharmacia Fine Chemicals) was prepared in a column (30cm x 2.6cm; Wright Scientific). This expensive, but re-usable gel was regenerated after use in the following way at a flow-rate of 24 ml/h. Approximately one bed volume of distilled water was pumped on to the column followed by one bed volume of absolute ethanol. Approximately two bed volumes of butan -1-ol were then pumped on to the column followed by one bed volume of absolute ethanol. Distilled water was pumped on to the column overnight (16h) before the column was finally equilibrated with one bed volume of Tris-HCl buffer (20mM; pH 7.25) that was 50% saturated with ammonium sulphate.

The pooled supernatant from the ammonium sulphate step was eluted (24 ml/h) on to the Phenyl Sepharose column. Fractions (6ml) were collected and assayed for GST activity. From previous experiments it had been observed that a reducing ammonium sulphate gradient did not remove GST from the Phenyl Sepharose column. When addition of the sample was completed, the column was eluted

with 400mM ammonium sulphate in Tris-HCl buffer (20mM; pH 7.25) for 4h followed by elution with Tris-HCl (20mM; pH 7.25) for a further 4h. Elution of GST was effected by the formation of an ethanediol gradient on the column. This was prepared by the continuous addition of a 55% solution of ethanediol in Tris-HCl buffer (20mM; pH 7.25) to a 250ml reservoir of Tris-HCl buffer (20mM; pH 7.25) at a flow-rate of 24 ml/h. Fractions (6ml) were collected and assayed for GST activity. Pooled samples containing GST activity were dialysed against three changes (each of 1 litre) of Tris-HCl buffer (50mM; pH 9.00) before GSH-affinity chromatography was carried out.

2.5.4. GSH-Affinity Chromatography.

Affinity chromatography is a type of adsorption chromatography in which the compound to be purified is separated from contaminating molecules by binding to a complementary ligand immobilised onto an inert matrix. When all unwanted material has been flushed through the support matrix, adsorption of the bound molecules is reversed.

Affinity chromatography can be used to selectively remove GST isoenzymes from other material. This is achieved by immobilising one of its substrates, reduced glutathione, onto a suitable matrix, epoxy-activated Sepharose 6B. Although highly selective in retaining the GST isoenzymes, this method is not selective for the individual isoenzymes.

Separation of the isoenzymes must therefore be made by another procedure before purification of specific isoenzymes can be achieved. GSH-affinity chromatography provided an effective method of purifying the various isoenzymes of GST following their separation on a Phenyl Sepharose column. The method used is essentially that of Simons and Vander Jagt (1977).

Approximately 4g epoxy-activated Sepharose 6B (Sigma Chemicals) was washed with 2L of distilled water through a sintered glass funnel until all traces of yellow had been removed from the Sepharose. The epoxy-activated Sepharose 6B was then washed with phosphate buffer (45m<u>M</u>; pH 7.00) prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate. Following this, epoxy-activated Sepharose 6B was washed out with the phosphate buffer into a glass stoppered tube. Nitrogen gas was passed through the suspension for 5 min. Four ml of a solution of 400mg of reduced glutathione in 4ml distilled water, adjusted to pH 7.00 with potassium hydroxide solution (1<u>M</u>), was added to the suspension gel. Nitrogen was passed through the gel for a further 2h and the tube containing the gel was rotated slowly for 17h. The coupled gel was washed with 500ml distilled water. The remaining active groups were blocked by returning the suspension gel to the glass tube and adding 60-70ml ethanolamine (1<u>M</u>) for 4h. Once prepared the suspension was packed into a column (Pharmacia 10cm x 1cm) and equilibrated with

Tris buffer (50m<u>M</u>; pH 9.00). The prepared sample containing the GST isoenzyme was pumped onto the column at a flow-rate of 10 ml/h followed by Tris buffer (50m<u>M</u>; pH 9.00) for 2h. The retained GST isoenzyme was eluted from the column using glutathione (5m<u>M</u>) in Tris buffer (50m<u>M</u>; pH 9.00). The GSH-affinity column was regenerated ready for use by pumping sodium chloride (1<u>M</u>) for 2h followed by Tris buffer (50m<u>M</u>; pH 9.00) for 16h.

2.6. <u>CHROMATOFOCUSING</u>.

The use of pH gradients to elute proteins adsorbed on to ion-exchange columns has generally proved to be problematical. This approach requires that the pH gradient is formed before elution of the column. Little separation of proteins is achieved unless the buffer has a high buffering capacity that can counteract the sudden large changes in pH that will occur as the proteins are eluted. Traditional buffers of high buffering capacity necessitate high ionic strengths. Such buffer systems are undesirable since the high ionic concentrations will automatically and indiscriminately remove all the less tightly bound proteins. If a buffer of low ionic strength is used, its intrinsically low buffering capacity becomes ineffective in producing a pH gradient because its effect is counteracted by the buffering properties of the adsorbed proteins on the matrix.

The technique of chromatofocusing first developed

by Sluyterman and Wijdenes (1977), overcomes this problem by the use of ampholyte-type buffers of high buffering power but low effective ionic strength. The exchanger matrix is polyethyleneimine-agarose which is similar to DEAE- adsorbents but also has continuously titratable groups throughout a wide range of pH values. Another important feature of chromatofocusing is that the pH gradient is formed within the column. The continuous titration of the groups on the matrix by the acidic ampholyte produces a steady pH gradient at low ionic strength. It should be noted however that proteins will elute in order of, and not at, their isoelectric points. There are three important factors that influence the pH at which proteins finally elute from a chromatofocusing column. Firstly, the Donnan potential that is established between the strong charge of the ampholyte buffer and the surface of the pores of the matrix causes proteins to elute at lower pH values than their isoelectric points. This happens when the overall charge on the bound proteins has become zero and as they diffuse away from the pores of the matrix, enter an environment of lower pH and become positively charged. They are immediately repelled by the positive charge of the matrix. This phenomenon occurs throughout the column. Secondly, the insolubility of a protein at its isoelectric point also forces its elution from a chromatofocusing column at a pH below the isoelectric point. This will occur when the protein precipitates at

its isoelectric point and then redissolves when the oncoming pH is lowered. The protein, now positively charged, is repelled from the matrix and will migrate further down the column. The elution pH in this situation is always slightly lower than the isoelectric point of the protein. These phenomena do not however occur in isolation from each other and the third factor, that of displacement effects, responsible for affecting the final elution pH of proteins, generally overrides the combined effects of the other two described above. Displacement effects occur as the pH of the system decreases causing relatively weak binding of the proteins to the matrix. The interaction between proteins and matrix becomes increasingly susceptible to the effect of the presence of competing ions namely the ampholytes. Eventually proteins may elute by a process similar to that of ampholyte displacement chromatography and elute at pH values higher than their isoelectric points.

The human GST include isoenzymes with a wide range of isoelectric points and for this reason chromatofocusing was chosen as a most suitable technique for studying the developmental changes in expression of the GST loci.

Advantages of this method include well defined separation of the different sets of GST1, GST2 and GST3 isoenzymes. The contribution of each set of isoenzymes can then be quantified. The method was simple to operate and comparatively fast in that a cytosol could be eluted and the column fractions collected in about 12h.

A disadvantage of the technique was the relative variability in the elution pH of some of the isoenzymes. Although the various isoenzymes could be readily identified by their order of elution from the column, the pH at which an enzyme eluted depended on the pH gradient. Care was taken to reproduce the experimental conditions exactly and as the identity of the eluted peaks was also checked by using starch-gel electrophoresis, classification did not present any difficulty. Elution of fractions of the same cytosol showed that the elution pH could vary ± 0.3 pH units although usually variation was much smaller. Another disadvantage was the relatively high expense incurred in the use of the ampholyte-type buffer (Poly-buffer, Pharmacia Fine Chemicals).

The primary aim of using this technique was to separate the isoenzymes of GST into their major groups; GST1 (near-neutral), GST2 (basic) and GST3 (acidic). Chromatofocusing allows two ranges of pH values to be developed. Firstly, a range approximating from pH 9.00 to pH 7.00 and secondly, a range approximating from pH 7.00 to pH 4.00. Initial experiments showed that the use of the range pH 9.00 to pH 7.00 resulted in poor resolution of the GST1 and GST3 isoenzymes. Further, there was a substantial loss (45%) in activity of these isoenzymes when this gradient was used. The range approximating pH 7.00 to pH 4.00 separated the three sets of isoenzymes but without good resolution of the basic (GST2) isoenzymes. There was good recovery

(greater than 90%) of activity using this pH range with good resolution of the GST1 isoenzymes. Since most of the foetal samples were of small size, it was only possible to develop one chromatofocusing profile for each of the cytosols. The range pH 7.00 to pH 4.00 was therefore chosen for the separation of the three sets of GST isoenzymes for all the tissue samples investigated.

Chromatofocusing was used to separate the isoenzymes of GST in cytosols of adrenal, kidney, liver and spleen tissue. Cytosols (1-3 ml; approximately 15mg of protein /ml)were eluted (30 ml/h; 4°C) from columns (40 x 1 cm; Pharmacia Fine Chemicals) containing Polybuffer Exchanger 94 (Pharmacia Fine Chemicals) equilibrated with start buffer (25mM imidazole buffer; pH 7.30). The pH gradient was formed using Polybuffer 74 (Pharmacia Fine Chemicals) adjusted to pH 4.00 with HCl (5M) and 2.5 ml fractions were collected.

The basic GST2 isoenzymes were not usually retained on the column although small amounts of activity eluted at values as low as pH 6.90. The near-neutral GST1 isoenzymes in liver eluted between pH 6.35 and pH 5.85 and the acidic GST3 isoenzymes eluted at values less than pH 5.20. When cytosol from the other tissues was studied these isoenzymes eluted at a slightly more alkaline pH. This probably reflects the variation in slope of the pH gradient rather than differences in structure of the enzymes from different tissues. Starch-gel electrophoresis

showed the GST1 isoenzymes to have identical electrophoretic mobilities and separate studies carried out in the laboratory using inhibitors support the suggestion that the GST1 isoenzymes from the different tissues are the same.

2.7. ION-EXCHANGE CHROMATOGRAPHY.

The basic GST2 isoenzymes in human liver tissue were first classified by Kamisaka et al. (1975). They used a combination of DEAE- and CM-cellulose to separate five isoenzymes with basic isoelectric points. These isoenzymes were termed α, β, γ, δ and ε in order of increasing isoelectric points. Since this publication the use of these two ion-exchangers has become the accepted method of separating the human GST2 isoenzymes (Stockman et al. 1985). Identification of the α, β, γ, δ and ε isoenzymes has been largely based on their order of elution from DEAE- and CM-cellulose and the elution concentration of Na^+. To allow comparison therefore of results with those of Stockman et al. (1985) these ion-exchangers were used in preference to the many other chromatographic media currently available.

2.7.1 DEAE-Cellulose Chromatography.

Cytosols (approx. 3 ml; approx. 15 mg of protein/ml) were eluted (30 ml/h; 4°C) from columns (30 x 1.6 cm; Wright Scientific). They contained DEAE-cellulose (Whatman DE52) equilibrated with Tris-HCl buffer (20mM; pH 7.30).

Fractions of 2.5 ml were collected. Flow-through fractions containing GST activity were pooled and dialysed against sodium phosphate buffer (10mM; pH 6.70) containing disodium EDTA (0.1mM) and reduced glutathione (1mM). Dialysis was performed without a change of buffer for 16h in a ratio of pooled fraction to buffer of 1 to 40.

2.7.2. CM-Cellulose Chromatography.

The dialysed pool from DEAE-Cellulose was eluted from a column (40 x 1.6 cm; Pharmacia Fine Chemicals) containing CM-cellulose (Whatman CM 52) equilibrated with sodium phosphate buffer (10mM; pH 6.70). The sodium phosphate buffer was pumped on to the column for 30 min following addition of the sample. A linear NaCl gradient (15-110mM Na^+) was then formed by eluting the column with sodium phosphate buffer containing increasing concentrations of sodium chloride. The flow-rate was 30 ml/h and fractions of 3ml were collected.

2.8. HYBRIDISATION STUDIES.

Dissociation of a multimeric enzyme into its constituent monomers followed by reassociation can often provide useful information concerning the number of monomers present in the enzyme as well as whether inter-allelic or inter-locus enzymes can be formed. The technique is generally simple since most multimeric proteins can be readily dissociated using guanidinium chloride or

urea. Concentrated solutions of sodium chloride and freezing have also been used for this purpose. The monomers are allowed to reassociate back into active enzymes by dialysing the dissociating agent into buffer. This approach was used to study the inter-convertibility of the basic and near-neutral GST isoenzymes.

2.8.1. GST2(Basic)Isoenzymes.

The basic isoenzymes in tissue cytosols were separated by elution from CM-cellulose and the fractions corresponding to α, β, γ, δ and ε were combined. The enzymes were dissociated into monomers by incubation (25°C; 20 min) in imidazole buffer (25mM; pH 7.3) containing guanidinium chloride (5M). Following incubation the mixtures were dialysed for 20h at 4°C against 4 changes (each of 2L) of phosphate buffer (10mM; pH 6.7) containing reduced glutathione (1mM) and disodium EDTA (0.1mM). The dialysed solutions were then eluted from CM-cellulose as described.

2.8.2. GST1(Near-neutral)Isoenzymes.

Cytosol (approx. 3 ml; approx. 20 mg protein/ml) from an adult subject with the GST1 2-1 phenotype was eluted from the chromatofocusing column. Three enzymes eluted between pH values of 6.35 and 5.85 and the fractions corresponding to the GST1 2 enzyme (elution pH 6.35), GST1 2-1 enzyme (elution pH 6.10) and GST1 1 enzyme (elution pH 5.85) were separately pooled. Dissociation of the dimers

was achieved by incubation (25°C; 20 min) in imidazole buffer (25mM; pH 7.30) containing guanidinium chloride (5M). The monomers were allowed to recombine by dialysis (20h; 4°C) against 4 changes (each of 2L) of 25mM imidazole buffer (pH 7.30) containing reduced glutathione (1mM) and disodium EDTA (0.1mM). The resulting dimeric enzymes were then eluted as described from the chromatofocusing column.

2.9. ANALYTICAL.

2.9.1. Assay of GST.

Although the GST isoenzymes have a broad substrate specificity with respect to the electrophile, the tripeptide, reduced glutathione (GSH) appears to be a mandatory second substrate (Chasseaud, 1979). The electrophile, 1-chloro-2, 4-dinitrobenzene (CDNB) was chosen as the other substrate since all the human GST isoenzymes so far identified have a high specific activity towards it. All procedures involving CDNB required care, particularly the wearing of disposable gloves, since CDNB is known to be markedly allergenic in some subjects if inhaled or placed on the skin. The assay for GST was essentially as described by Habig et al. (1974). It has the advantages of simplicity and ease of automation. The latter was an important consideration since the developmental investigations entailed some 16,000 assays.

A discrete analyser (Coulter Electronics Ltd) was used for the assay. The CDNB reagent was prepared by dissolving

61 mg CDNB in 5 ml absolute ethanol; 1 ml of this solution was added to 49 ml of sodium phosphate buffer (100m<u>M</u>; pH 6.45) and mixed. Reduced glutathione (46mg) was then dissolved in 10ml distilled water. The sample and the working CDNB reagent were dispensed into a disposable cuvette and to this was added the glutathione. Volumes were measured to give a working substrate concentration for CDNB of 1m<u>M</u> and for glutathione 1m<u>M</u>. The assay mixture was incubated at 30°C and absorbances read at 6 second intervals over a 30 second time period. Since these substrates demonstrate a small non-catalytic reaction, a substrate blank was always carried out and the appropriate blank correction was made to the enzyme assays.

During the project a number of separate stock quality controls were prepared from liver cytosols. They were suitably diluted with Tris-HCl buffer (20m<u>M</u>; pH 7.20) to give a range of values of GST activity that varied from approximately 1.5 to 2.5ΔA_{340}/min/ml. Quality control samples (300 µl) were dispensed into 1ml plastic containers and stored at -40°C before use. The mean between-batch coefficient of variation was found to be 6.5%.

2.9.2. <u>Glutathione Peroxidase Assay</u>.

Glutathione peroxidase activity was measured using a discrete analyser (Kem-O-Mat, Coulter Electronics, Ltd, Luton, Bedfordshire). Enzyme assays were carried out at 30°C in Tris buffer (50m<u>M</u>; pH 7.40) containing disodium

EDTA (1.0mM), reduced glutathione (1.0mM), NADPH (200μM), cumene hydroperoxide (200μM) and 1-2 units glutathione reductase per cuvette.

2.9.3. Protein Determination.

The protein concentrations of cytosols were determined at 25°C using a centrifugal analyser (Centrifichem; Baker Instruments Ltd). The reagent used was a modified biuret (Skeggs & Hockstrasser, 1964) and blanking was carried out for background colour and turbidity. The batch to batch coefficient of variation for levels greater than 5 g/L was not greater than 6%. Cytosols found to have a protein content of less than 5 g/L were concentrated using either a vacuum dialysis system (Prodimem) or an Amicon filtration system, and re-analysed for protein concentration and GST activity. The results were used to express GST activity in cytosols as μmol/min per mg protein.

2.9.4. Sodium Assays.

Sodium ion concentrations were measured using a flame photometer (Flame Photometer 243, Instrumentation Laboratory, Kelvin Close, Birchwood, Warrington, U.K.). The system was calibrated using aqueous standards. The coefficient of variation was calculated using control samples and was found to be 0.7%.

RESULTS

3.1. STARCH-GEL ELECTROPHORESIS STUDIES OF BASIC (GST2) ISOENZYMES IN ADULT TISSUES.

3.1.1. Preliminary Studies.

The first stage of my research project was to establish a starch-gel electrophoretic method to study the GST isoenzyme composition of human tissues. The method described in the Materials and Methods section represents a hybrid of those described by Board (1981a) and Scott and Wright (1980) for human erythrocytes.

In preliminary experiments a series of cytosols from human livers was examined to ensure that electrophoretic patterns were reproducible, not subject to edge effects and also assess the sensitivity of the system.

Figure 3 shows an example of the pattern obtained when a single cytosol was studied. Distinct bands of GST activity were obtained and there was no evidence of edge effects. This figure shows the results of running samples at a constant current of 23 mA per gel. This current resulted in the migration of one group of isoenzyme bands towards the cathode and the migration of a second set towards the anode. When the samples were examined using a lower current (7 mA per gel) the cathodally migrating isoenzymes were seen as an unresolved group of bands close to the origin. The band with anodal mobility was much closer to the origin and

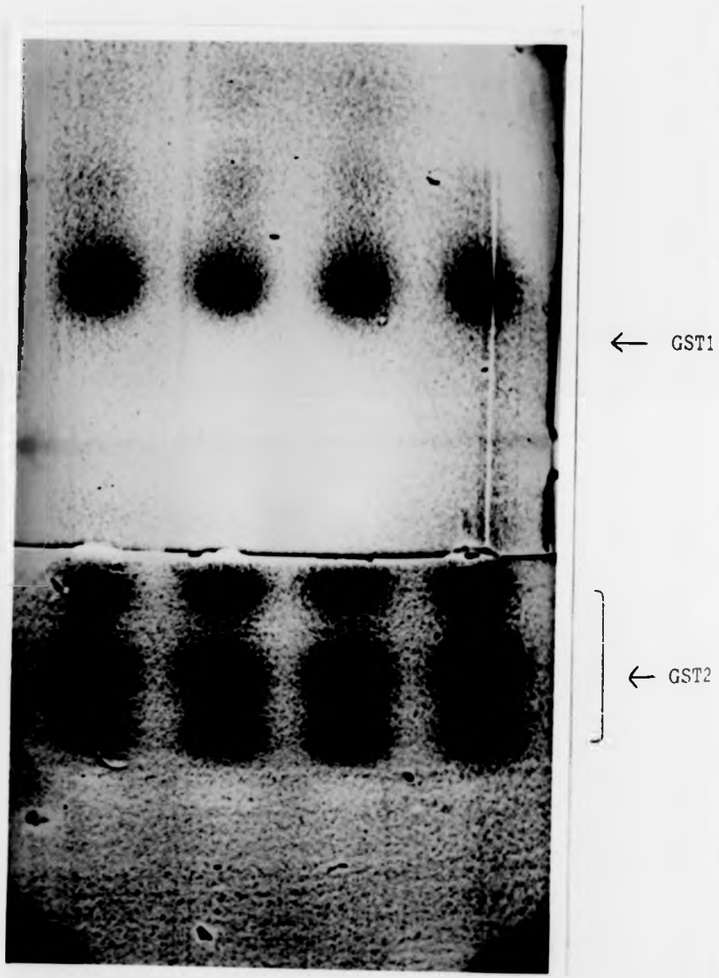
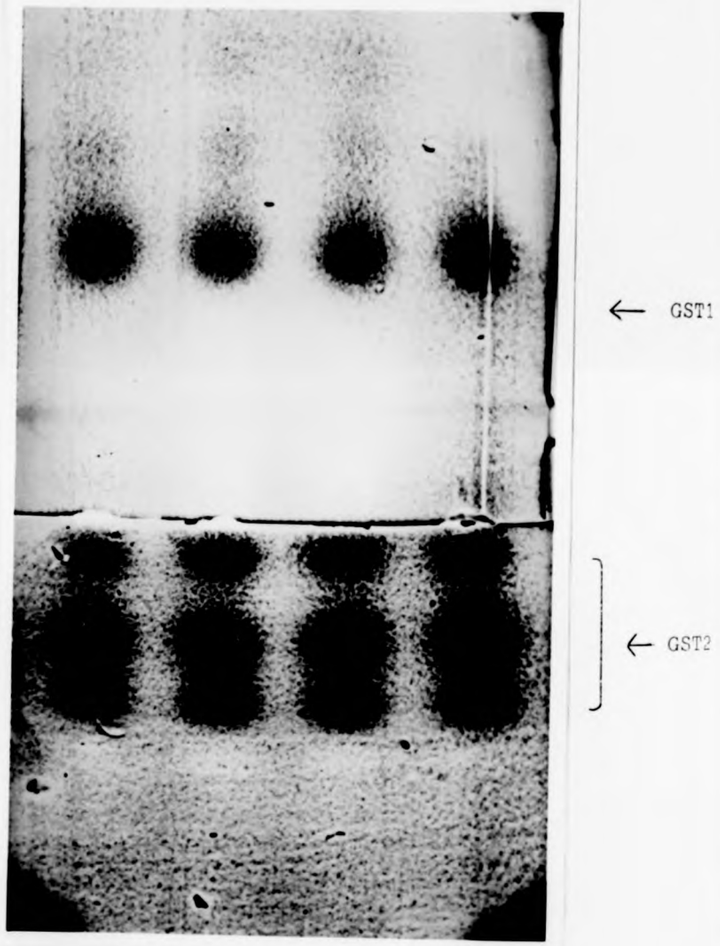


Figure 3.

Figure 3. Starch-gel electrophoresis of cytosol from the same individual demonstrating that the technique was not subject to "edge effects".

The subject is a GST1 1 phenotype.



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The subject is a GST1 1 phenotype.

a further set of isoenzymes with fast anodal mobility was also seen (figure 4).

These results are similar to those described by Board (1981a) in his original publication. The set of isoenzymes that demonstrated cathodal mobility correspond to the products of the GST2 locus, the isoenzyme with intermediate anodal mobility to the products of the GST1 locus and the more acidic isoenzymes with fast anodal mobility to the products of the GST3 locus.

3.1.2. Examination of basic GST Patterns in Liver.

Having established that the starch-gel procedure was working satisfactorily and giving results similar to those described by Board (1981a) a series of 65 adult liver cytosols was examined to determine the extent of variation within the isoenzyme sets. Board (1981a) proposed that the basic GST2 isoenzymes demonstrated genetical variation. This variation arose from the presence of two alleles so that the population could be divided into heterozygotes and homozygotes for these genes. A further interesting observation concerned the relatively high incidence (19 out of 198) of samples in the Board (1981a) study that demonstrated no GST2 activity. He suggested that the absence of GST2 activity in these samples was a consequence of inactivation in degraded samples and they were subsequently not included in his calculations.

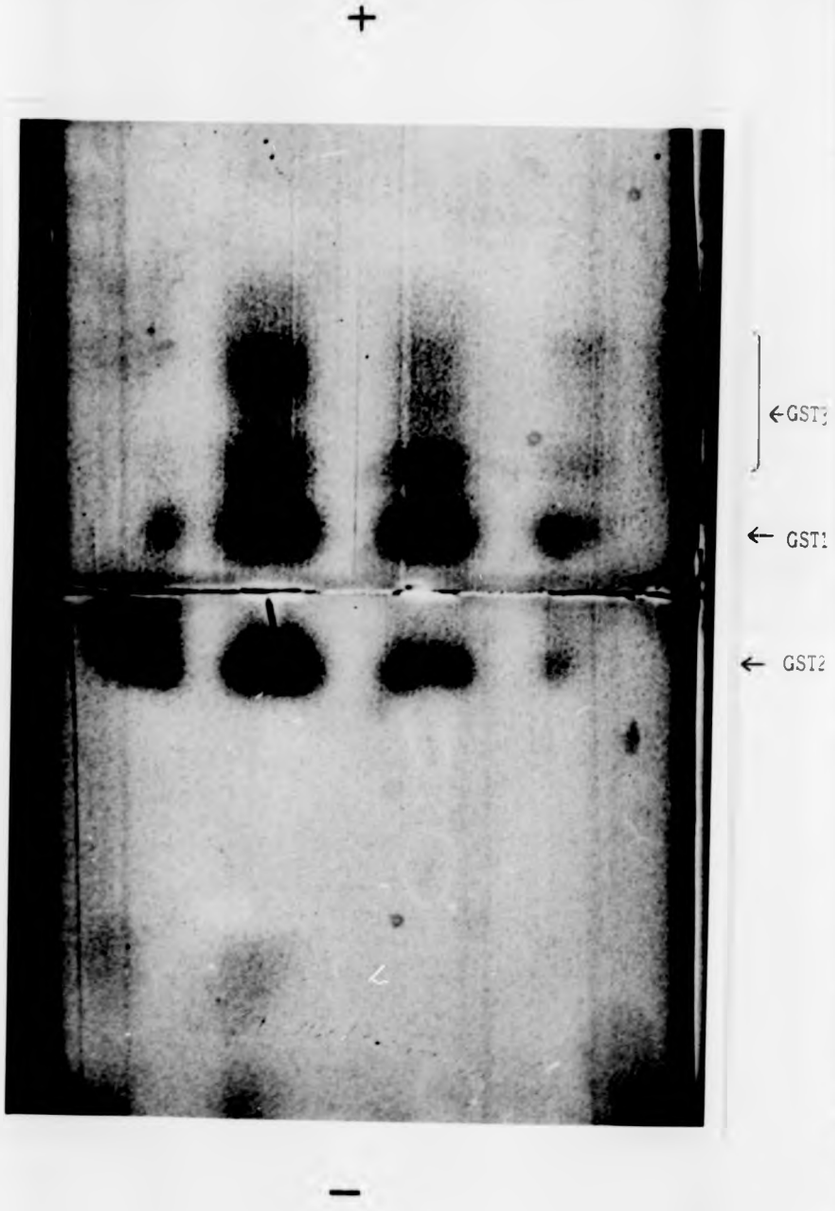

Figure 4.

Figure 4. **Starch-gel electrophoresis of cytosols.** Left to right adult liver, from one sub adrenal adenoma, adrenal and kidney med from a different subject. A current of per gel was used to demonstrate anodall migrating GST3. These are not visible 23mA per gel because of their fast mobi (compare figure 3 in which a cytosol fr different subject has been developed at 23mA per gel).

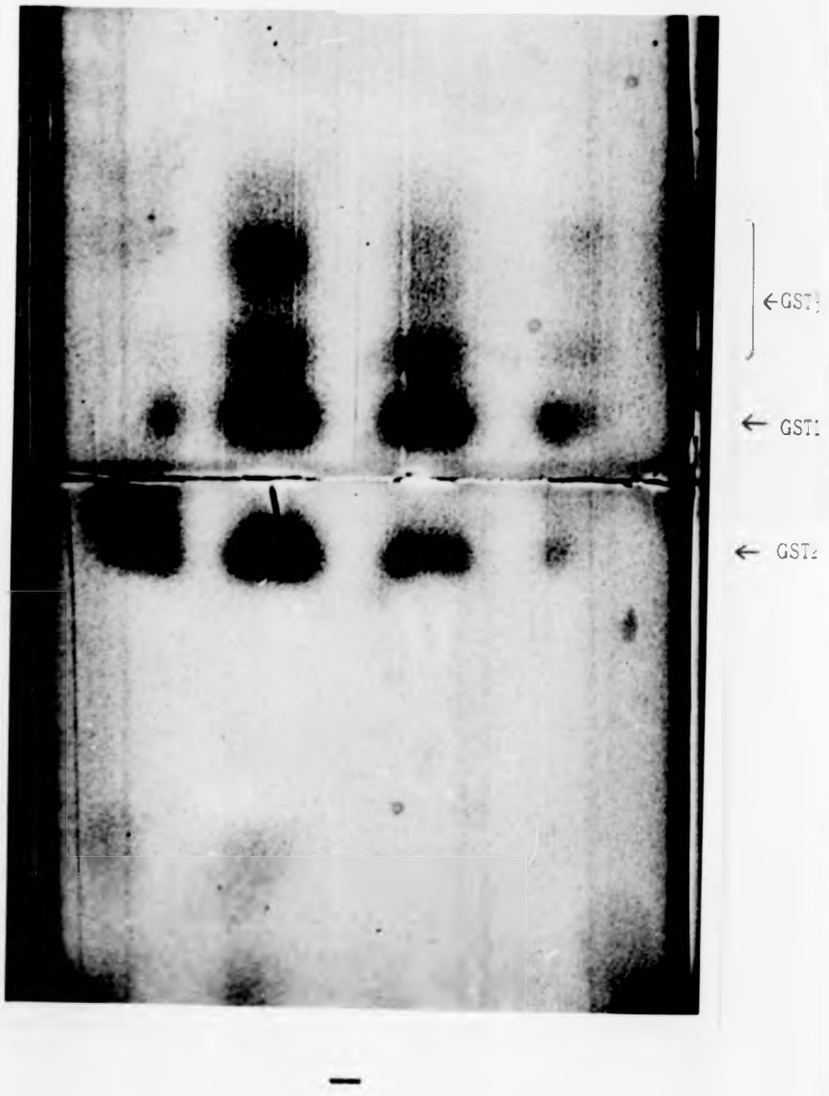

Figure 4.

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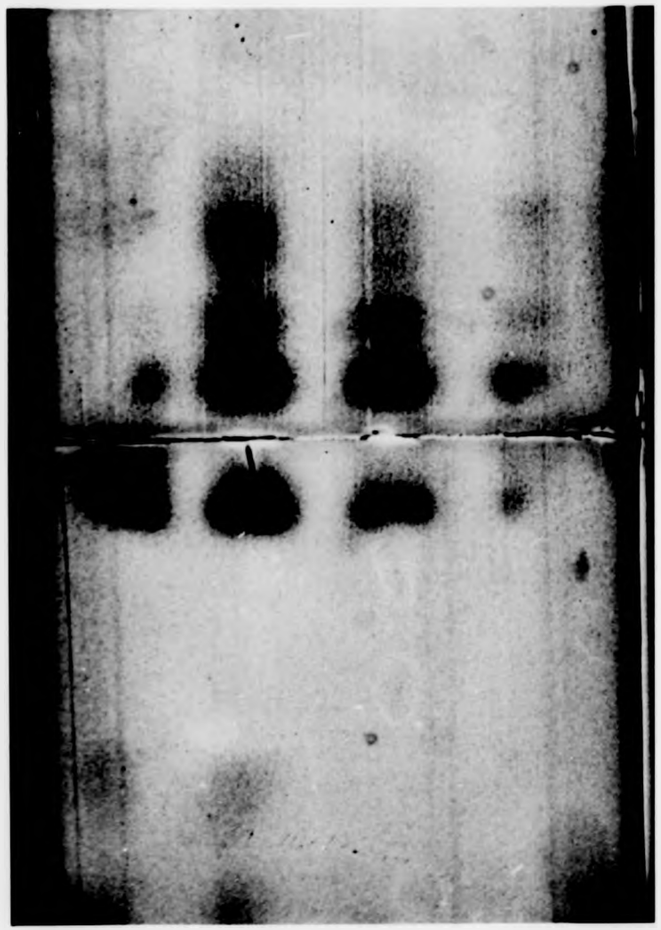

Figure 4.

Figure 4. **Starch-gel electrophoresis of cytosols.** Left to right adult liver, from one subject, adrenal adenoma, adrenal and kidney medulla from a different subject. A current of 7mA per gel was used to demonstrate anodally migrating GST3. These are not visible using 23mA per gel because of their fast mobility (compare figure 3 in which a cytosol from a different subject has been developed at 23mA per gel).

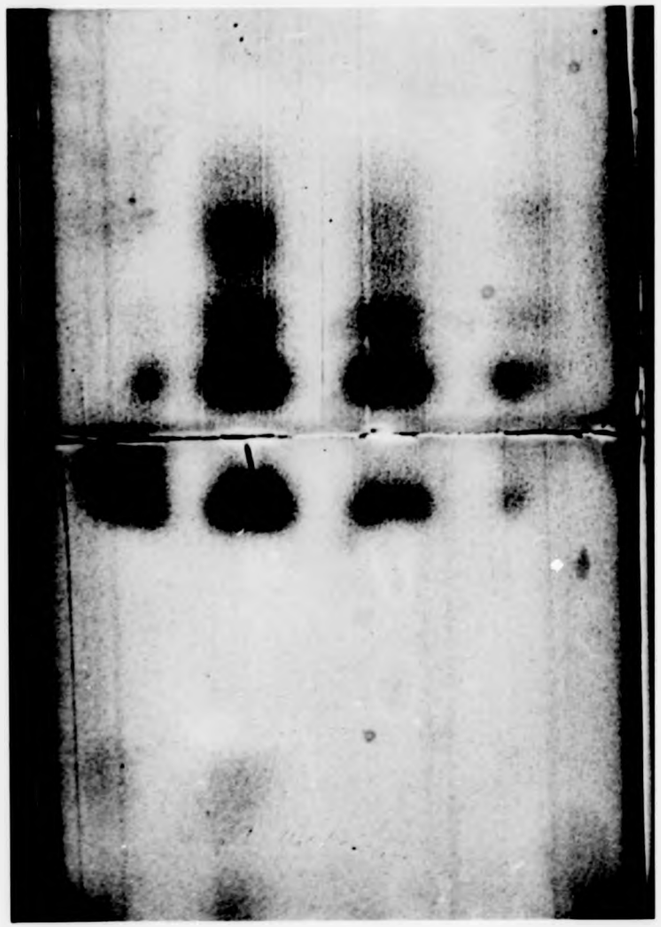

Figure 4.

Figure 4. **Starch-gel electrophoresis of cytosols.** Left to right adult liver, from one subject, adrenal adenoma, adrenal and kidney medulla from a different subject. A current of 7mA per gel was used to demonstrate anodally migrating GST3. These are not visible using 23mA per gel because of their fast mobility (compare figure 3 in which a cytosol from a different subject has been developed at 23mA per gel).

Examples of starch-gel zymograms obtained from our local Caucasian population are shown in figures 5 and 6. A variety of patterns was seen. Twelve of the 65 subjects demonstrated three bands of activity; these comprised a slow cathodal band, an intermediate cathodal band and also a band with fast cathodal mobility. This is the pattern expected for a heterozygous phenotype. In 5 further samples only a single band of fast cathodal mobility was observed. This single band would have been expected from one of the two possible homozygous phenotypes of the proposed two allele model. However, the other phenotype, that is the one identified by a single band of slow cathodal mobility was not seen. With the exception of one subject that demonstrated no GST2 activity, every subject demonstrated the fast cathodal band either alone or with one or with two slower bands.

In 47 samples two bands of GST2 activity were observed, a fast band and also a band with intermediate cathodal mobility. In these samples the band with slow mobility was not seen.

There was some evidence to suggest variation between samples since although the fast band usually demonstrated the same mobility in different subjects, both this and the band with intermediate mobility demonstrated slightly slower mobility than usual in one subject (figure 7).

The original proposal that two alleles are found at this locus and that some individuals will be GST2 1

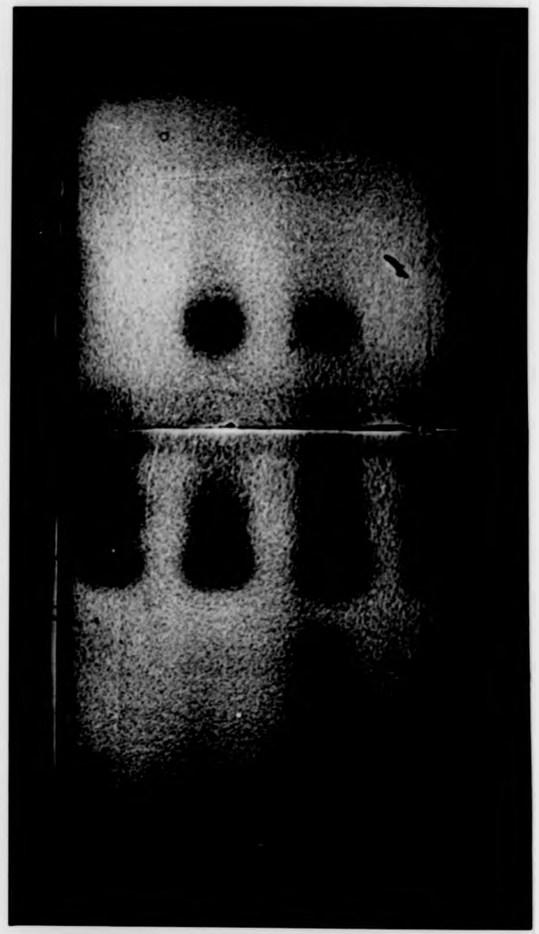

Figure 5.

Figure 5. Starch-gel electrophoresis of adult liver cytosols demonstrating the variety of patterns observed for basic GST isoenzymes.

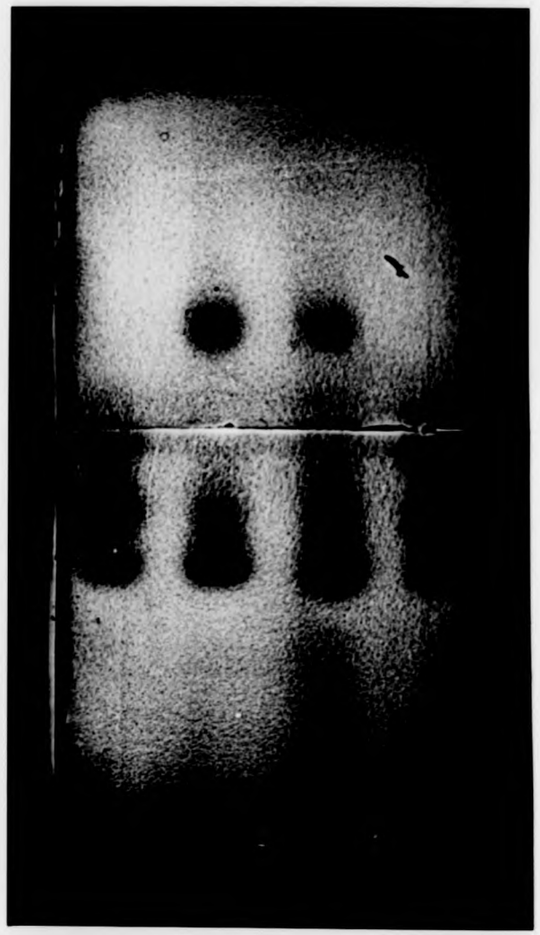

Figure 5.

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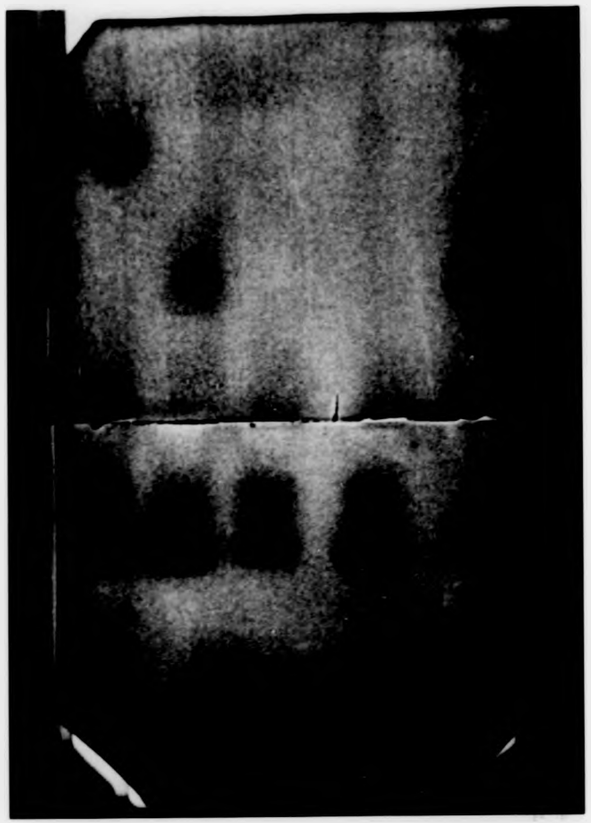


Figure 6.

Figure 6. Starch-gel electrophoresis of adult liver cytosols demonstrating the variety of patterns observed for basic GST isoenzymes.

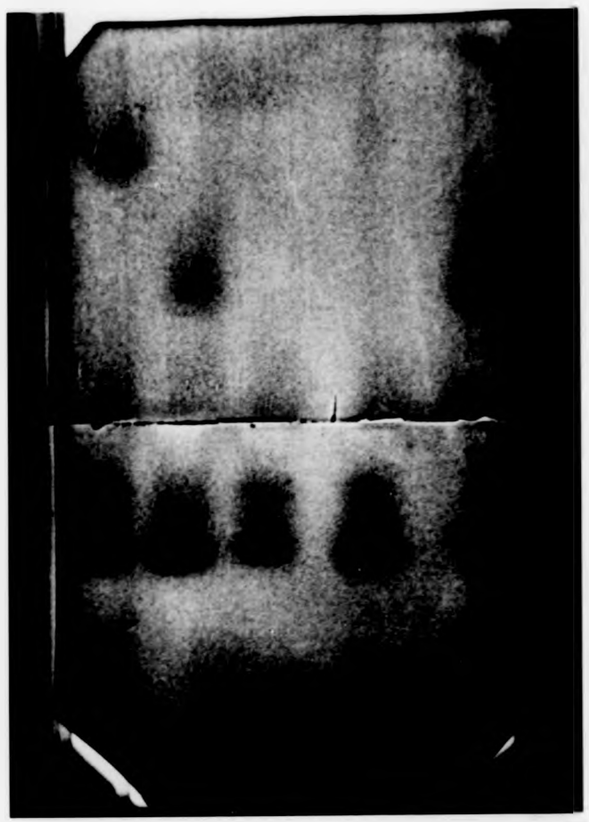
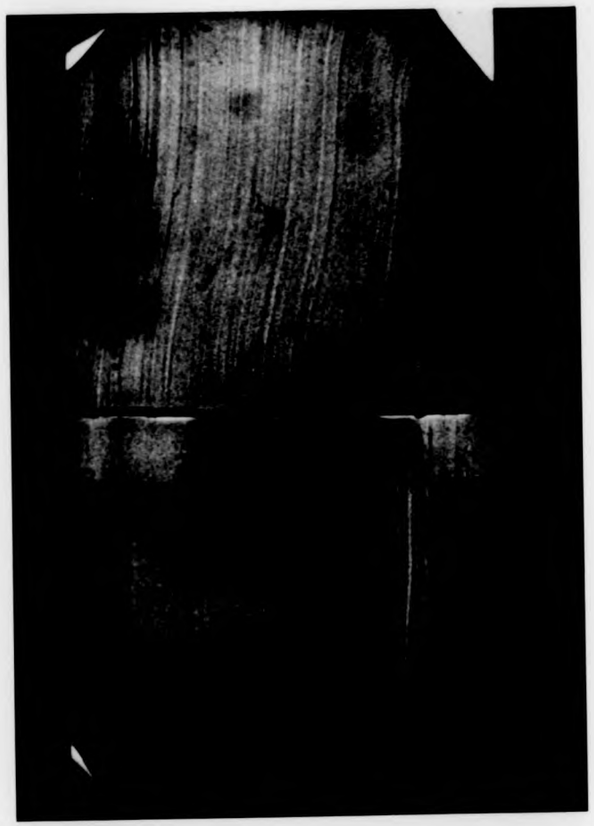


Figure 6.

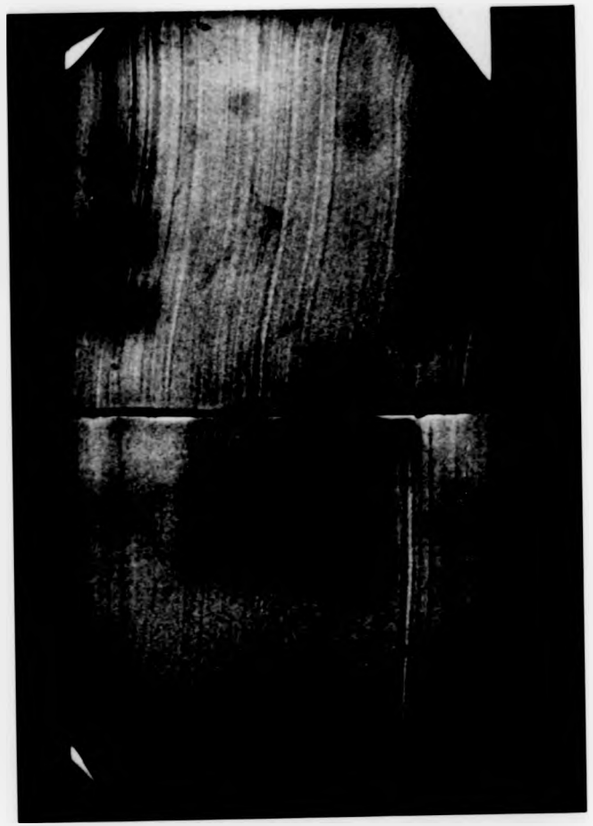
Figure 6. Starch-gel electrophoresis of adult liver cytosols demonstrating the variety of patterns observed for basic GST isoenzymes.



(i) (ii) (iii) (iv)

Figure 7.

Figure 7. Starch-gel electrophoresis of a GST1 2-1 phenotype (i) and (iv) and two individuals with the GST1 0 (null) phenotype. Note that the individual represented by (i) and (iv) was the only subject in which GST2 was not expressed. Subjects (ii) and (iii) show dissimilar mobilities of GST2.



(i) (ii) (iii) (iv)

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Figure 7. Starch-gel electrophoresis of a GST1 2-1 phenotype (i) and (iv) and two individuals with the GST1 0 (null) phenotype. Note that the individual represented by (i) and (iv) was the only subject in which GST2 was not expressed. Subjects (ii) and (iii) show dissimilar mobilities of GST2.

(GST2*1/GST2*1), other individuals GST2 2 (GST2*2/GST2*2) and the remainder GST2 1-2 (GST2*1/GST2*2) does not seem tenable from my data and analysis of the number of proposed heterozygotes could not achieve Hardy-Weinberg equilibrium.

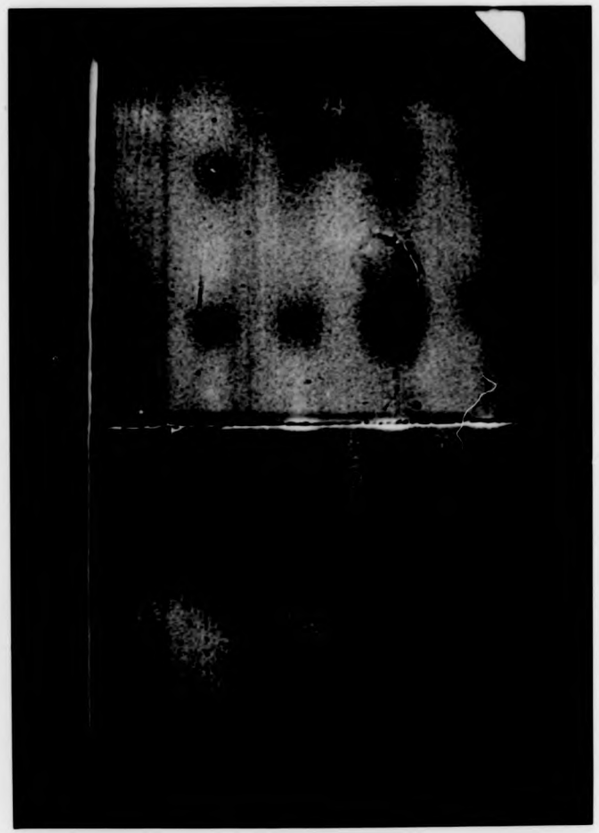
Only one individual who demonstrated an absence of GST2 was found. Unfortunately only liver tissue was obtained from this subject and it was not therefore possible to compare the liver zymogram with that of other tissues. However, the sample was collected within six hours of death and was therefore well within the time limit that had been set. The post-mortem report indicated that this subject had died after suffering acute myocardial infarction and there was no evidence of other disease or the administration of any drugs. The absence of GST2 activity in this individual is unlikely therefore to be an artefact and may truly reflect a genetic trait. If this is correct, the trait is uncommon since during the course of this thesis nearly two hundred liver samples have been examined and only this one example of a putative null phenotype, at this locus was found. To further investigate this subject I asked Dr. J. D. Hayes of the Department of Clinical Chemistry, University of Edinburgh to determine the concentration of B_1 and B_2 monomers in the cytosol by using his radioimmunoassay method (Stockman et al., 1985). The concentration of both monomers was near the

detection limit of his assay and these data were interpreted as supporting the hypothesis that the absence of GST2 isoenzymes in this subject resulted from a failure to synthesise significant amounts of B_1 and B_2 monomers.

3.1.3. Examination of the Basic GST in Other Tissues.

Examination of liver cytosols indicated that the genetic model previously proposed was incorrect. It was important therefore to determine whether the isoenzyme pattern was a constant individual characteristic in other tissues from the same subject. Tissue sets studied comprised adrenal, cardiac muscle, kidney, skeletal muscle, spleen and stomach. The rationale for studying these cytosols is that if the liver pattern is genetically determined, at least some of these other tissues would be expected to demonstrate similar patterns.

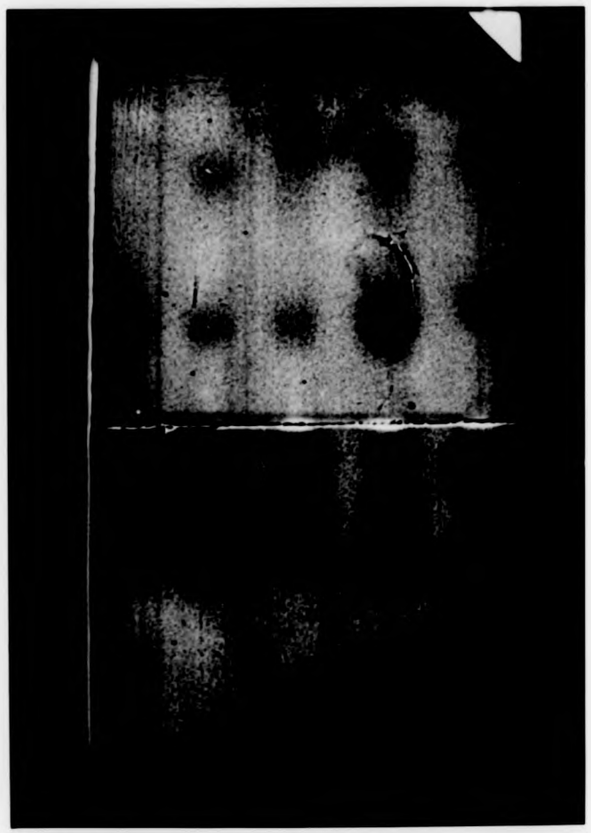
Figure 8 shows the starch-gel pattern obtained after electrophoresis of liver, kidney, adrenal and spleen cytosols from one subject. The anodally migrating activity shows that this subject was a GST1 2 phenotype and as expected, at the current used, some evidence of GST3 isoenzymes could be seen, since they are strongly expressed in the non-hepatic tissues. Each of the tissues expressed cathodally migrating enzyme activity and figure 8 shows that this iso-



(i) (ii) (iii) (iv) (v)

Figure 8.

Figure 8. Starch-gel electrophoresis (23 mA per gel) of (i) liver, (ii) kidney cortex, (iii) kidney medulla, (iv) adrenal and (v) spleen cytosols from the same individual.



(i) (ii) (iii) (iv) (v)

Figure 8.

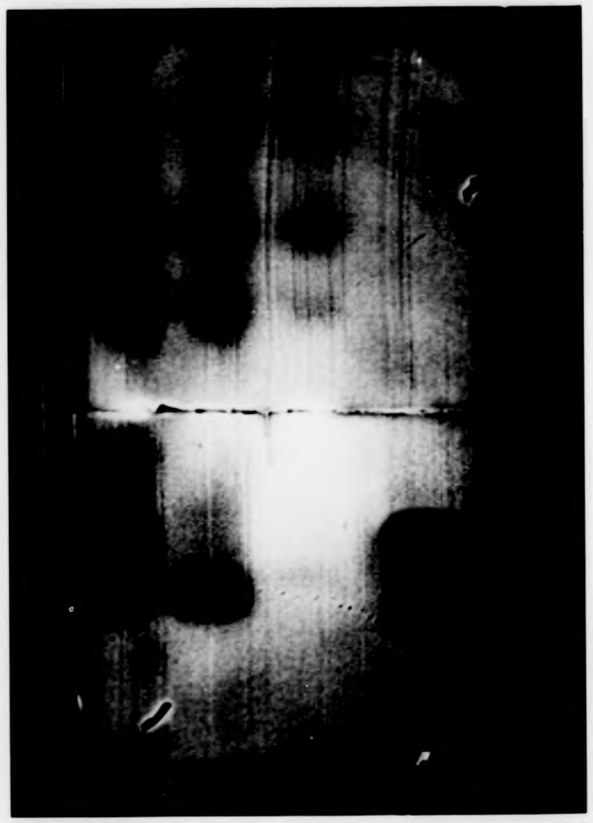
Figure 8. Starch-gel electrophoresis (23 mA per gel) of (i) liver, (ii) kidney cortex, (iii) kidney medulla, (iv) adrenal and (v) spleen cytosols from the same individual.

enzyme set was prominant in liver, adrenal and kidney but only weakly present in spleen. It can also be seen that whilst liver and kidney cortex and kidney medulla demonstrated similar electrophoretic patterns in that three isoenzyme bands can be seen, adrenal is characterised by an intense band with fast cathodal mobility and less activity migrating with intermediate and slow cathodal mobility.

The difference in zymogram patterns between liver and adrenal was confirmed by studying further samples. All the adrenal cytosols demonstrated a strongly stained band with fast mobility but much less activity of slower mobility. Figure 9 shows an example of an individual with a GST1 2-1 phenotype and it is clear that whilst the GST1 isoenzyme pattern is a constant individual characteristic in liver, adrenal and stomach, the GST2 patterns are different in the three tissues.

In the kidney samples examined, no differences between cortex and medulla were detected. The samples demonstrated either two or three bands with cathodal mobility although in some cases resolution between them was very poor. Whilst the basic isoenzymes were detected in all samples of kidney cytosol obtained from adults, the level of expression appeared to be rather weaker than that in the corresponding liver samples.

Figures 8 to 12 show examples of spleen and



(i) (ii) (iii)

Figure 9.

Figure 9. Starch-gel electrophoresis of (i) liver, (ii) adrenal, (iii) stomach cytosols from the same heterozygous individual demonstrating different expression of the basic GST isoenzymes.

(i) (ii) (iii)

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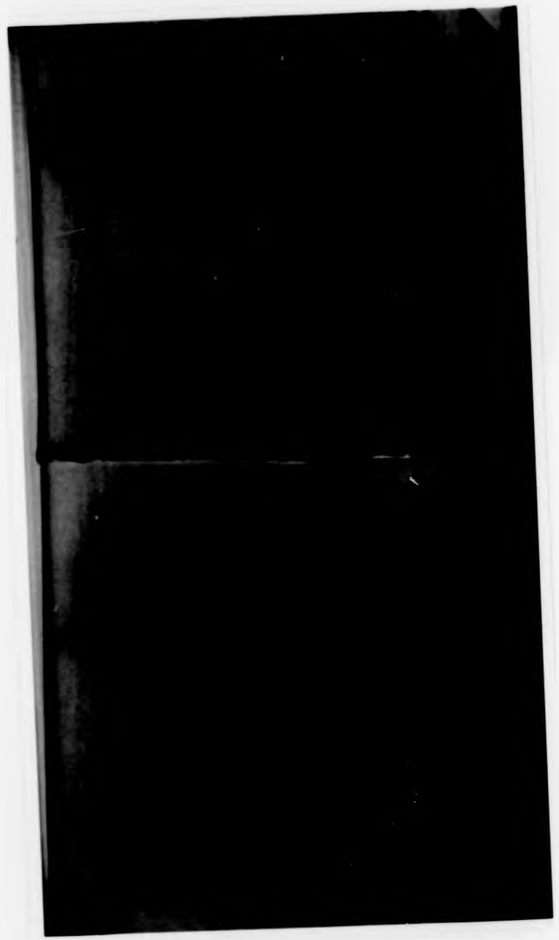
Figure 9.

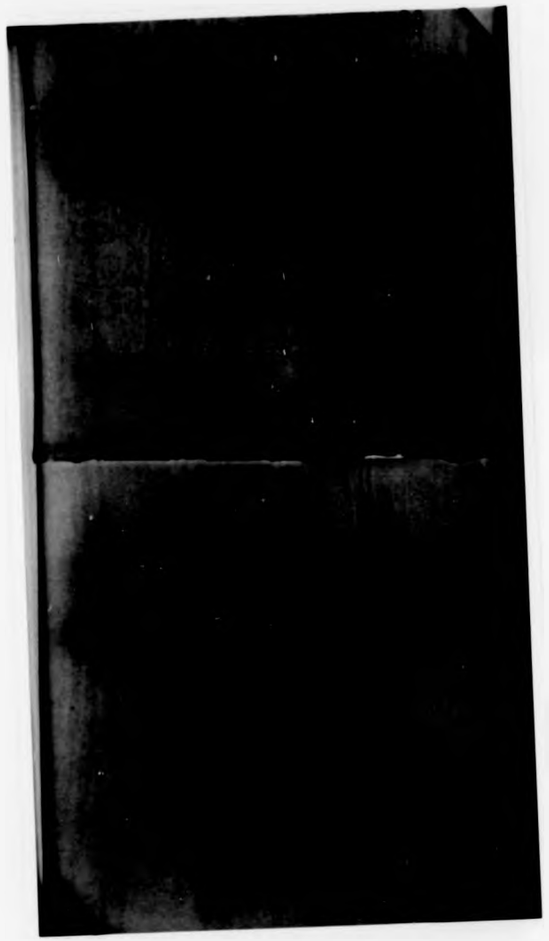
Figure 9. Starch-gel electrophoresis of (i) liver, (ii) adrenal, (iii) stomach cytosols from the same heterozygous individual demonstrating different expression of the basic GST isoenzymes.



(i) (ii) (iii) (iv) (v)

Figure 10.

Figure 10. Starch-gel electrophoresis of (i) & (ii) liver, (iii) kidney, (iv) adrenal cytosols from the same individual and (v) spleen cytosol from a different individual.



(i) (ii) (iii) (iv) (v)

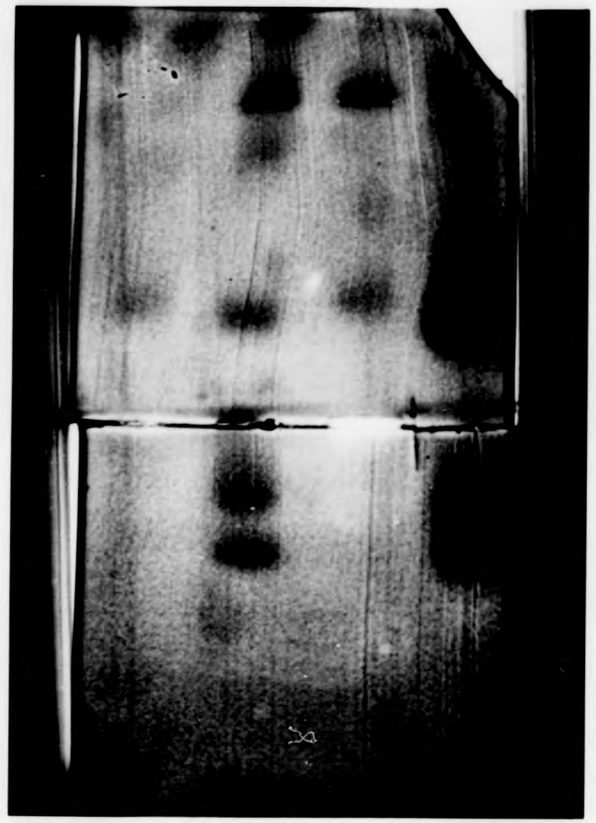
Figure 10.

Figure 10. Starch-gel electrophoresis of (i) & (ii) liver, (iii) kidney, (iv) adrenal cytosols from the same individual and (v) spleen cytosol from a different individual.

(i) (ii) (iii) (iv)

Figure 11

Figure 11. Starch-gel electrophoresis of (i) stomach, (ii) kidney, (iii) pancreas and (iv) liver cytosols from the same individual.



(i) (ii) (iii) (iv)

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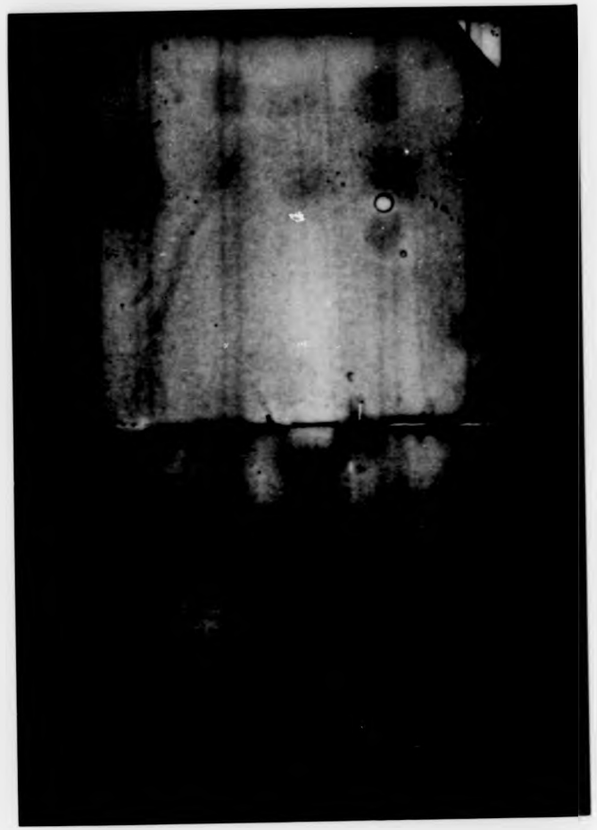
Figure 11

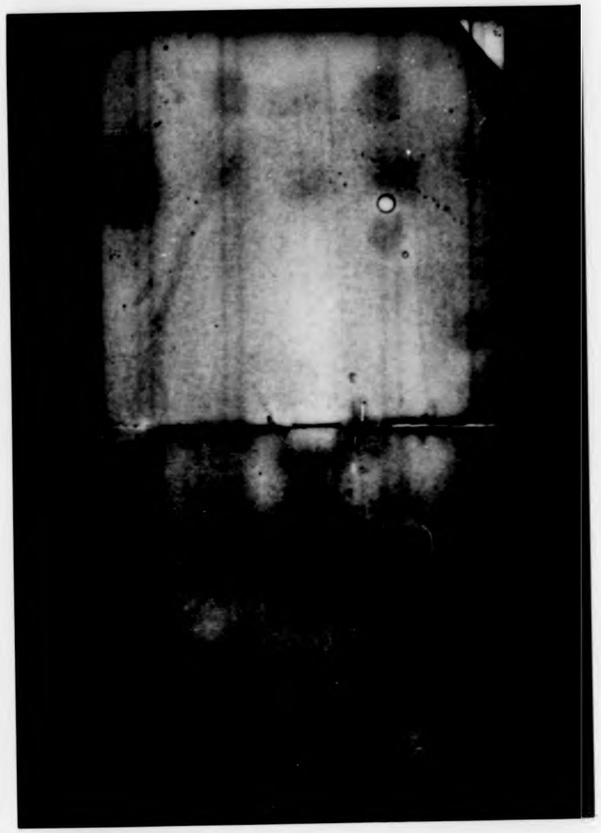
Figure 11. Starch-gel electrophoresis of (i) stomach, (ii) kidney, (iii) pancreas and (iv) liver cytosols from the same individual.



(i) (ii) (iii) (iv) (v)

Figure 12

Figure 12. Starch-gel electrophoresis of (i) liver (ii) stomach (iii) kidney (iv) spleen (v) adrenal from the same adult individual.



(i) (ii) (iii) (iv) (v)

Figure 12

Figure 12. Starch-gel electrophoresis of (i) liver (ii) stomach (iii) kidney (iv) spleen (v) adrenal from the same adult individual.

stomach cytosols that demonstrated very weak expression of GST2 isoenzymes and these isoenzymes were also very weakly expressed in three samples of pancreas that were examined.

Cytosols obtained from skeletal muscle was also examined for the presence of GST2 isoenzymes and figure 13 shows that this tissue also weakly expressed this isoenzyme set. Interestingly skeletal muscle did not appear to express GST1 isoenzymes in significant amounts. GST2 isoenzymes were not detected in platelets or erythrocytes.

The results obtained from the starch-gel studies are summarised in Table 2.

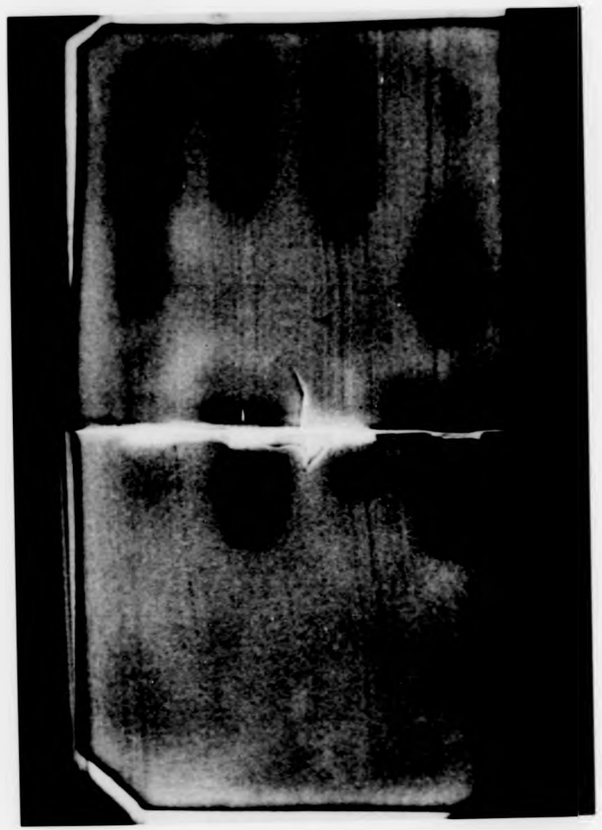
3.2. SEPARATION OF THE BASIC (GST2) ISOENZYMES IN LIVER CYTOSOLS.

The starch-gel electrophoresis patterns described in the previous section demonstrated that there are at least three isoenzymes with basic isoelectric points present in liver cytosol from adults. Further isoenzymes might be expected since, in the only systematic examination of human liver GST, Kamisaka *et al*. (1975) were able to demonstrate the five isoenzymes designated α, β, γ, δ and ε. A combination of different ion-exchangers was used by this group to resolve the GST in cytosol and this approach has also been adopted by Hayes *et al*. (1983) and Stockman *et al*. (1985). In a

(i) (ii) (iii) (iv)

Figure 13.

Figure 13. Starch-gel electrophoresis of (i), (ii) and (iii) skeletal muscle and (iv) liver cytosol.



(i) (ii) (iii) (iv)

Figure 13.

Figure 13. Starch-gel electrophoresis of (i), (ii) and (iii) skeletal muscle and (iv) liver cytosol.

Summary of the results obtained from starch-gel electrophoresis of various adult tissue cytosols.

+ strong expression

± variable expression from weak to non-expression

- not expressed

Tissue	GST1 Near-neutral	GST2 Basic	GST3 Acidic
Erythrocytes	-	-	+
Adrenal	±	+	±
Cardiac Muscle	±	±	+
Kidney	+	+	+
Liver	+	+	±
Skeletal Muscle	±	±	+
Spleen	+	±	+
Stomach	+	±	+

Table 2

later section of this thesis I have also used this approach to study the basic isoenzymes in different foetal, neonatal and adult tissues. However, I considered that it would be useful to develop an alternative purification scheme to that published by Kamisaka et al.(1975) so that identification of basic isoenzymes could be made using different separative techniques. The scheme makes use of anion-exchange chromatography, ammonium sulphate precipitation, hydrophobic interaction chromatography and affinity chromatography. The scheme is shown in Table 3.

The first step in the purification scheme was the elution of a liver cytosol from DEAE-Sephadex and figure 14(a) shows the elution profile obtained. Since the sample studied was from an individual of a GST1 0 phenotype, all the GST activity eluted in the "flow-through" fractions. These fractions were also found to have glutathione peroxidase activity. Activity was partially resolved into two peaks. The electrophoretic profiles showed that the first peak of activity (B1) contained enzyme activity that migrated with fast cathodal mobility whereas the second peak (B2) that eluted from the ion-exchange column contained activity with intermediate cathodal mobility. It appears therefore that the DEAE-Sephadex step is able to resolve the two main bands of basic GST activity. The zymograms also showed the absence of GST1 activity in this sample and

Purification scheme for GST2 isoenzymes.

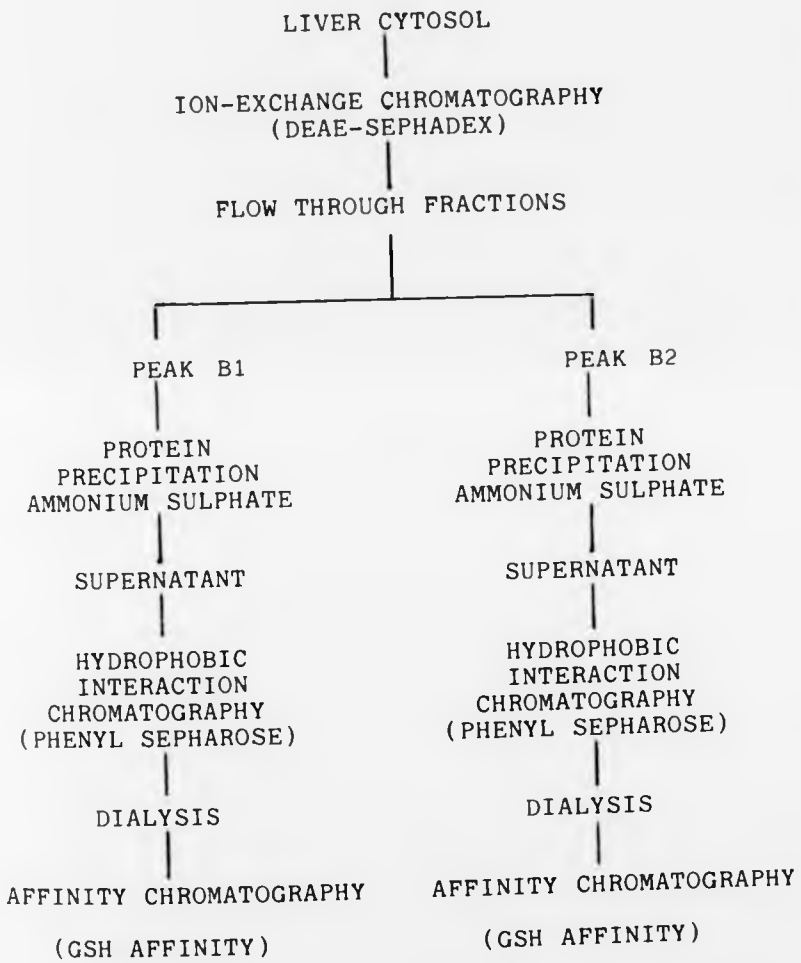


TABLE 3

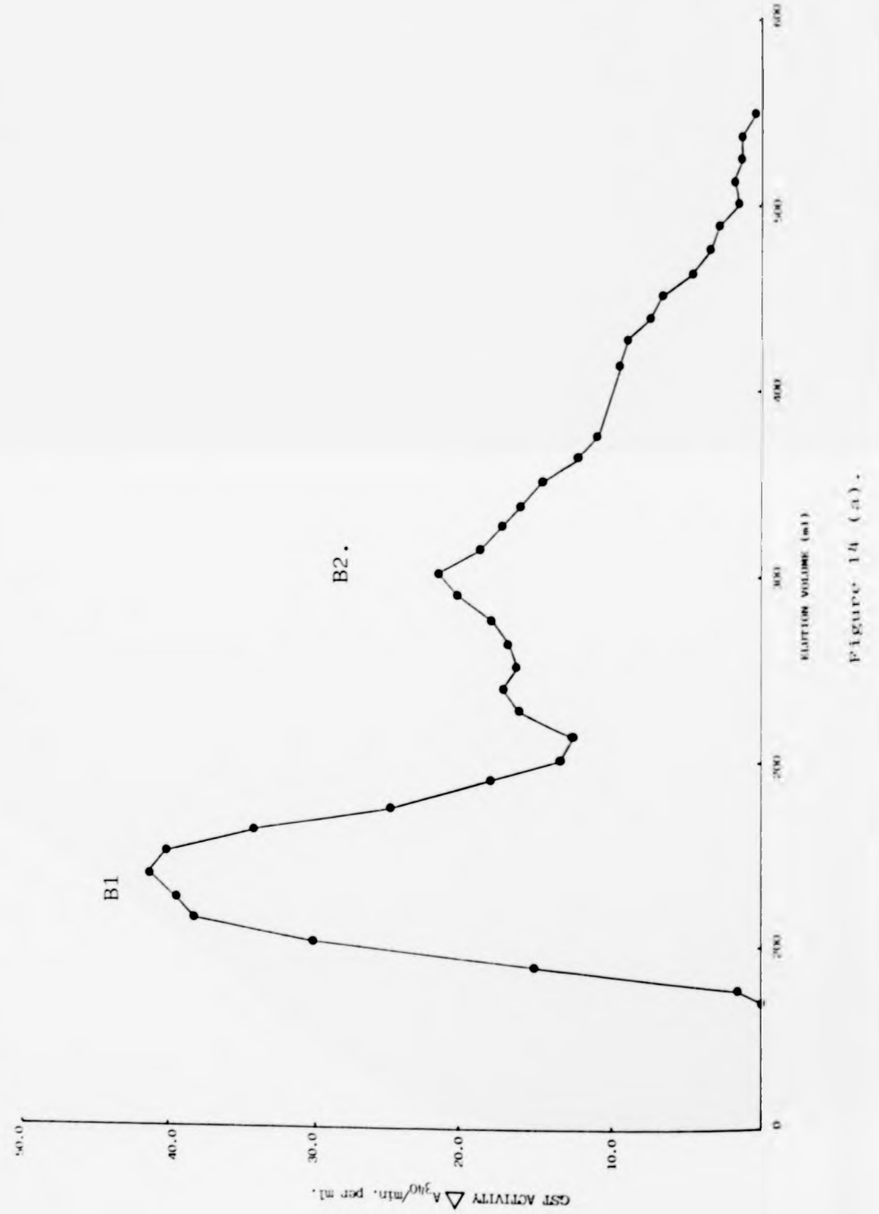


Figure 14 (a).

Figure 14(a) Elution profile of adult liver cytosol applied to a DEAE-Sephadex column equilibrated with 20mM Tris HCl buffer (pH 7.20) at 4°C.

confirm that the activity eluted from the ion-exchanger is the product of the GST2 locus.

The B1 and B2 pooled fractions were then separately treated with ammonium sulphate (50% saturation). The resulting precipitate was discarded since it contained no significant GST activity. The supernatant was applied to a column of Phenyl Sepharose. Figure 14 (b) and (c) shows the elution profiles of B1 and B2. The isoenzyme in the B1 fractions eluted as a single symmetrical peak of activity (B1P) that migrated on the starch-gel electrophoresis with fast cathodal mobility. Most of the activity in the B2 fractions also eluted from the Phenyl Sepharose column as a single peak B2P that appeared to contain two isoenzyme forms. Examination of the fractions from this peak showed that GST activity migrated with intermediate cathodal mobility. A small amount of activity eluted in the "flow-through" fractions from the Phenyl Sepharose column but the amount was too small to allow further analysis (figure 14(c)).

Following hydrophobic interaction chromatography, fractions from B1P and B2P were dialysed and eluted from GSH-affinity columns. Figure 14(d) shows the elution profiles obtained. B1P was eluted as two peaks; the first was a broad unresolved peak that was eluted before application of the GSH. The second peak was eluted with GSH and was found to migrate with fast cathodal mobility on starch-gel. The fractions obtained

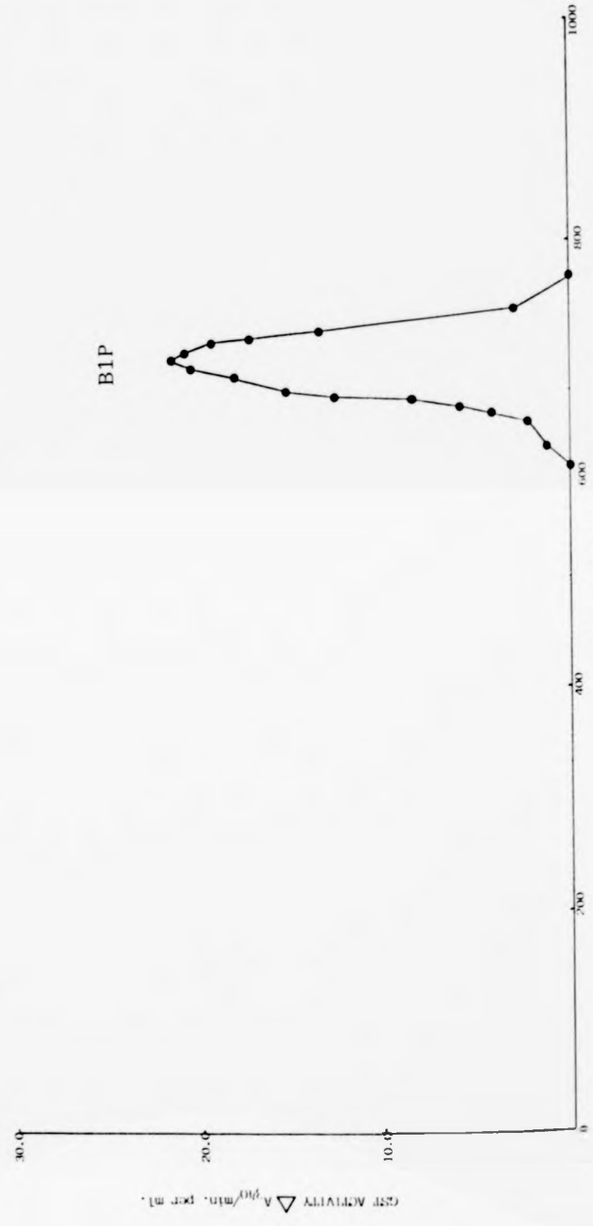


Figure 14 (b).

Figure 14(b) Elution profile of peak B1 (DEAE-Sephadex) from a Phenyl Sepharose column.

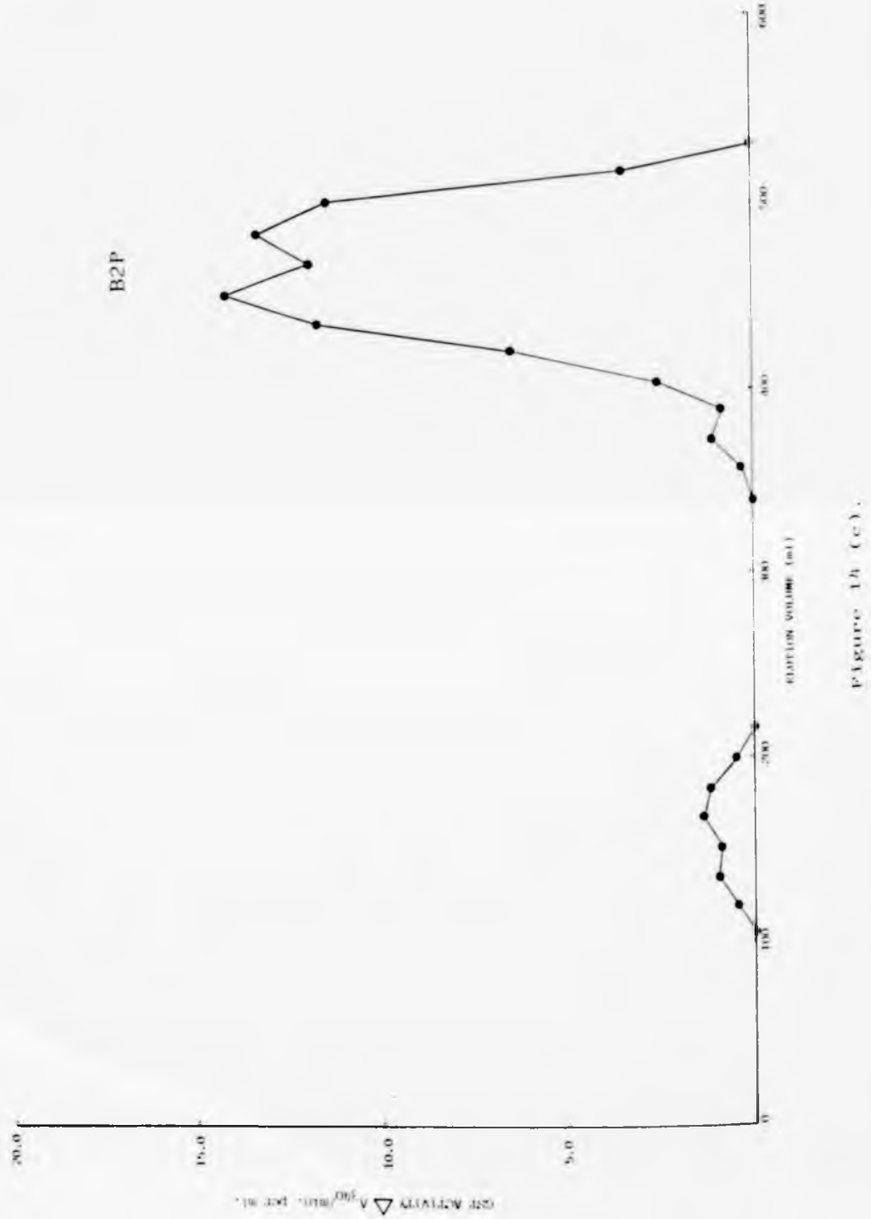


Figure 14 (c).

Figure 14(c) Elution profile of peak B2 (DEAE-Sephadex) from a Phenyl Sepharose column.

Figure 14(d) Elution profile of peak B1P (Phenyl Sepharose) from a GSH affinity column.

from the B2P also eluted as two peaks from the GSH-affinity column. The first peak, eluted in the "flow-through" fractions $B2PA_1$, contained about the same amount of activity as that eluted with GSH ($B2PA_2$) fig. 14(e).

Examination of the B1PA, $B2PA_1$ and $B2PA_2$ peaks using SDS discontinuous polyacrylamide gel electrophoresis showed that each was homogenous and contained the same single monomer.

The separation scheme described has not exhaustively attempted to separate the different basic isoenzymes present in liver cytosols and it is possible that whilst the final peaks were homogenous with respect to proteins other than GST, they still contained other GST isoenzymes that would not be identified by using GSH-affinity chromatography. It is interesting however that five isoenzyme forms were identified, the same number of forms reported by Kamisaka et al. (1975). Whereas other forms of GST may be present, it seems unlikely that a large amount of undetected variation exists within this isoenzyme set.

3.3. DEVELOPMENT OF THE GST ISOENZYMES.

The ontogeny of the GST in humans was studied by comparing the expression of the isoenzymes in tissue sets obtained from individuals during development. In the first series of experiments, the expression of the

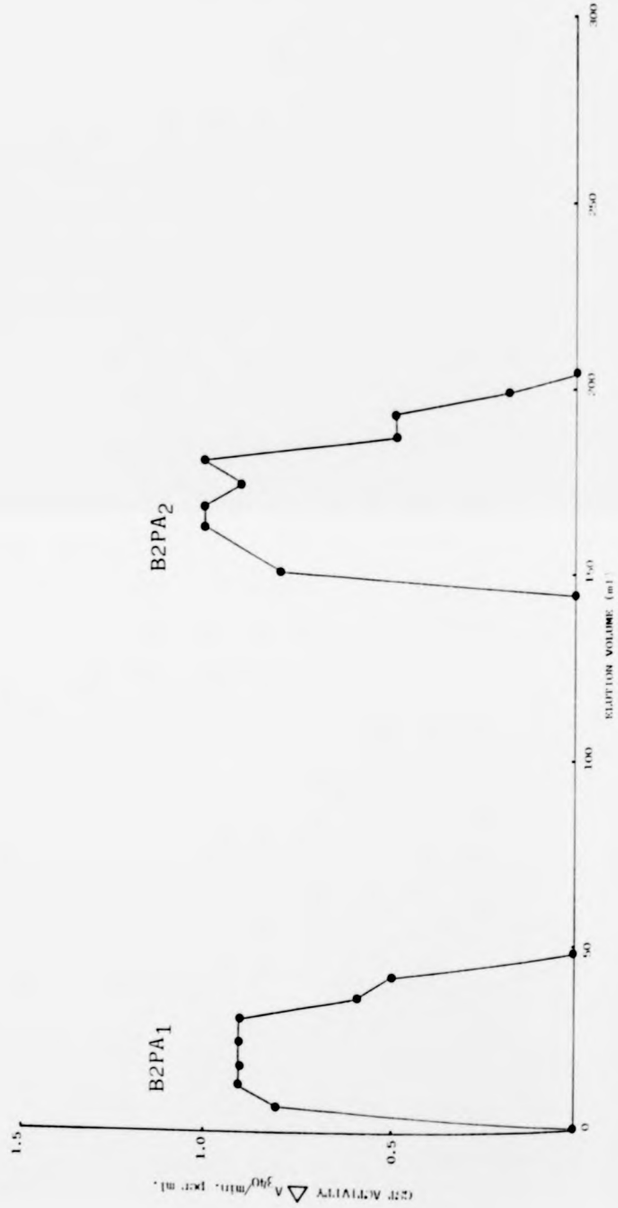


Figure 14 (e).

Figure 14(e) Elution profile of peak B2P (Phenyl Sepharose) from a GSH affinity column.

loci was surveyed using starch-gel electrophoresis. The expression of the different isoenzyme sets (basic, near-neutral and acidic) was then quantified using chromatofocusing. Further, more detailed investigation of the basic isoenzymes was carried out using ion-exchange chromatography.

3.3.1. Starch-gel Electrophoresis Studies of GST Development.

Studies in adults have shown that liver strongly expresses the basic and near-neutral isoenzymes and this tissue was therefore studied to obtain a reference for the ontogeny of the basic isoenzymes.

3.3.2. Basic Isoenzymes.

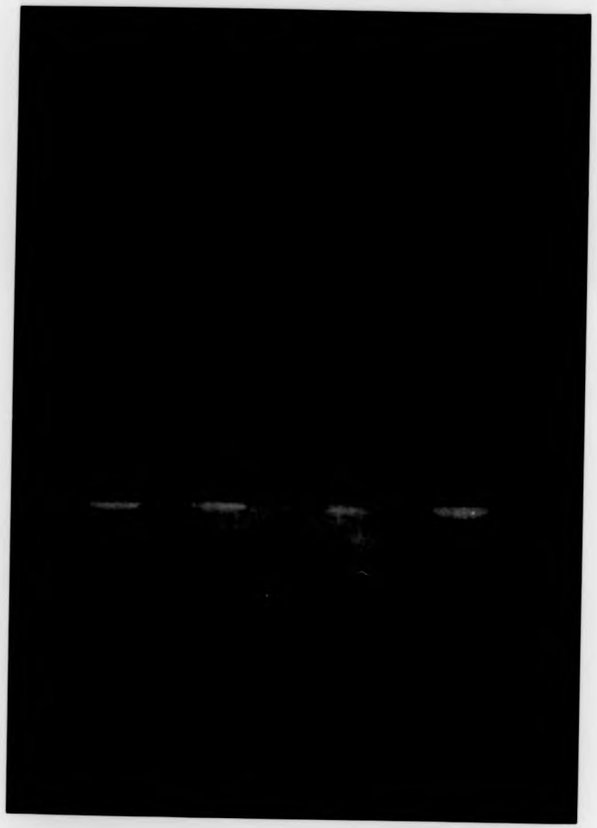
Liver cytosols were prepared from subjects of ten weeks gestation and thereafter up to about one year post-natal age. A total of one hundred liver samples were studied and in each of these the GST2 isoenzymes were found to be strongly expressed. The electrophoretic patterns were similar to those seen in adults; a band with fast cathodal mobility was always seen, the band with intermediate cathodal mobility was present in about 50% of the samples but the band with slowest cathodal mobility was not seen. The mobility of the bands of fast and intermediate cathodal mobility was the same as corresponding bands in adult cytosols.

Basic isoenzymes were also consistently identified in adrenal cytosol obtained during development. The zymogram patterns of these samples were identical to those seen in adult adrenal in that only one band with fast cathodal mobility was present. This band had the same mobility as the corresponding band in the adult tissue.

Although strongly expressed in kidney cytosol obtained from adults, the ontogeny of the basic isoenzymes in this tissue was surprising. They were either not present or only weakly expressed in the foetal and neonatal samples studied (figures 15 and 16). They were first strongly expressed at about 45 weeks post-natal age as two bands of fast and intermediate cathodal mobilities and with intensities similar to those found in adult samples.

The basic isoenzymes were found to be weakly expressed in diaphragm, heart and spleen from adults and, as expected, their expression during development was also weak. Figure 17 and 18 show the starch-gel electrophoresis patterns after 17 days and 17 weeks post-natal age. Both individuals appear to be unable to express the basic isoenzyme in diaphragm or heart muscle although acidic isoenzymes were present in high activity.

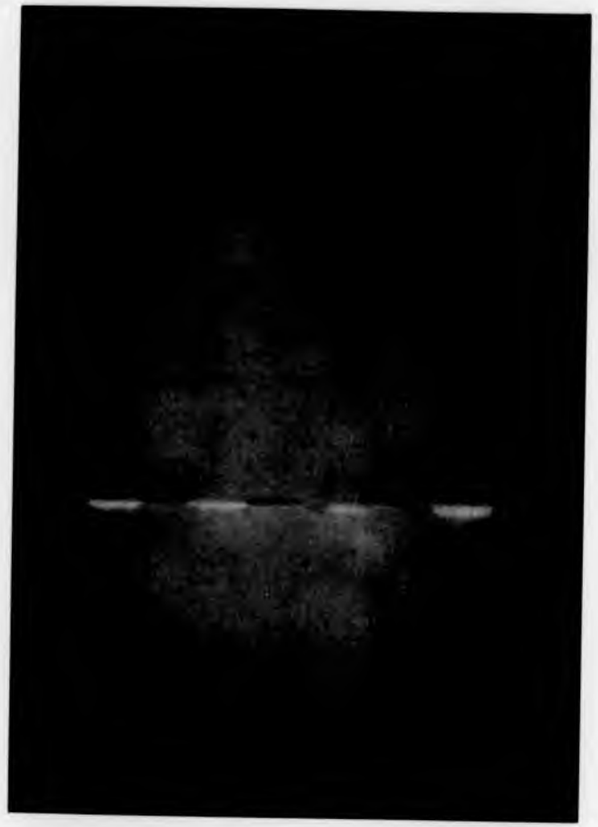
At the end of each starch-gel electrophoresis run it was necessary to record the results using a



(i) (ii) (iii) (iv)

Figure 15.

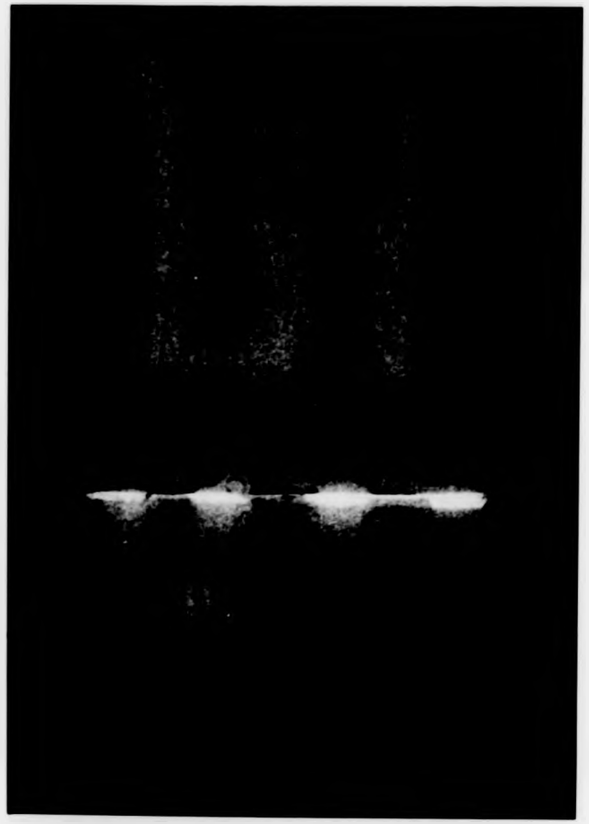
Figure 15. Starch-gel electrophoresis of kidney cytosols (i) 39 weeks gestation, (ii) 31 weeks gestation, (iii) 28 weeks gestation (iv) 40 weeks gestation, post-natal age 17 days.



(i) (ii) (iii) (iv)

Figure 15.

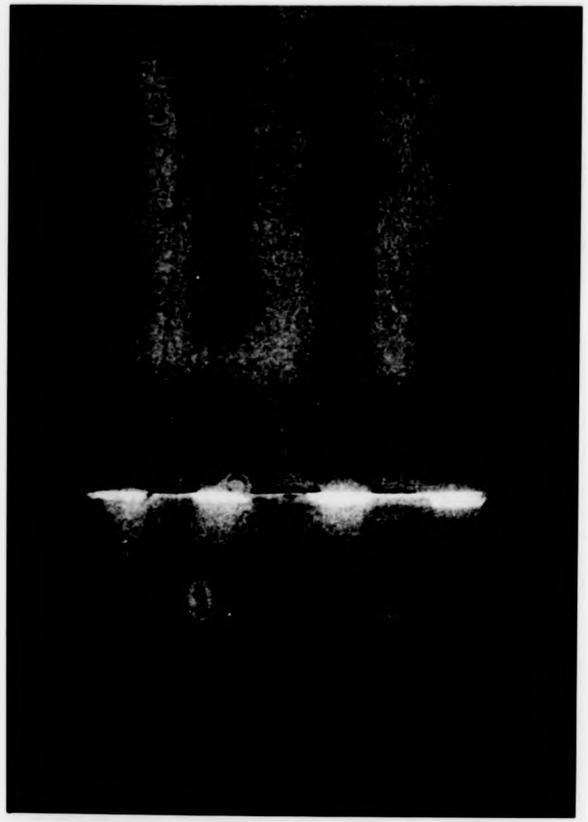
Figure 15. Starch-gel electrophoresis of kidney cytosols (i) 39 weeks gestation, (ii) 31 weeks gestation, (iii) 28 weeks gestation (iv) 40 weeks gestation, post-natal age 17 days.



(i) (ii) (iii) (iv)

Figure 16.

Figure 16. Starch-gel electrophoresis of cytosols from (i) kidney 25 weeks gestation, (ii) kidney 26 weeks gestation, (iii) kidney 40 weeks gestation, post-natal age 9 weeks. (iv) adult liver.



(i) (ii) (iii) (iv)

Figure 16.

Figure 16. Starch-gel electrophoresis of cytosols from (i) kidney 25 weeks gestation, (ii) kidney 26 weeks gestation, (iii) kidney 40 weeks gestation, post-natal age 9 weeks. (iv) adult liver.

(i) (ii) (iii) (iv)

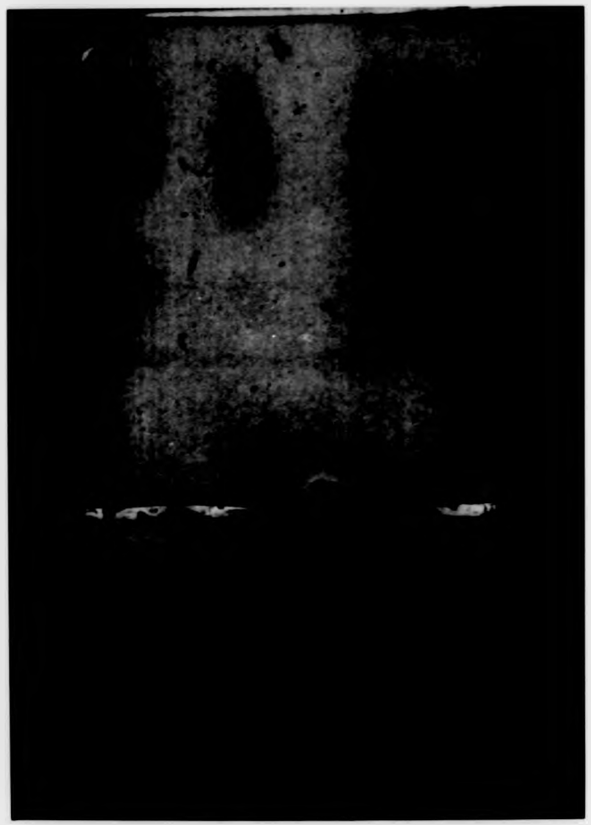
Figure 17.

Figure 17. Starch-gel electrophoresis of (i) heart, (ii) diaphragm, (iii) adrenal and (iv) liver cytosols from the same individual 40 weeks gestation, post-natal age 17 days (GST1 0 phenotype).

(i) (ii) (iii) (iv)

Figure 17.

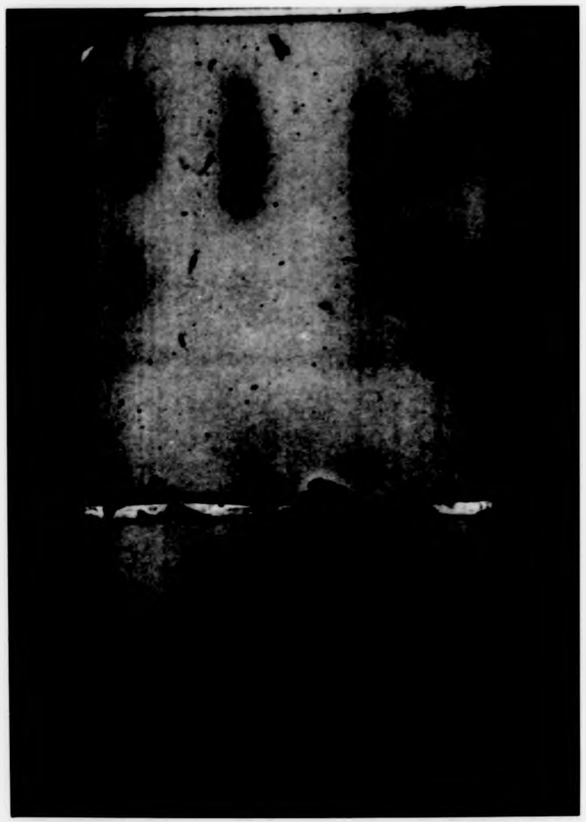
Figure 17. Starch-gel electrophoresis of (i) heart, (ii) diaphragm, (iii) adrenal and (iv) liver cytosols from the same individual 40 weeks gestation, post-natal age 17 days (GST1 0 phenotype).



(i) (ii) (iii)

Figure 18.

Figure 18. Starch-gel electrophoresis of (i) spleen, (ii) heart, (iii) diaphragm cytosols from the same individual 40 weeks gestation, post-natal age 17 weeks.



(i) (ii) (iii)

Figure 18.

Figure 18. Starch-gel electrophoresis of (i) spleen, (ii) heart, (iii) diaphragm cytosols from the same individual 40 weeks gestation, post-natal age 17 weeks.

Polaroid camera. In many cases it was not possible to obtain a negative and since some of the photographs were not suitable for reproduction they were redrawn by an artist (figure 19). This figure shows the results of three further starch-gel electrophoresis experiments and figure 20 shows the results obtained when tissue sets from four individuals were studied.

The results of the starch-gel electrophoresis experiments are summarised in figure 21. They have been presented in block diagram format and are intended to show typical results obtained at the different stages of development. They show that there was essentially no change in the pattern of basic isoenzymes in liver, that expression in adrenal increases after the first twenty weeks of gestation and that expression in kidney of this isoenzyme set is weak.

3.4. USE OF CHROMATOFOCUSING TO QUANTIFY GST ISOENZYME SETS DURING DEVELOPMENT.

The data described in the previous sections have shown marked changes in the expression of the different isoenzyme sets. Some of these changes were easily identifiable since an isoenzyme strongly expressed in the earliest stages of development was down-regulated in the perinatal period. The acidic isoenzymes in liver cytosol provide an example of this phenomenon. In other cases however, changes in expression appear to happen

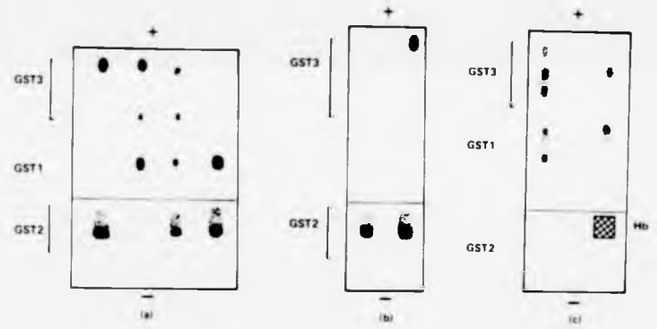
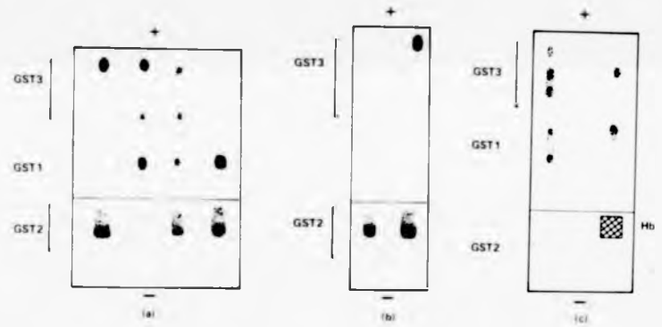


Figure 19.

Figure 19 Starch-gel electrophoresis patterns of Tissue cytosols.

Starch-gel electrophoresis was performed as described in the Materials and Methods. (a) left to right - liver: 15 weeks gestation; spleen, kidney; 40 weeks gestation, postnatal age 45 weeks (phenotype GST1 2); liver: adult (phenotype GST1 2). (b) left to right - liver: 40 weeks gestation, postnatal age 40 weeks (phenotype GST1 0); liver, 11 weeks gestation. (c) left to right - kidney: 25 weeks gestation, postnatal age 1 day; erythrocytes: adult.



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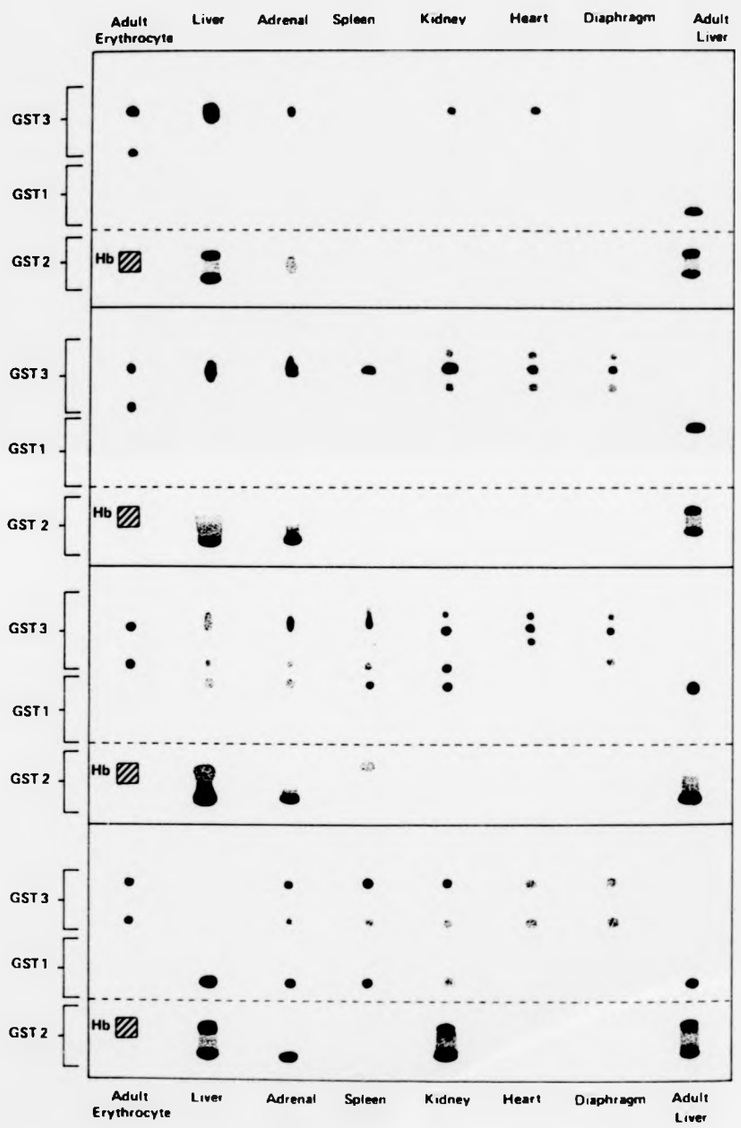

Figure 20.

Figure 20 Starch-gel electrophoresis patterns of tissue cytosols.

Starch-gel electrophoresis was performed as described in the Materials and Methods section. Results show (top to bottom) tissues from: foetus (14 weeks gestation) and adult liver (GST1 2 phenotype); Neonate (25 weeks gestation) and adult liver (GST1 1 phenotype); neonate (31 weeks gestation) and adult liver (GST1 1 phenotype) and infant (40 weeks gestation, postnatal age 45 weeks) and adult liver (GST1 2 phenotype). The electrophoretic pattern of the GST3 isoenzymes of human erythrocytes is also shown.

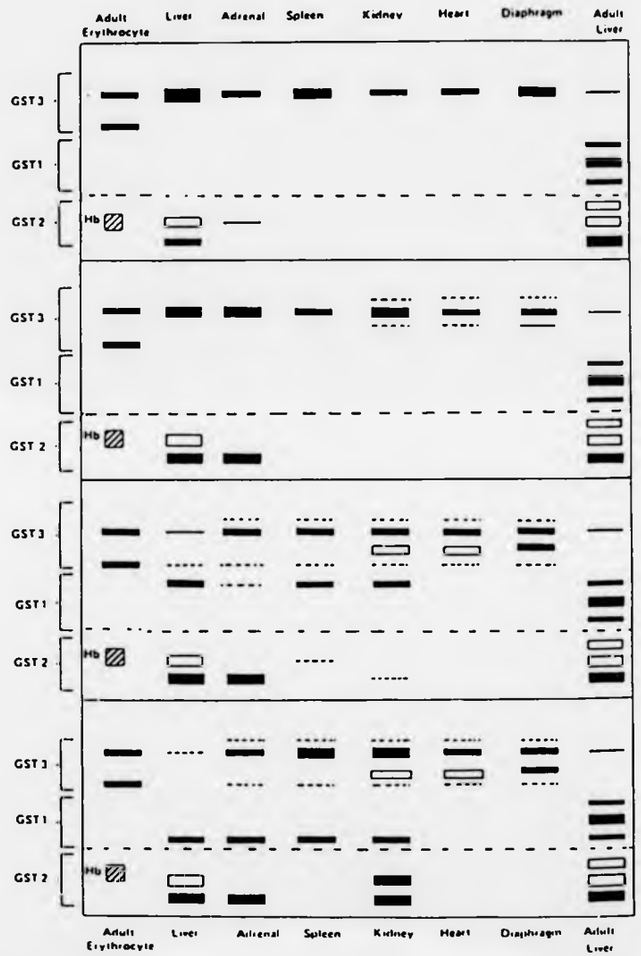

Figure 21.

Figure 21 Block diagram format of starch-gel electrophoresis profiles to show typical results of the developmental expression of GST isoenzymes in different tissues.

Top to bottom:

First developmental group 10-20 weeks gestation.

Second developmental group 21-30 weeks gestation.

Third developmental group 31-42 weeks gestation.

Fourth developmental group 2-67 weeks post-natal age.

Patterns from the third group are from a GST1 1 phenotype. Patterns from the fourth group are from a GST1 2 phenotype. A representative adult (GST1 2-1 phenotype) pattern is shown on the right for comparison.

Isoenzymes that were a constant individual characteristic are shown as filled blocks. Those that were usually, but not invariably, present are shown by open blocks. Relative activities of these isoenzymes are shown by the area of the blocks.

gradually and it was not easy to determine when isoenzyme sets were first expressed or first down-regulated. This is an expected drawback of the use of a qualitative technique such as starch-gel electrophoresis. To obtain quantitative data a further series of experiments was performed in which the isoenzyme sets in tissue cytosols were separated by use of chromatofocusing. Preliminary experiments showed that good separation of the sets was achieved using the conditions described in the Materials and Methods section and although only partial resolution of the basic isoenzymes was achieved, this approach was considered preferable to the use of ion-exchangers which were more time-consuming and offered less effective resolution. Preliminary experiments suggested that rather better resolution of the basic isoenzymes would be achieved by using a gradient between pH 9.0 and pH 4.0, however, comparison of the profiles obtained using this range and the range described in Materials and Methods (pH 7.3 to pH 4.0) showed that the use of the more alkaline range resulted in considerable loss of both the acidic and near-neutral isoenzymes (figure 22a,b).Subsequent experiments were performed therefore using a gradient of pH 7.3 to pH 4.0. It is interesting to note that Koskelo and Icen (1984) also found a substantial loss of activity of acidic GST when they chromatofocused adult human kidney cytosol with a gradient of pH 9.6 to pH 4.0

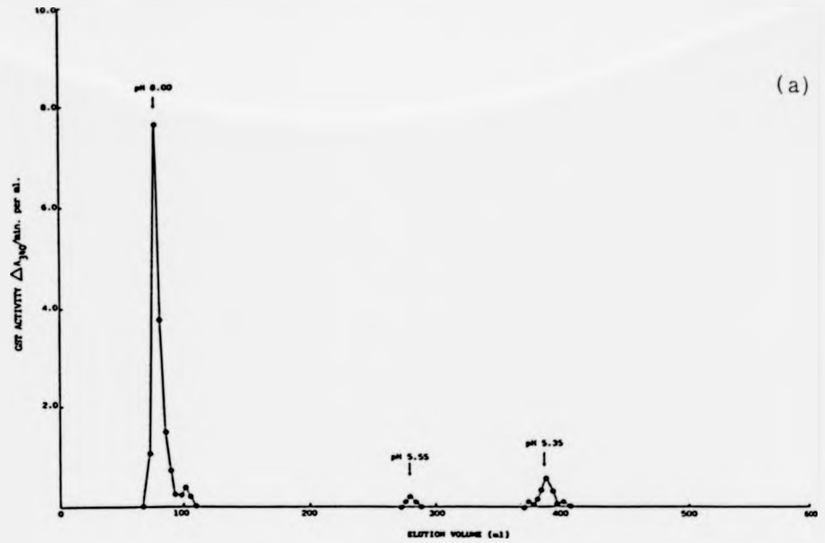

Figure 22.

Figure 22(a) Chromatofocusing profile of a foetal liver cytosol developed using a pH gradient of pH 9-4.

Figure 22(b) Chromatofocusing profile of the same foetal liver cytosol developed using a pH gradient of pH 7-4.

3.4.1. Chromatofocusing of Adult Liver Cytosol.

To assess the resolving power of the chromatofocusing system, cytosol from an adult with the GST1 2-1 phenotype was eluted from the column and each of the peaks obtained was examined using starch-gel electrophoresis. Figure 23 shows the elution profile obtained as well as the corresponding electrophoretic pattern. The basic isoenzymes eluted first as three peaks of activity. The first peak was not retained by the column and corresponded to the isoenzyme with fast cathodal mobility. The second peak eluted at pH 6.85 and corresponded to the isoenzyme of intermediate cathodal mobility. A third peak of activity that corresponded to the isoenzyme of slow cathodal mobility eluted at a pH of 6.80. Three isoenzymes classified as the products of the GST1 locus were readily identified on the starch-gels and corresponding enzymes were eluted from the column as shown in figure 23. The GST1 2 isoenzyme eluted at pH 6.35, the interallelic hybrid isoenzyme (GST1 1/GST1 2) at pH 6.10 and the GST1 1 isoenzyme at pH 5.85. A small peak of activity was also eluted at pH 5.05 and this corresponded to the GST3 isoenzyme on the starch-gel (data not shown). The resolution achieved was considered to be satisfactory and subsequent experiments, in which about one hundred cytosol samples were similarly chromatofocused, confirmed that the

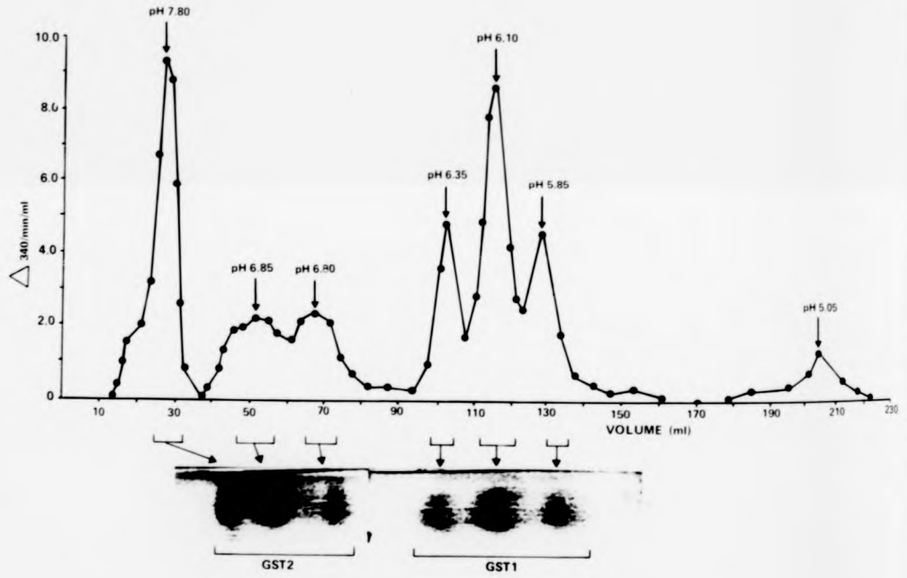

Figure 23.

Figure 23 Chromatofocusing profile of an adult liver cytosol from a GST1 2-1 heterozygous individual. The corresponding starch-gel electrophoresis pattern is also shown.

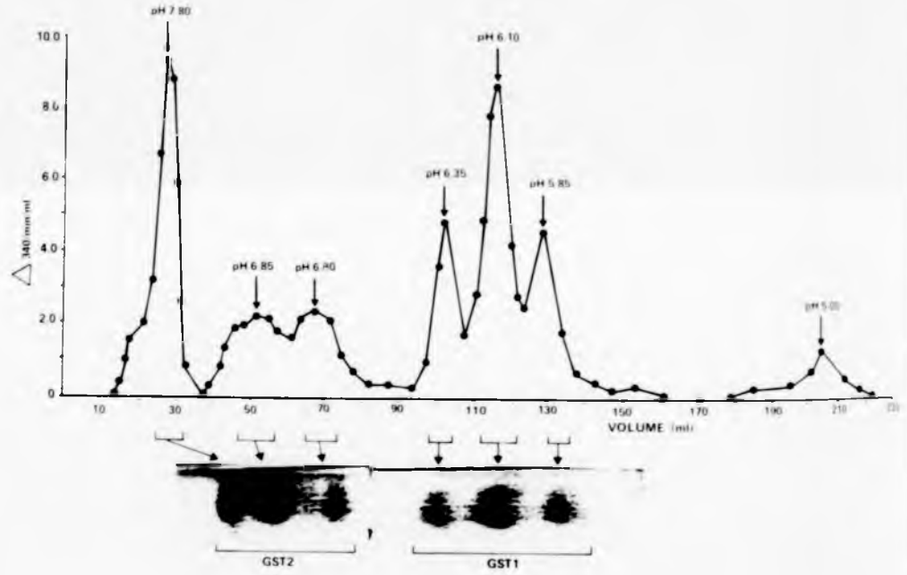

Figure 23.

Figure 23 Chromatofocusing profile of an adult liver cytosol from a GST1 2-1 heterozygous individual. The corresponding starch-gel electrophoresis pattern is also shown.

separation of the isoenzyme sets was always acceptable. Some variation in elution pH was noted but this was generally small and in cases of doubt starch-gel electrophoresis was used to classify isoenzymes as products of either GST1, GST2 or GST3.

A further eight cytosols from adult liver were also examined using chromatofocusing to determine the percentage contribution of each of the three isoenzyme sets to total cytosol activity. Figure 24 shows the profiles of cytosols from individuals with GST1 0, GST1 1 and GST1 2 phenotypes. In each case the basic isoenzymes eluted at the expected pH values although it is clear from the patterns that only partial resolution of this set has been achieved. The near-neutral isoenzymes however were well separated from the basic and acidic isoenzymes and they also eluted at the pH values expected from the data shown in figure 23. The subject with the GST1 0 phenotype demonstrated no GST activity in the fractions that eluted between pH 6.40 and pH 5.80. Table 4 shows the percentage contribution of the isoenzyme sets to total cytosol activity. The GST1 isoenzyme set appears to contribute about 40% of activity in cytosol of subjects with the GST1 1 or GST1 2 phenotypes and in the single subject with the GST1 2-1 phenotype approximately 50% activity. Previously, studies from our laboratory have suggested that most subjects with the GST1 1 and GST1 2 phenotypes will be hetero-

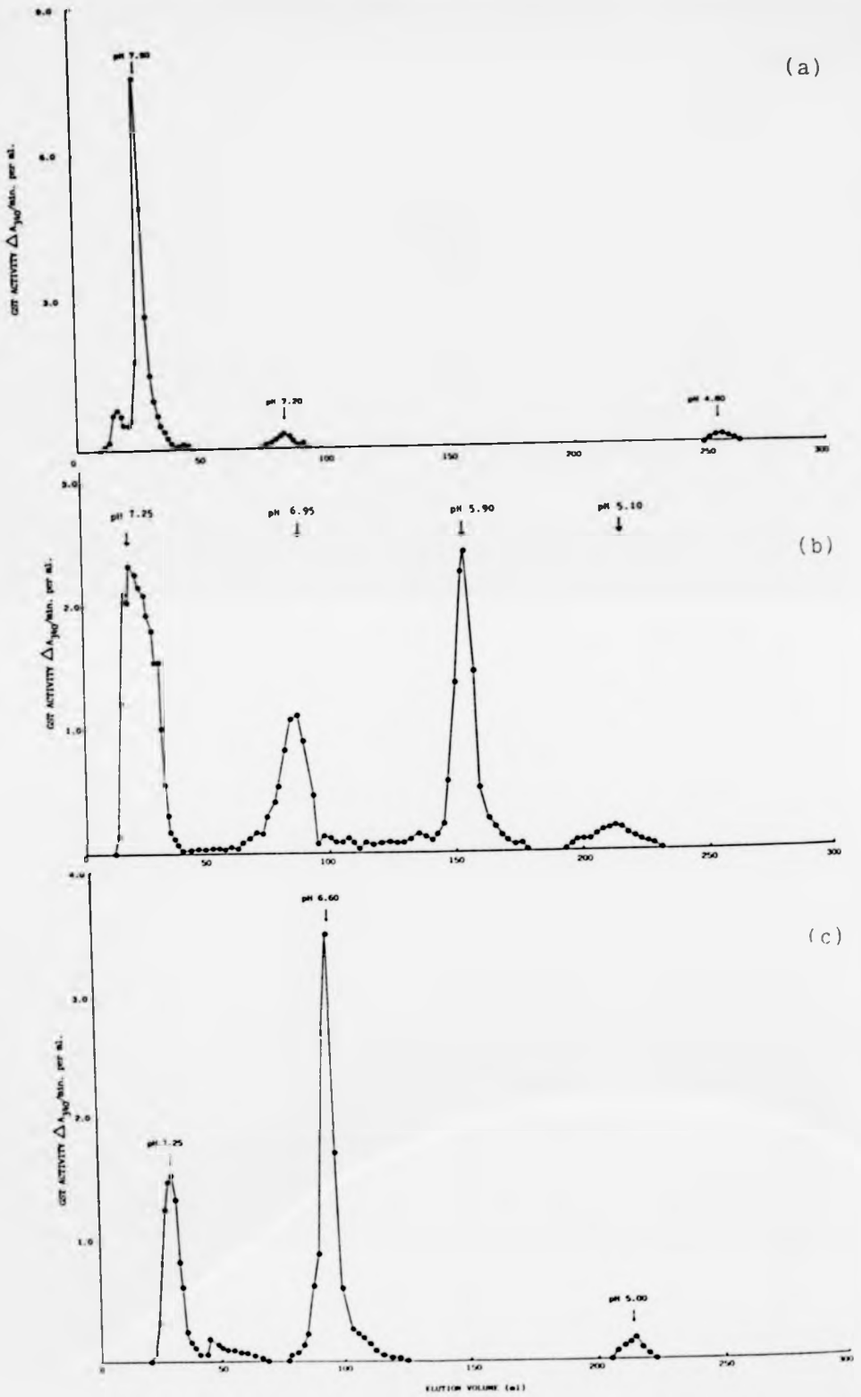

Figure 24.

Figure 24(a) Chromatofocusing profile of an adult liver cytosol GST1 0 (null) phenotype.

24(b) Chromatofocusing profile of an adult liver cytosol GST1 1 phenotype.

24(c) Chromatofocusing profile of an adult liver cytosol GST1 2 phenotype.

Percentage contribution of GST isoenzyme sets to activity in cytosol from subjects with different GST phenotypes. Data show mean ± SD with number of samples in parentheses.

	GST1	GST2	GST3
GST1 0	2.4 ± 4.1(3)	92.1 ± 11.9(3)	5.5 ± 7.8(3)
GST1 1	36.5 (2)	61.5 (2)	
GST1 2	47.2 ± 15.8(3)	51.3 ± 16.7(3)	1.6 ± 1.4(3)
GST1 2-1	49.6 (1)	44.4 (1)	6.1 (1)

Table 4.

zygous for the GST1*1, GST1*2 and GST1*0 alleles. Thus about one in eight of individuals with the GST1 1 and GST1 2 phenotypes will be homozygotes (GST1*1/GST1*1 or GST1*2/GST1*2). It would be expected that these homozygotes would show more GST1 activity than those of the heterozygous genotypes and some of the variation seen within these phenotypes may result from the presence of homo- and heterozygotes.

The results of these chromatofocusing experiments were used as indices of full expression of the GST isoenzyme sets. Similar experiments were carried out using adrenal, kidney and spleen cytosols obtained from adults and these are described below.

3.4.2. Chromatofocusing Studies of GST Development.

A series of cytosols obtained from adrenal, kidney, liver and spleen at different stages of development were chromatofocused in the same way as the adult tissues described previously. The starch-gel electrophoresis studies had already indicated that no specifically fetal isoenzyme forms of GST are present in humans and the chromatofocusing profiles were therefore interpreted in the same way as those of adults. The results of these experiments are described below for each of the isoenzyme sets which have been considered separately.

Since the starch-gel electrophoresis studies had indicated that there were changes in the expression of

the different isoenzyme sets it was considered important to assay total cytosol activity before chromatofocusing to determine whether developmental changes could be identified. Table 5 shows activity at different stages of development in various tissues. In each of these, GST activity demonstrated marked inter-individual variation and it was not possible therefore to identify any trends in activity associated with development. It is possible that trends in activity do occur, but because of the wide variation these would be masked unless they were at least an order of magnitude. The results of the chromatofocusing experiments are presented as the percentage contribution of each of the isoenzyme sets to total activity.

3.4.3. Development of the Basic Isoenzymes ($\alpha, \beta, \gamma, \delta, \varepsilon$; GST2)

Basic GST isoenzymes were expressed in all foetal, neonatal and infant liver cytosols studied; the earliest was from an individual of 10 weeks gestation. Figure 25 shows a chromatofocusing profile obtained after elution of a 14 week gestation liver cytosol. Starch-gel electrophoresis revealed that the component with the fastest cathodal mobility was always present. This electrophoretic band and the band with intermediate cathodal mobility was observed in about 50% of the specimens.

Figure 26 (i-v) shows chromatofocusing profiles from different gestational and post-gestational ages. Although

GST activity in foetal, neonatal and infant tissues.

GST activity was determined in tissue cytosols obtained during development.

The results shown are mean ± S.D. with the number of samples shown in parenthesis.

GST activity (μmol/min per mg cytosol protein)

	10-20 weeks	21-30 weeks	31-40 weeks	40 weeks onwards.
Adrenal	0.45 ± 0.23(9)	0.42 ± 0.14(4)	0.85 ± 0.48(4)	0.42 ± 0.28(8)
Diaphragm	-	0.17 ± 0.06(4)	0.17 ± 0.06(3)	0.27 ± 0.18(9)
Heart	0.27 ± 0.70(9)	0.37 ± 0.20(5)	0.27 ± 0.05(4)	0.50 ± 0.52(6)
Kidney	0.39 ± 0.80(9)	1.05 ± 0.93(5)	0.28 ± 0.02(4)	0.66 ± 0.46(9)
Liver	0.63 ± 0.27(14)	0.43 ± 0.21(12)	0.60 ± 0.19(6)	1.32 ± 1.65(16)
Spleen	0.28 ± 0.20(6)	0.24 ± 0.19(6)	0.18 ± 0.09(4)	0.29 ± 0.27(9)

Table 5.

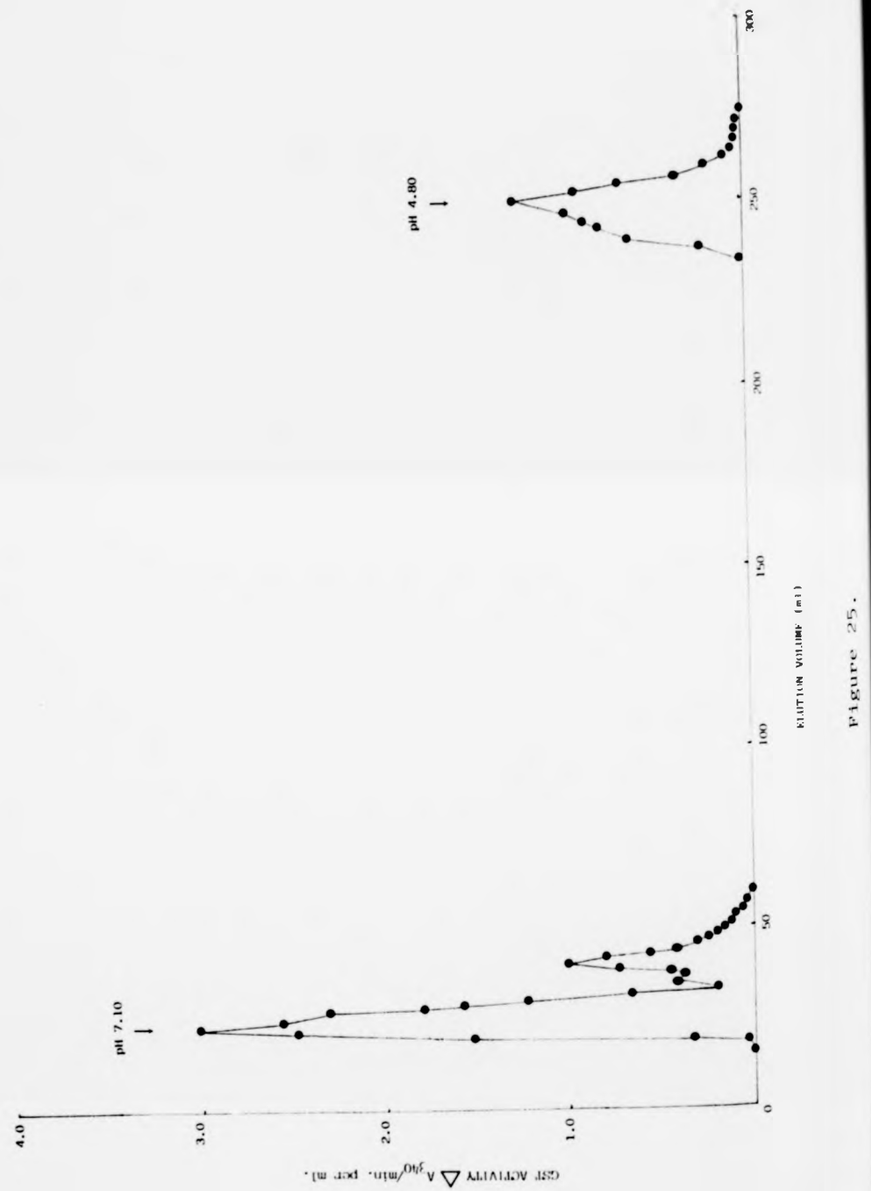

Figure 25.

Figure 25 Chromatofocusing profile of a foetal liver cytosol 14 weeks gestational age.

Figure 26 (i).

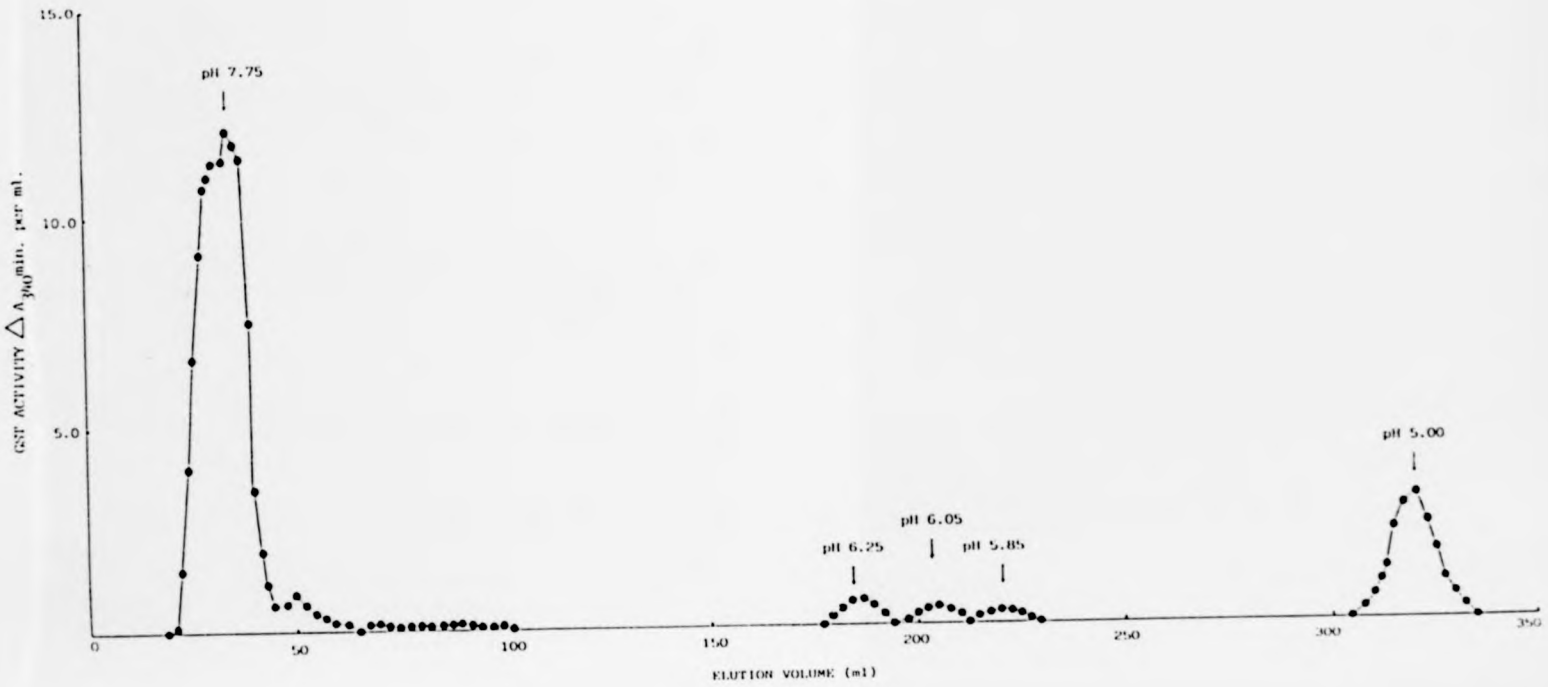

Figure 26 (ii).

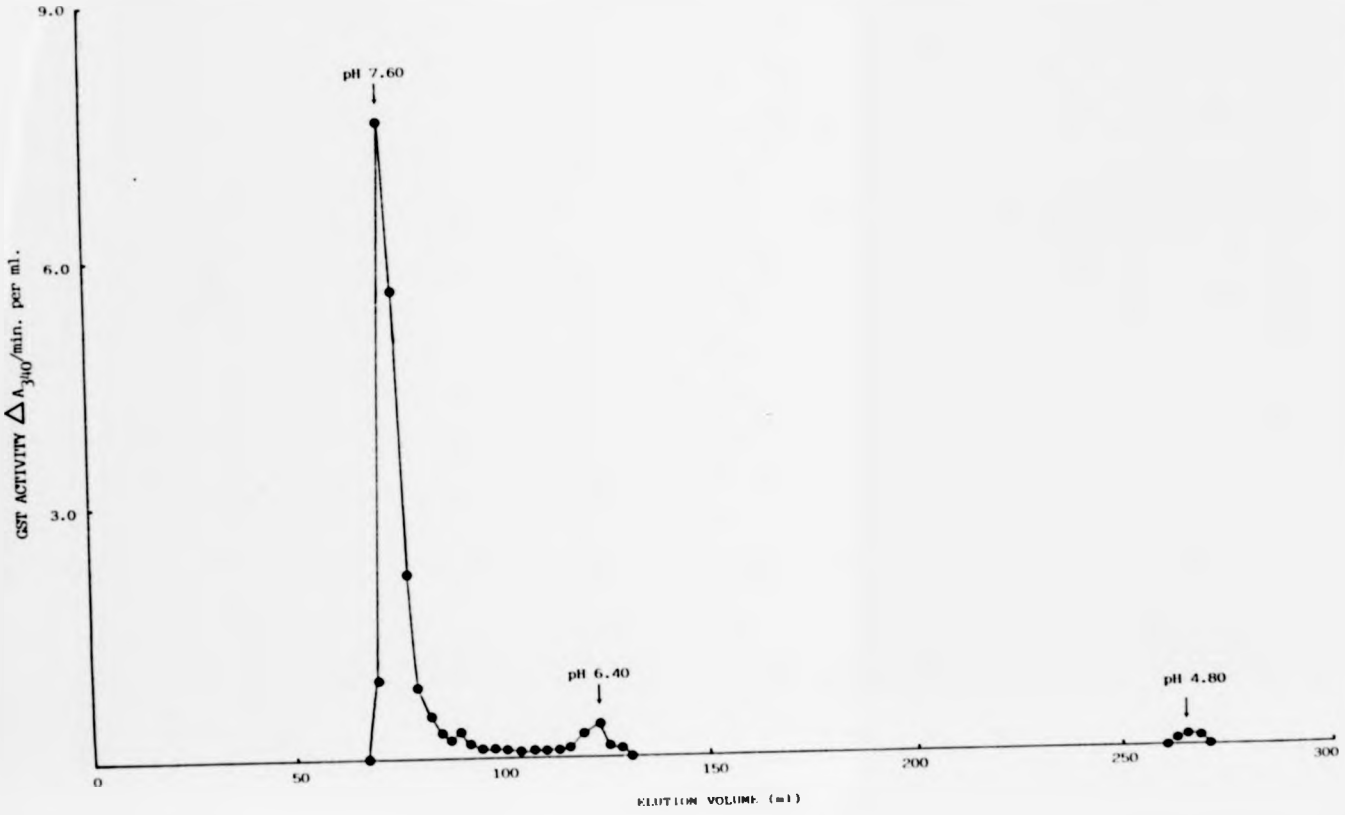

Figure 26 (iii).

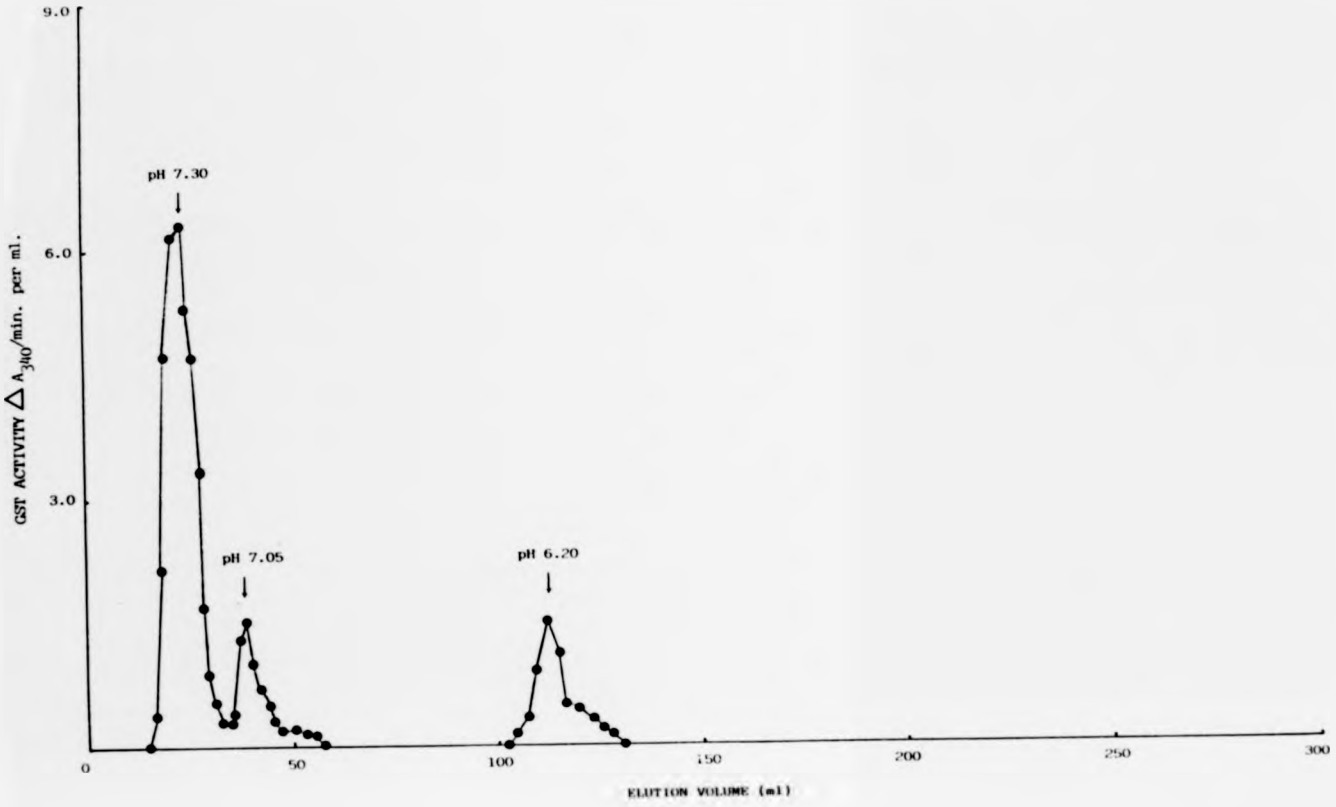

Figure 26 (iv).

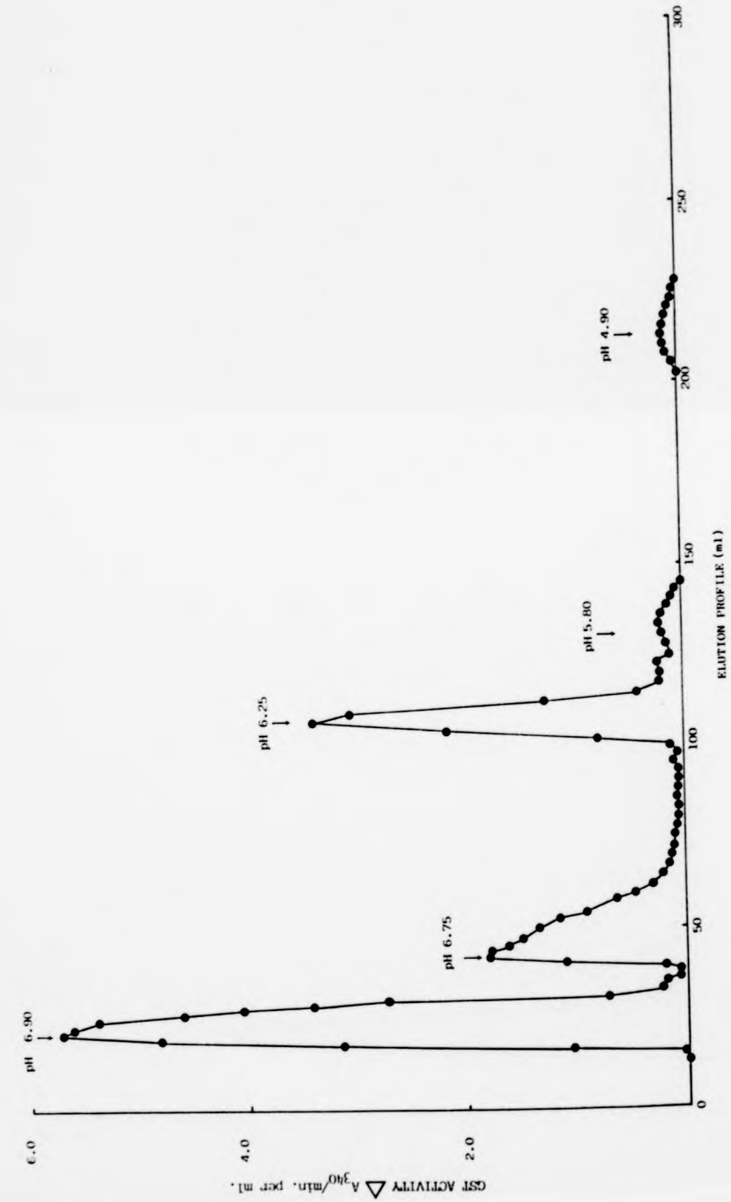

Figure 26 (v).

Figure 26 Chromatofocusing profile of foetal liver cytosols.

(i) 25 weeks gestation.

(ii) 31 weeks gestation.

(iii) 39 weeks gestation (GST1 2)

(iv) Neonatal liver cytosol 40 weeks gestation 12 weeks postnatal age (GST1 2)

(v) Infant liver cytosol 40 weeks gestation 45 weeks postnatal age (GST1 2)

resolution of the basics is incomplete, there is evidence of increasing complexity of the set during post-natal development (figure 26 (iv) and 26 (v)). This observation is in agreement with data from CM-cellulose studies described later.

The percentage contribution of the basic GST isoenzymes to total activity was determined and figure 27 shows that this set was consistently strongly expressed from about 10 weeks gestation.

Figure 28 shows the chromatofocusing results of adrenal cytosols obtained from the same individuals. The basic isoenzymes were also consistently strongly expressed from early in gestation.

The basic isoenzymes from adrenal cytosols usually eluted from chromatofocusing columns as a single peak. However, figure 28 (v) shows two fully resolved basic peaks of GST activity in an individual of 45 weeks post-natal age. A similar pattern (not shown) was seen in a subject of 61 weeks post-natal age. It is possible that the basic GST from adrenal demonstrate a similar, age-related increase in complexity to that of liver.

Figure 29 shows that the percentage contribution of the basic isoenzymes in adrenal was similar to that of liver. Expression was consistently high during development.

Figure 30 (i-v) shows chromatofocusing profiles of corresponding kidney samples from the same individuals. The basic GST isoenzymes are seen to be weakly expressed during gestation, only at 45 weeks post-natal age is there a major contribution of basic GST to total cytosol activity (figure

resolution of the basics is incomplete, there is evidence of increasing complexity of the set during post-natal development (figure 26 (iv) and 26 (v)). This observation is in agreement with data from CM-cellulose studies described later.

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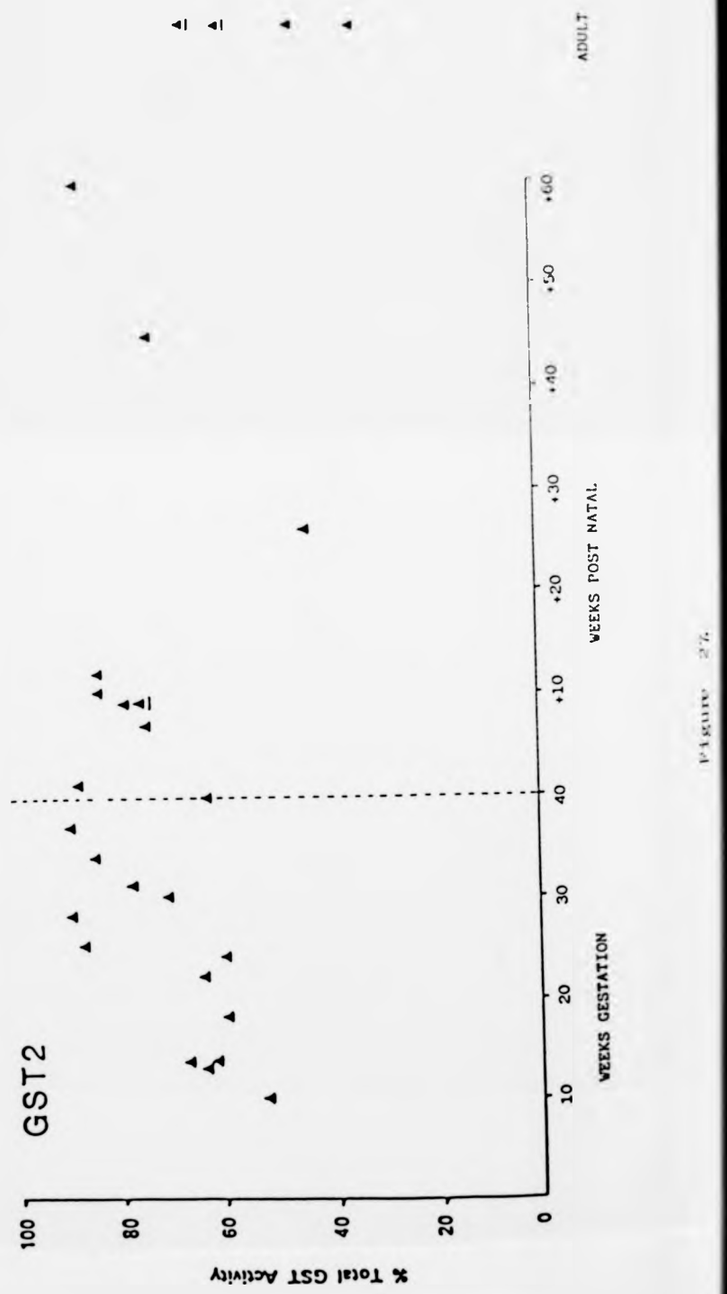


Figure 27 Percentage contribution of basic GST isoenzymes to total GST activity in liver cytosol throughout development. Typical adult contributions are shown on the right.

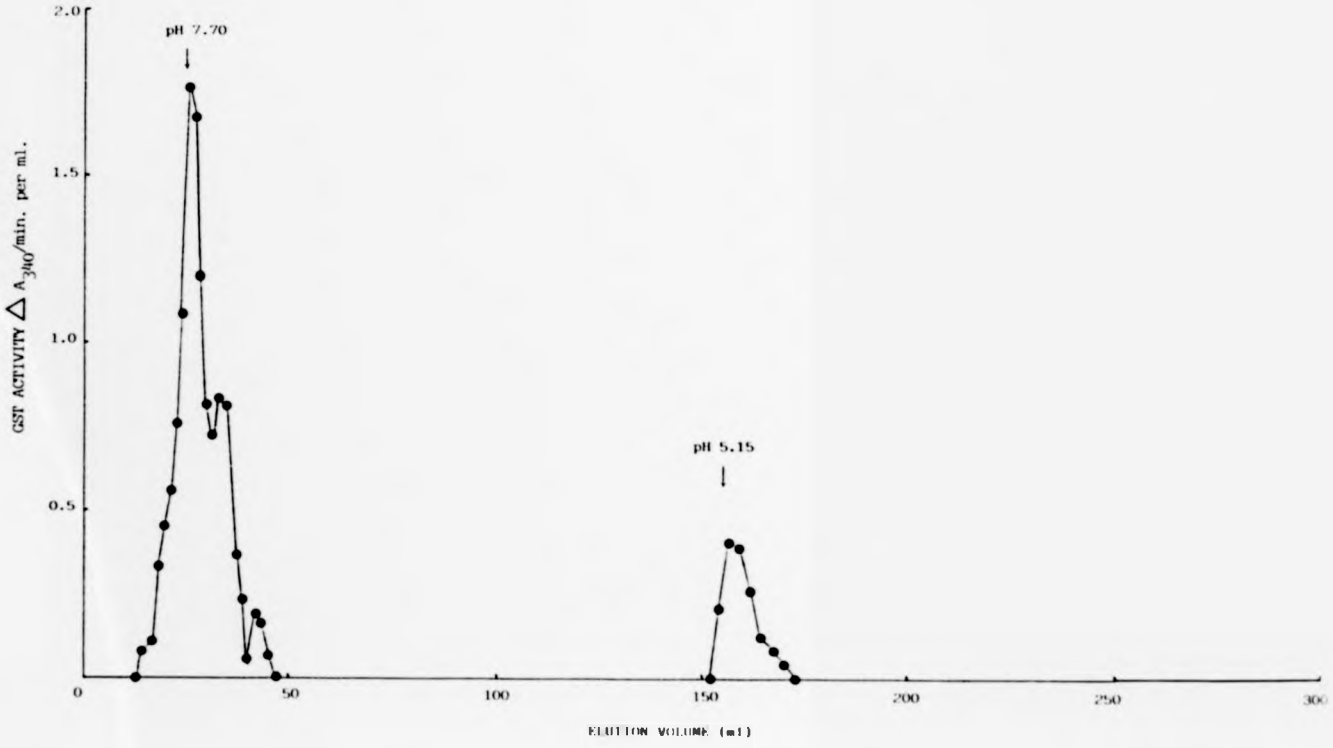


Figure 28 (i).

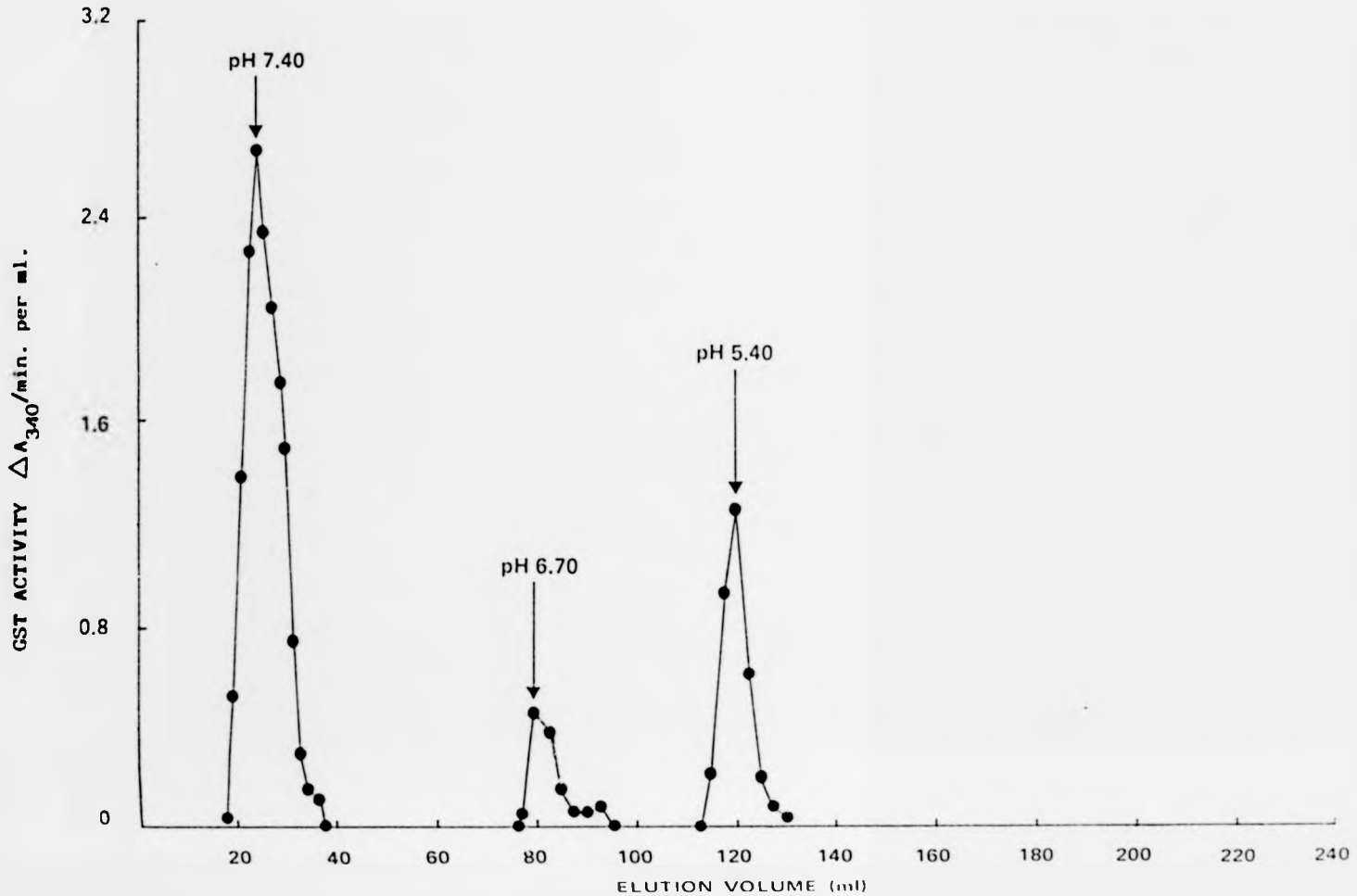


Figure 28 (11).

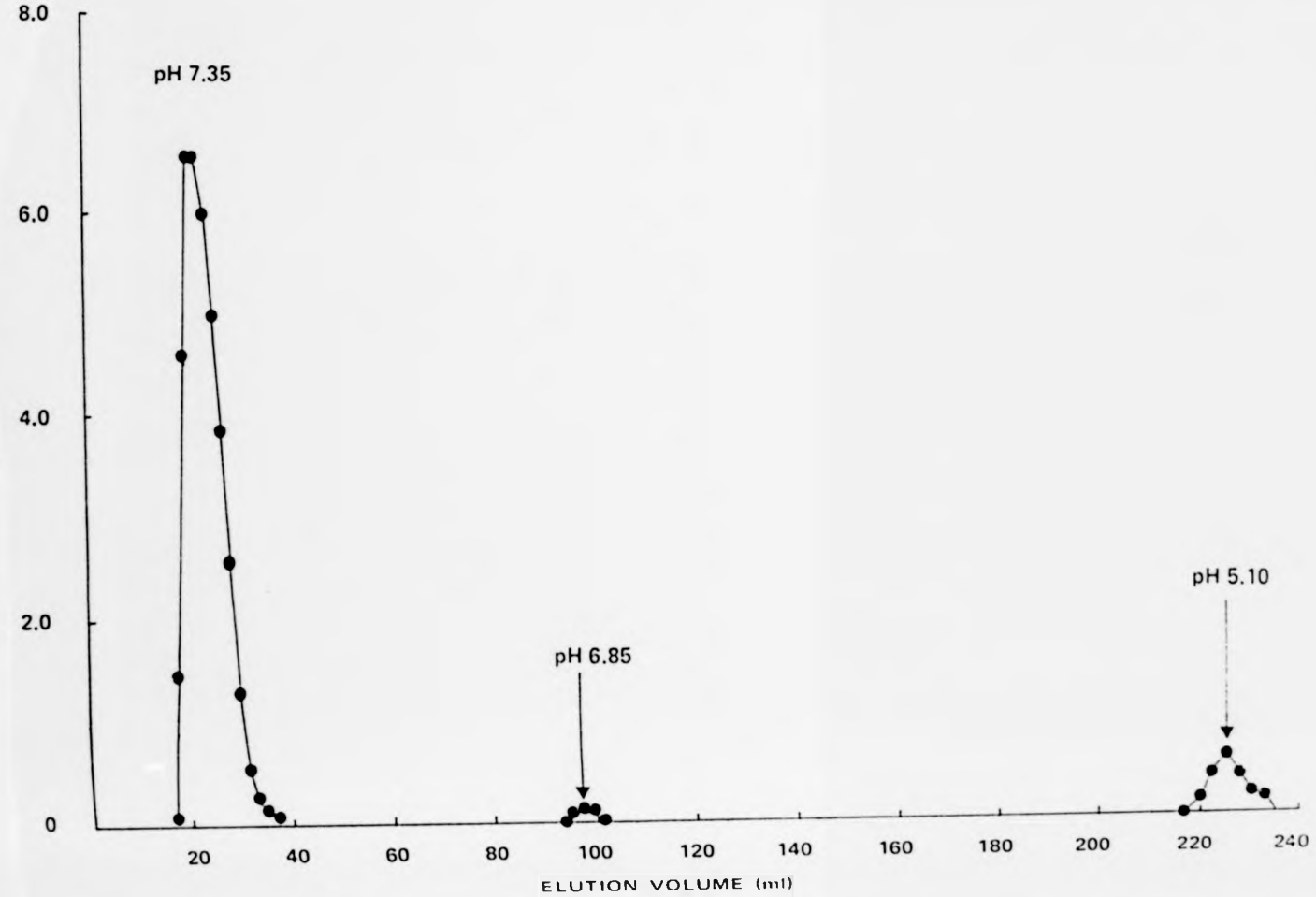


Figure 28 (111).

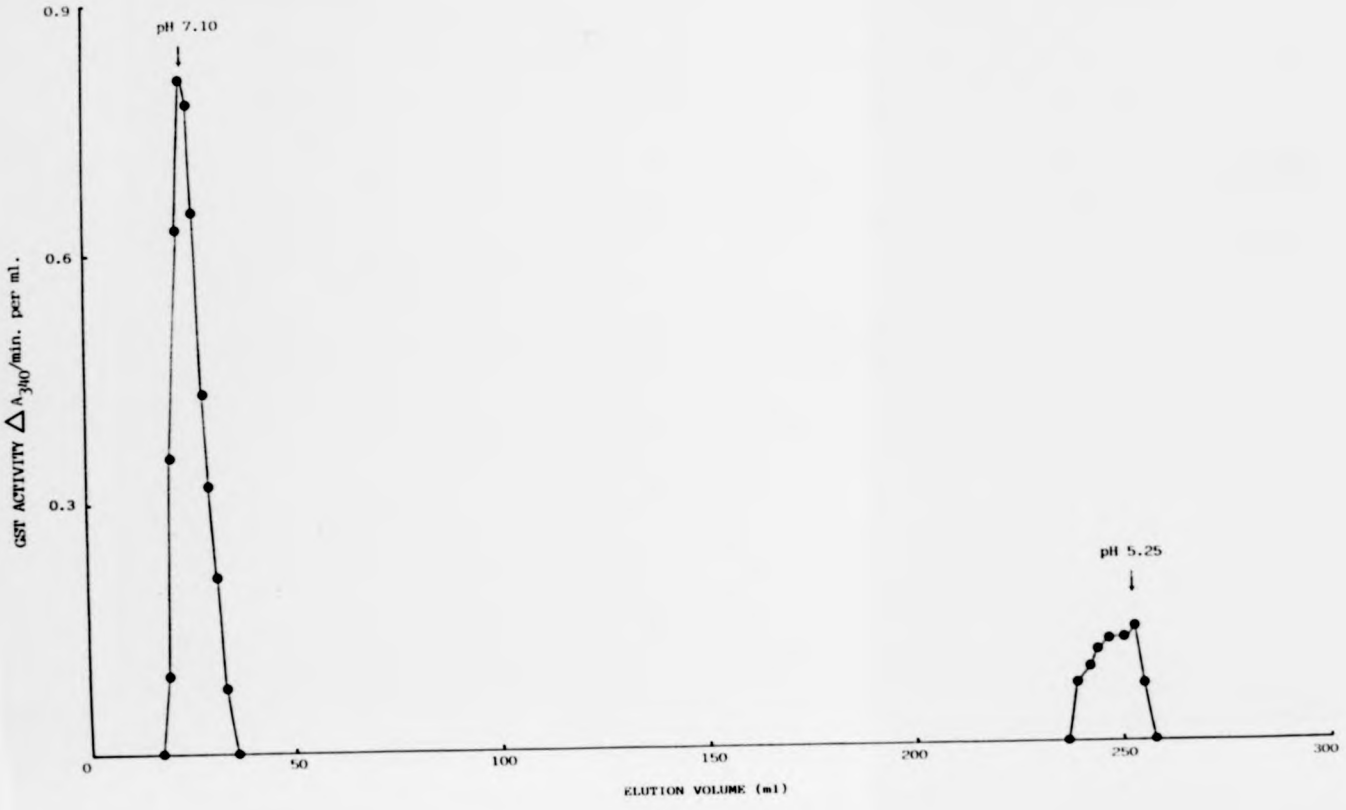


Figure 28 (iv).

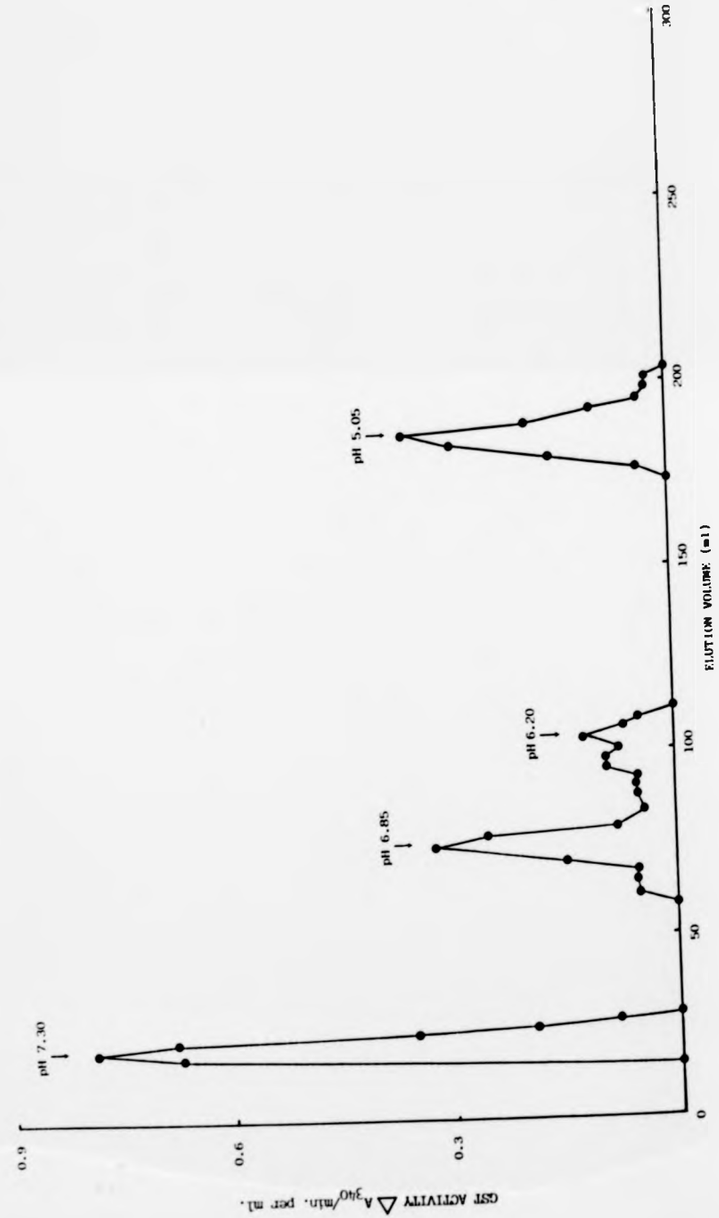


Figure 28 (v).

Figure 28 Chromatofocusing profile of;

(i) foetal adrenal cytosol 25 weeks gestation.

(ii) foetal adrenal cytosol 31 weeks gestation.

(iii) foetal adrenal cytosol 39 weeks gestation.

(iv) neonatal adrenal cytosol 40 weeks gestation 12 weeks postnatal age.

(v) infant adrenal cytosol 40 weeks gestation 45 weeks postnatal age.

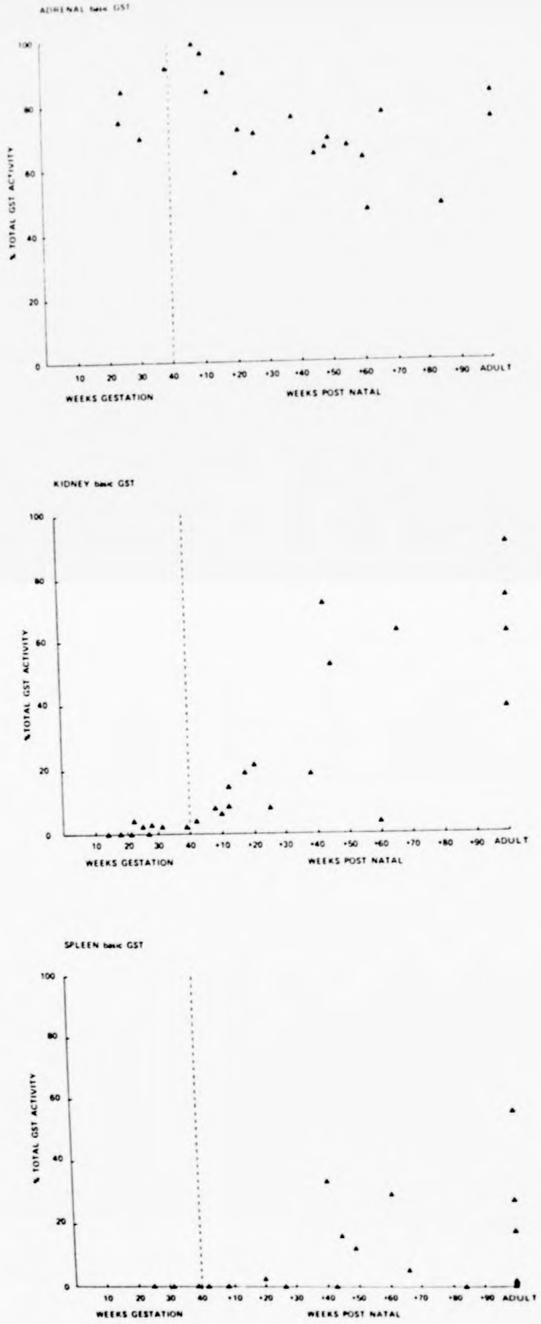


Figure 29.

Figure 29 Percentage contribution of basic GST isoenzymes to total GST activity in adrenal, kidney and spleen cytosols throughout gestation and neonatal development. Typical adult contributions are shown on the right for comparison.

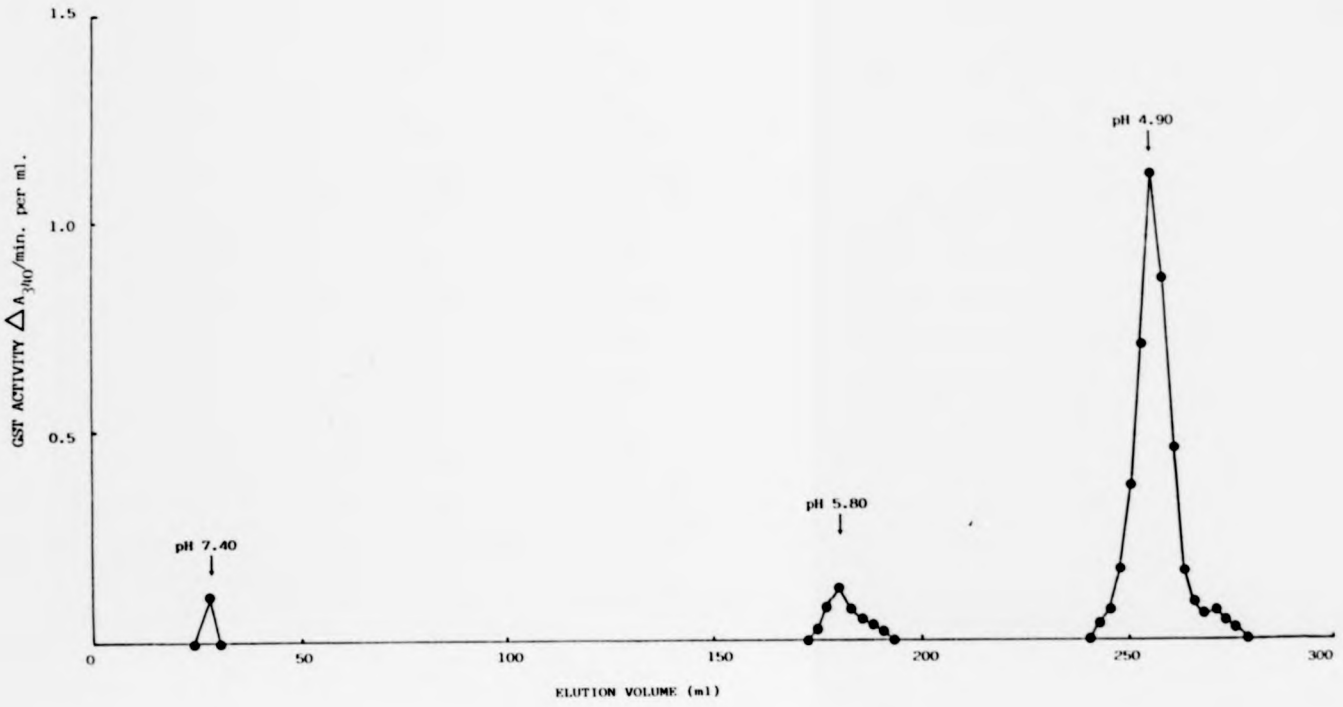


Figure 30 (i).

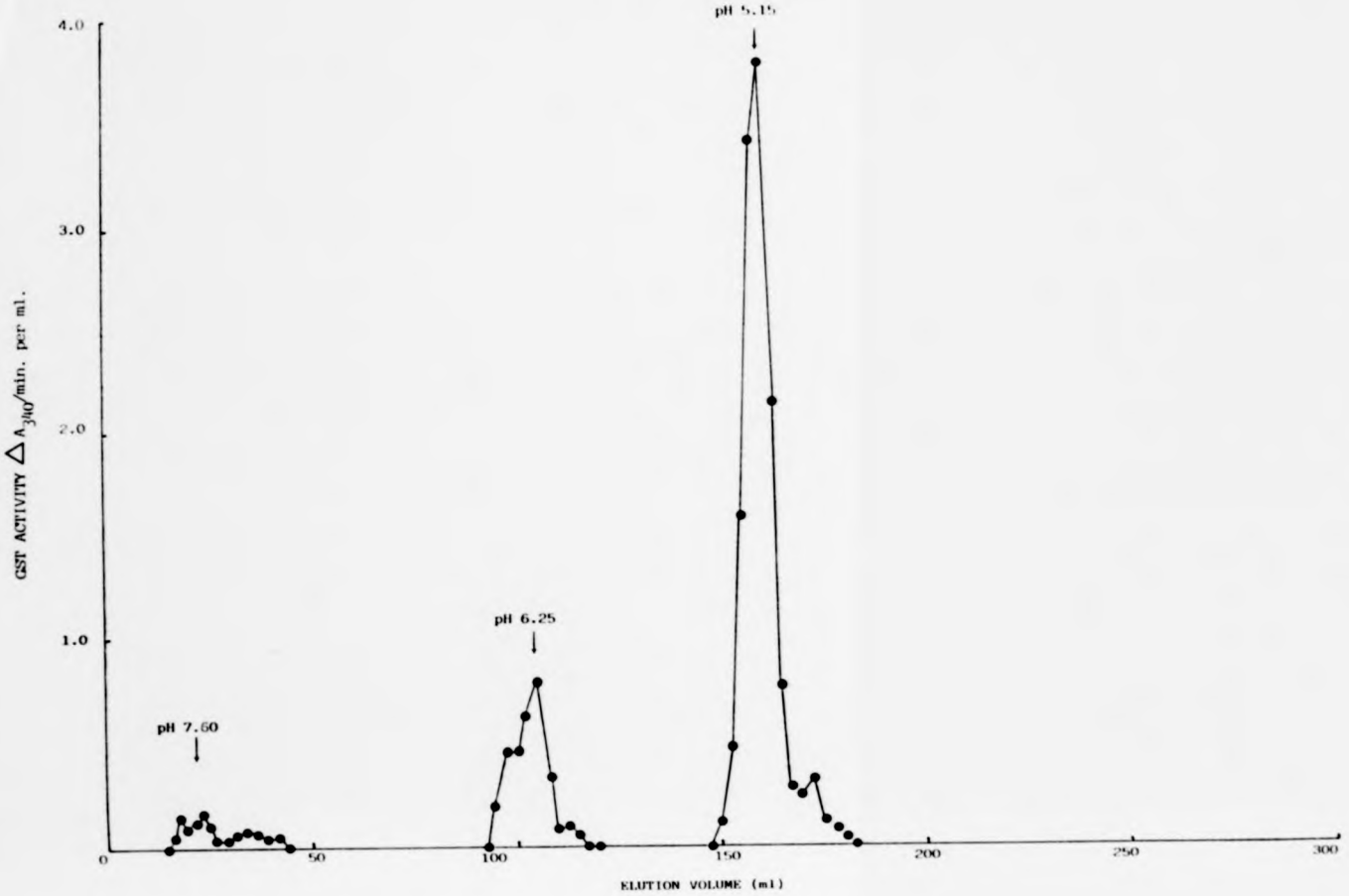


Figure 30 (ii).

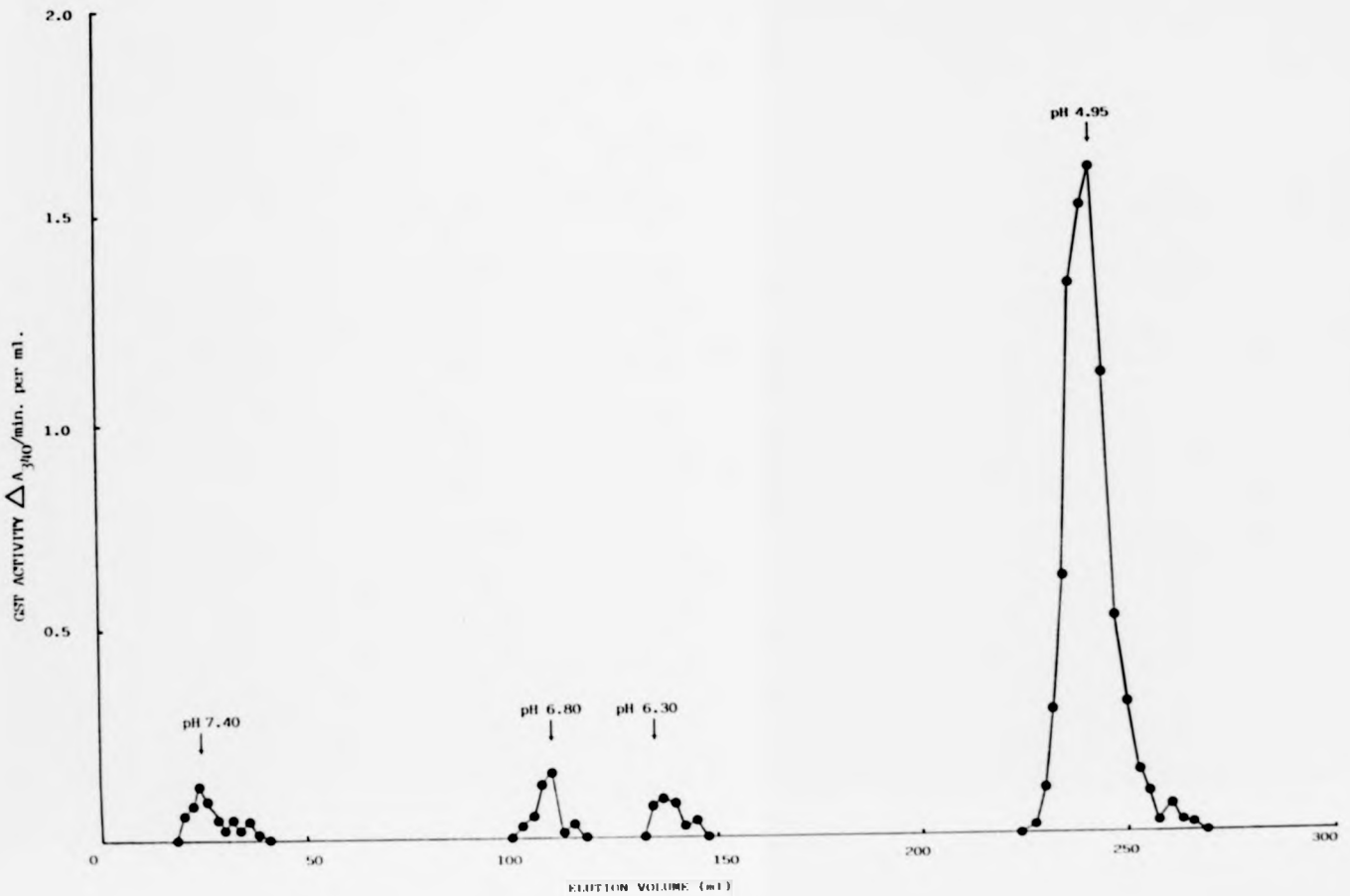


Figure 30 (iii).

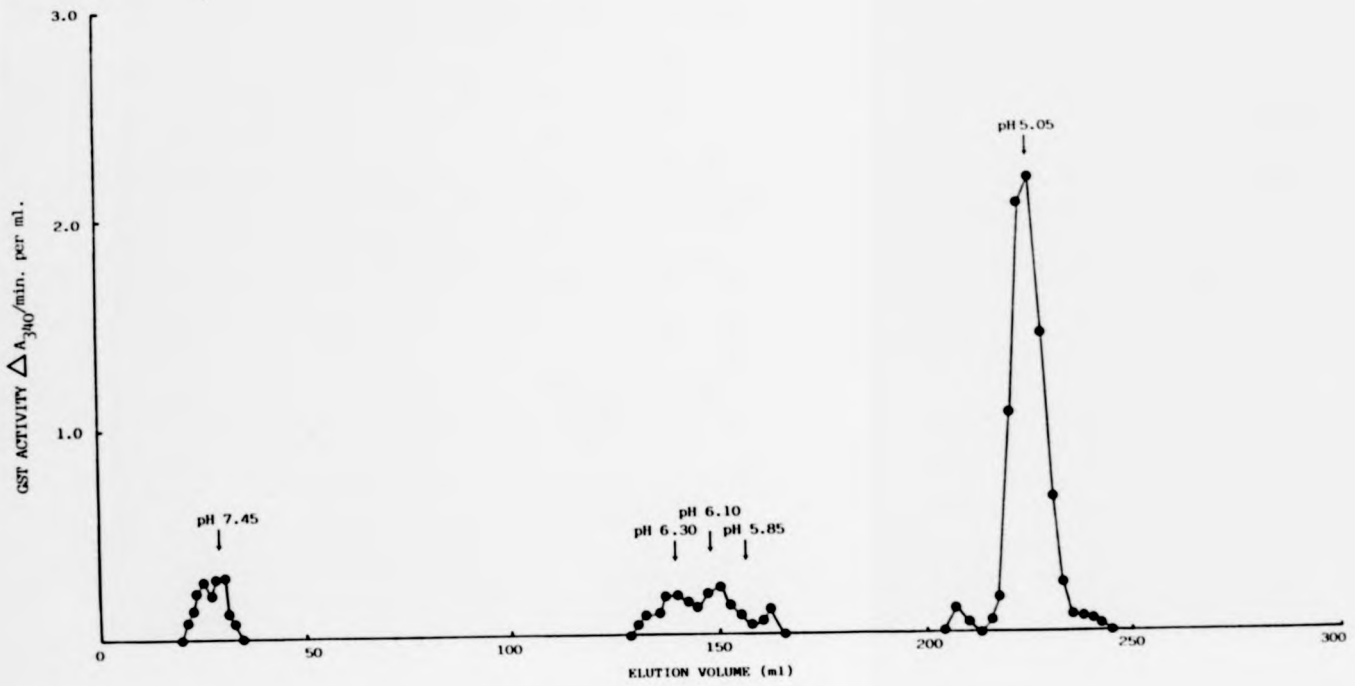


Figure 30 (iv).

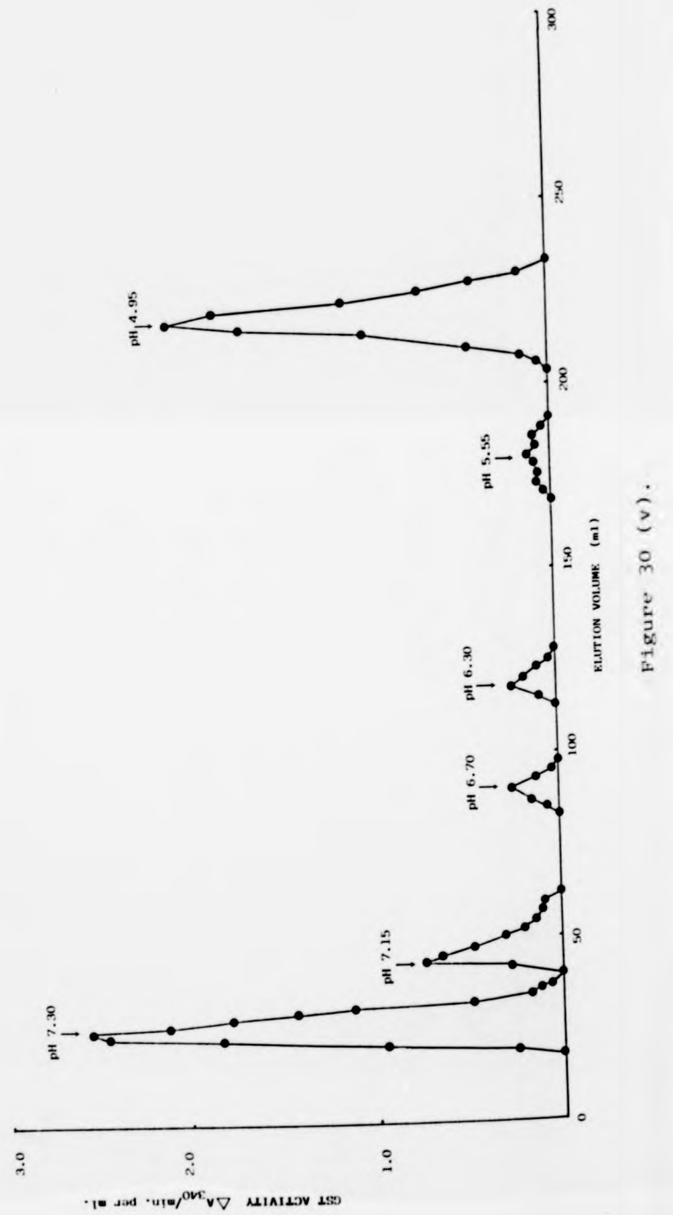


Figure 30 (v).

Figure 30 Chromatofocusing profile;

(i) kidney cytosol 25 weeks gestational age.

(ii) kidney cytosol 31 weeks gestational age.

(iii) kidney cytosol 39 weeks gestational age.

(iv) kidney cytosol 40 weeks gestation
12 weeks postnatal age.

(v) kidney cytosol 40 weeks gestation
45 weeks postnatal age.

30 (v)). Following birth however, there is a progressive increase in basic GST expression until adult levels are achieved at about one year of age. This can be seen from data shown in figure 29 and there is evidence that, in kidney, stimulus for expression of the basic GST occurs at birth.

The chromatofocusing profiles shown in figure 31 (i-v) demonstrate that basic GST was not usually expressed in spleen until one year after birth. The data shown in figure 29 confirm that adult levels of expression are achieved at about one year post-natal age but no developmental pattern is demonstrated. Starch-gel electrophoresis had previously shown that there was only weak expression of basic GST in spleen in most adult subjects.

3.4.4. Development of the Near-neutral Isoenzymes, (μ; GST1).

Starch gel electrophoresis suggests that the near-neutral isoenzymes are the products of three alleles; GST1*0 GST1*1 and GST1*2. It is believed therefore, that the near-neutral isoenzyme (μ) identified by Warholm et al. (1983) corresponds to the isoenzyme in subjects with the GST1 1 or GST1 2 phenotypes (Strange et al., 1984). However, apart from the data obtained from starch-gel electrophoresis, there is no evidence to support the hypothesis that the isoenzymes in individuals with the GST1 1 and GST1 2 phenotypes are homodimers and that subjects with the GST1 2-1 phenotype not only produce these two homodimeric isoenzymes but also a heterodimeric

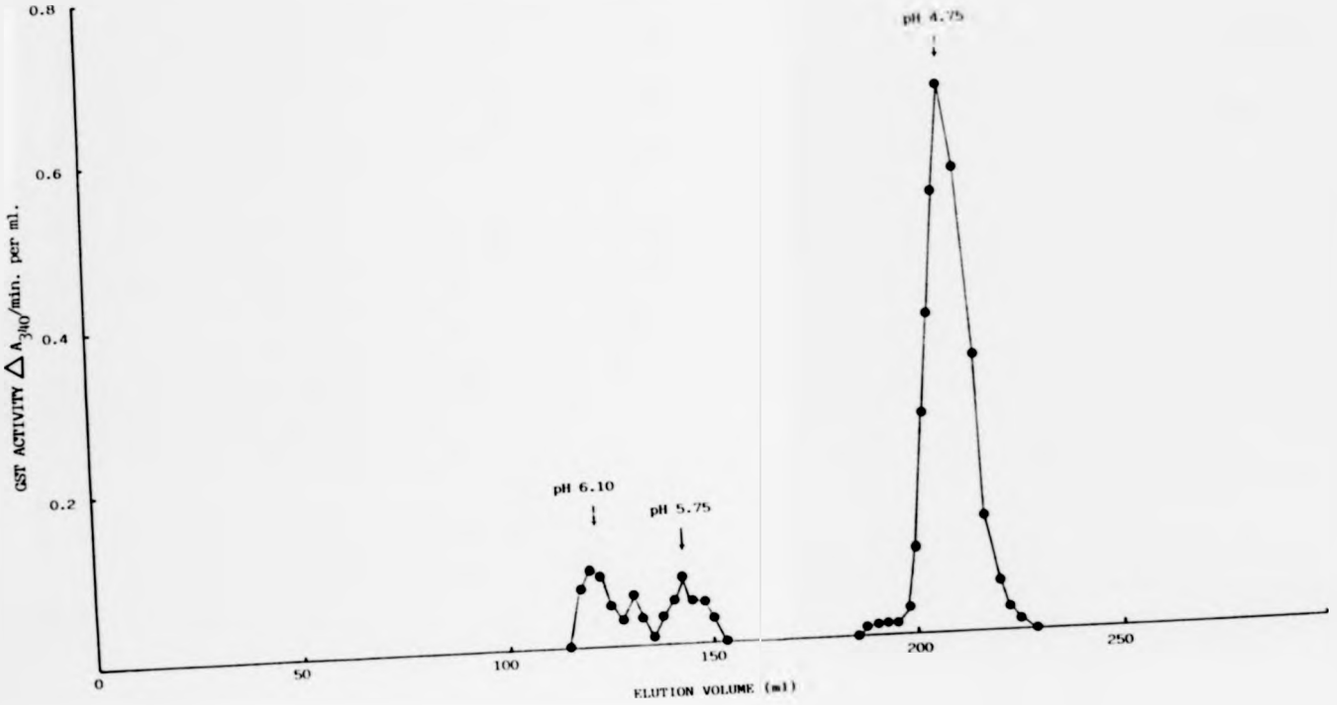


Figure 31 (i).

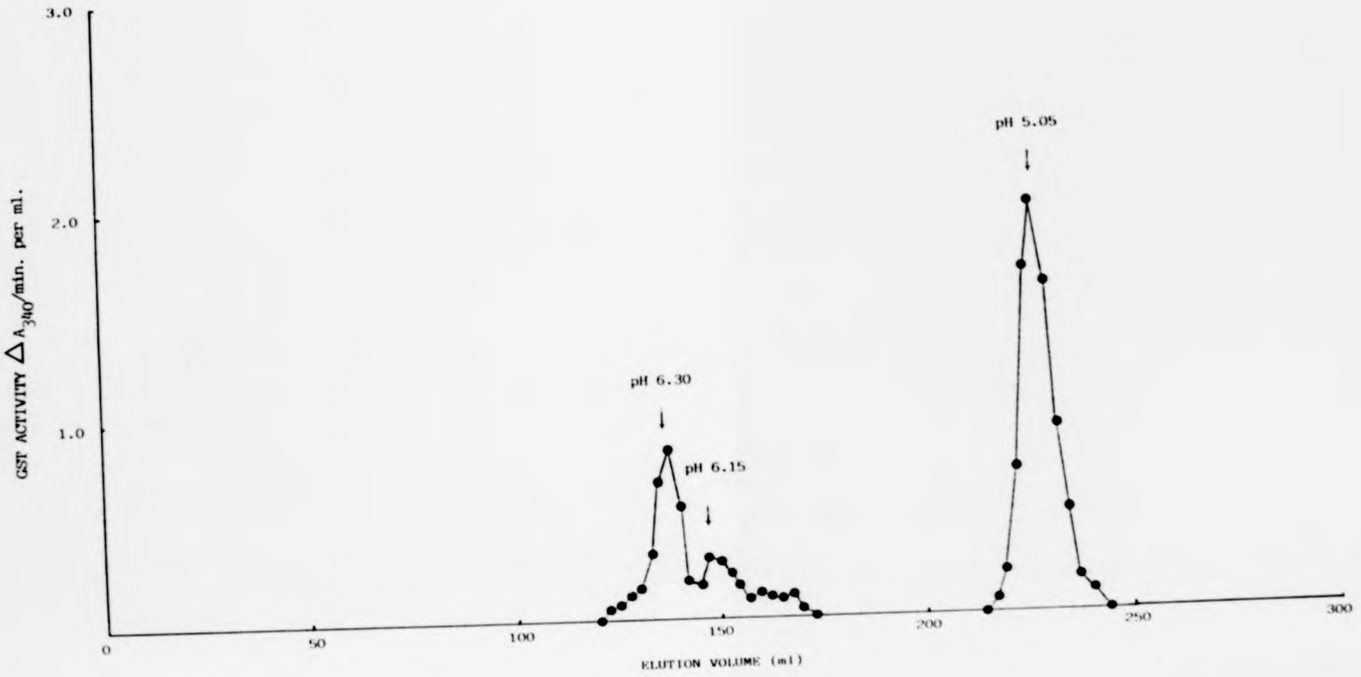


Figure 31 (ii).

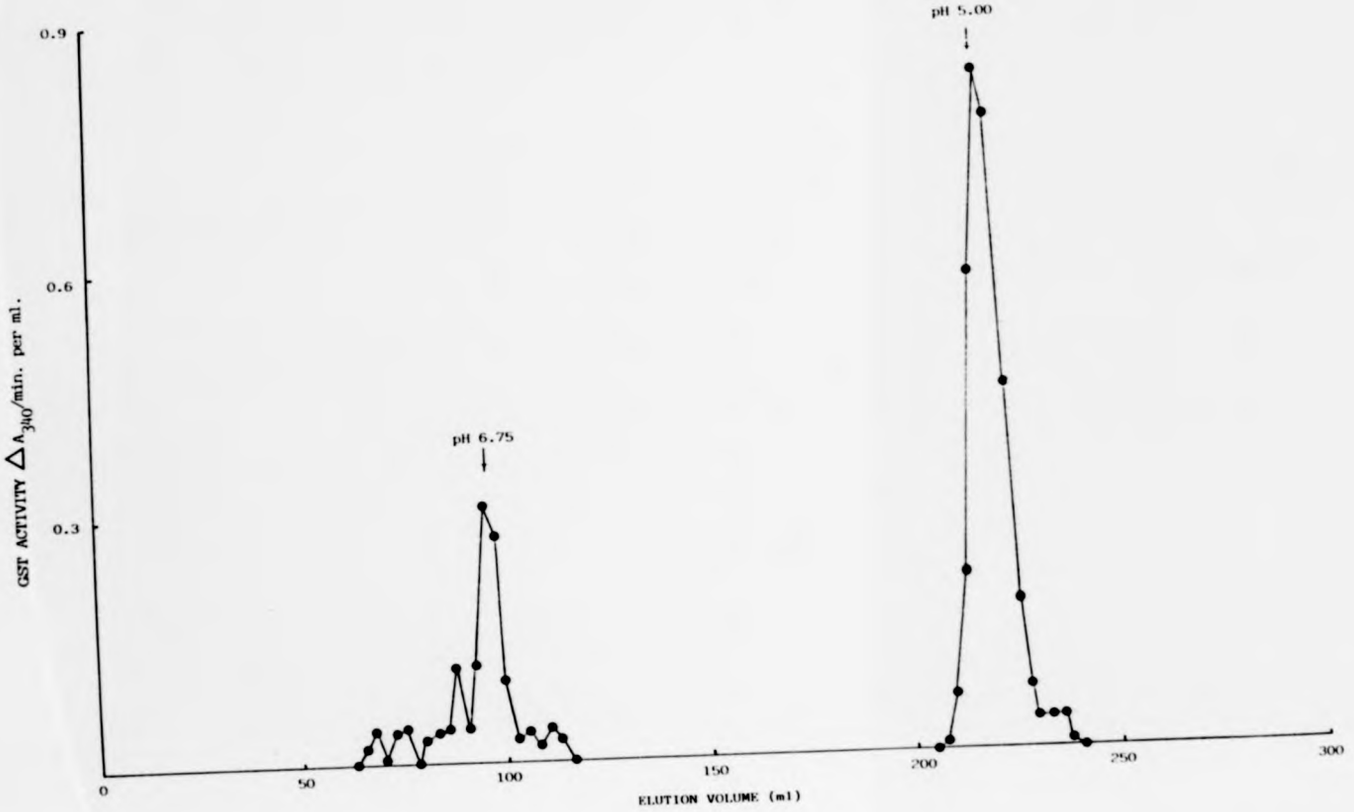


Figure 31 (iii).

Figure 31 (iv).

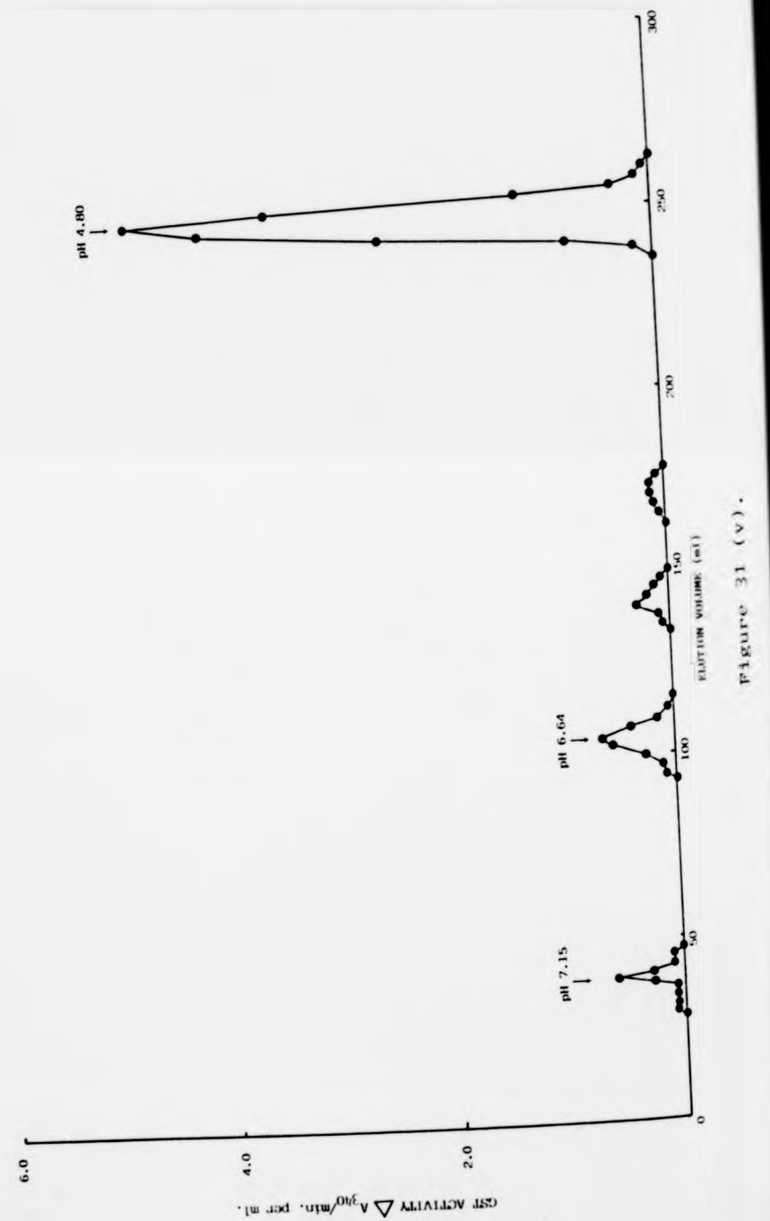


Figure 31 (v).

Figure 31 Chromatofocusing profile of;

(i) foetal spleen cytosol 25 weeks gestation

(ii) foetal spleen cytosol 31 weeks gestation.

(iii) foetal spleen cytosol 39 weeks gestation.

(iv) neonatal spleen cytosol 40 weeks gestation 12 weeks postnatal age.

(v) infant spleen cytosol 40 weeks gestation 45 weeks postnatal age.

form composed of one of each of the two monomers. In order to test the hypothesis therefore, *in vitro* hybridisation studies were carried out. The purpose of these experiments was to confirm that two monomeric forms are produced at this locus that result in active dimeric enzymes. The ontogeny of the two monomers could therefore be studied.

The chromatofocusing experiments previously described (figure 23) demonstrated that it was possible to separate the three GST1 isoenzymes present in cytosol from an individual with the GST1 2-1 phenotype. Each of these isoenzymes was isolated and a series of hybridisation experiments performed. In the first experiment the two homodimeric isoenzymes (*GST1*1*/*GST1*1* and *GST1*2*/*GST1*2*) were mixed, incubated with guanidinium chloride, dialysed and re-chromatofocused. Figure 32 shows that the two original isoenzymes are still present together with the expected hybrid (*GST1*1*/*GST1*2*) isoenzyme.

Similarly, dissociation followed by recombination of the monomers of the GST1 2-1 isoenzyme (*GST1*1*/*GST1*2*) resulted in the formation of three peaks of activity that correspond to the GST1 2,GST1 2-1 and GST1 1 isoenzymes.

The development of the near-neutral isoenzymes was also studied by chromatofocusing. As shown in figures 19 to 21 starch-gel electrophoresis studies had already indicated that this isoenzyme set is not usually expressed before 30 weeks gestation (Strange *et al*., 1985) and this was confirmed by the data using chromatofocusing.

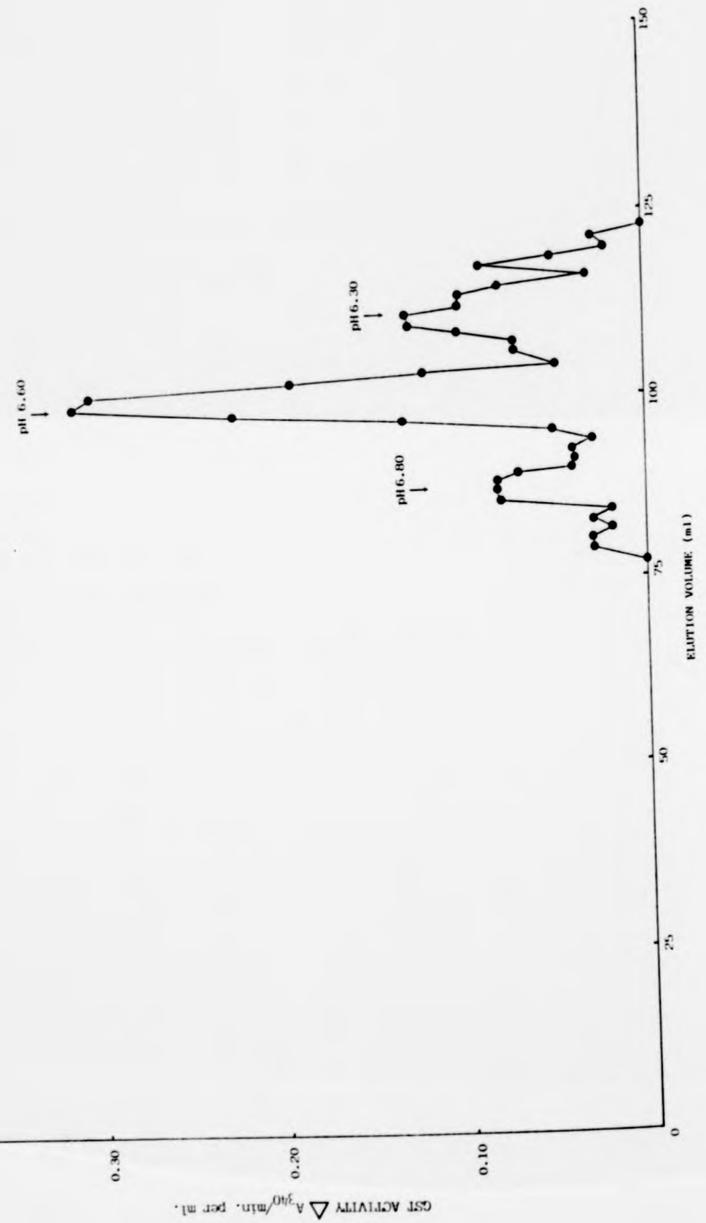


Figure 32. Chromatofocusing profile of reassociated allelic products GST1 1 and GST1 2 from a heterozygote individual of phenotype GST1 2-1.

Figure 32 Chromatofocusing profile of reassociated allelic products GST1 1 and GST1 2 from a heterozygote individual of phenotype GST1 2-1.

Figure 26(i) demonstrates no near-neutral activity in liver, but interestingly spleen from the same subject does demonstrate near-neutral GST activity. After about 30 weeks gestation there is a steady progressive increase in the expression of near-neutral GST activity in liver. This is seen from data presented in figure 33.

This figure also shows the GST1 phenotype of those cytosol samples where a classification could be made confidently. In five samples obtained before 30 weeks gestation, a phenotype was not assigned because no GST1 activity was demonstrable either by starch-gel electrophoresis or by assay after chromatofocusing. In two other cases the chromatofocusing and electrophoresis data were consistent with a GST1 2-1 phenotype even though the level of activity was very low (5% to 10% of total). In samples obtained later in development the percentage contribution of these isoenzymes to total cytosol activity was similar in individuals demonstrating GST1 1, GST1 2 and GST1 2-1 phenotypes.

The development of the GST1 isoenzymes was also studied in adrenal cytosol and since in many subjects tissue sets were obtained, it was possible to compare expression in different tissues from the same individual. Some of the chromatofocusing profiles are shown in figure 28 (i-v). The profiles demonstrate variable expression of the near-neutral isoenzymes. The genetical polymorphism of GST1 can usually be readily demonstrated in adult adrenal

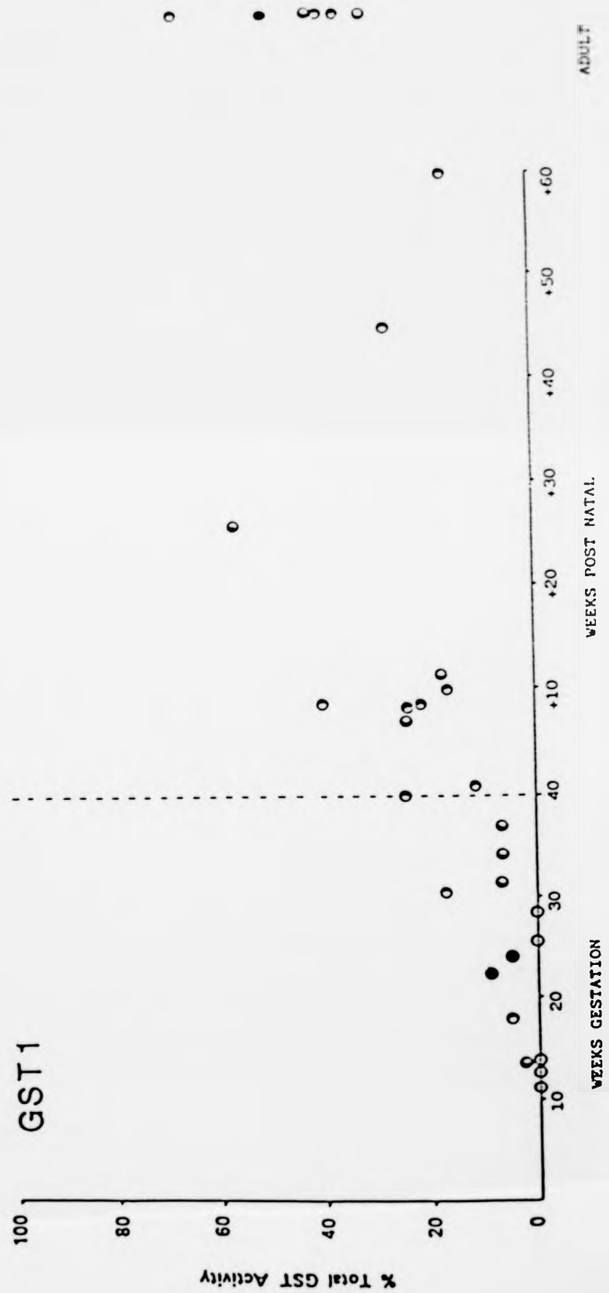


Figure 33.

Figure 33 Percentage contributions of near-neutral GST1 isoenzymes to total GST activity in liver cytosol throughout gestation and neonatal development. Typical adult contributions are shown on the right for comparison.

The phenotypes of each subject is given as:

- ○ No assignment
- ◐ GST 1 1
- ◑ GST 1 2
- ● GST 1 2-1

but expression of these isoenzymes during development was variable in both foetal and neonatal tissues. The percentage contribution of this set to total cytosol activity during development is summarised in figure 34. This shows no expression even in some subjects who have demonstrated GST1 1 and GST1 2 phenotypes by starch-gel electrophoresis or chromatofocusing of liver cytosol. Figure 28 (iv) compared with the corresponding liver profile (figure 26 (iv) is an example of this phenomenon. The ontogeny of this locus was difficult to study in adrenal because such small amounts of tissue were available and the level of activity was at the detection limits of the enzyme assay.

Development of the near-neutral isoenzymes in kidney cytosol is summarised in figure 34. The isoenzymes were identified in kidney after 22 weeks gestation and after this time the GST1 phenotype was as predicted from previous studies of liver cytosol from the same subject. The contribution of these enzymes to total cytosol activity was variable and no developmental trend was observed (figure 34).

The elution of the near-neutral isoenzymes in kidney was more complex than that of the isoenzymes in liver or adrenal samples from the same subject; two further isoenzymes eluted after the expected GST1 peak. Figure 35 shows a representative example of the elution of a kidney cytosol from a GST1 1 phenotype. Although the amounts of activity eluted were small, this profile was a consistent feature of cytosols with this phenotype. Studies using starch-gel electrophoresis showed that the GST1 1 isoenzyme

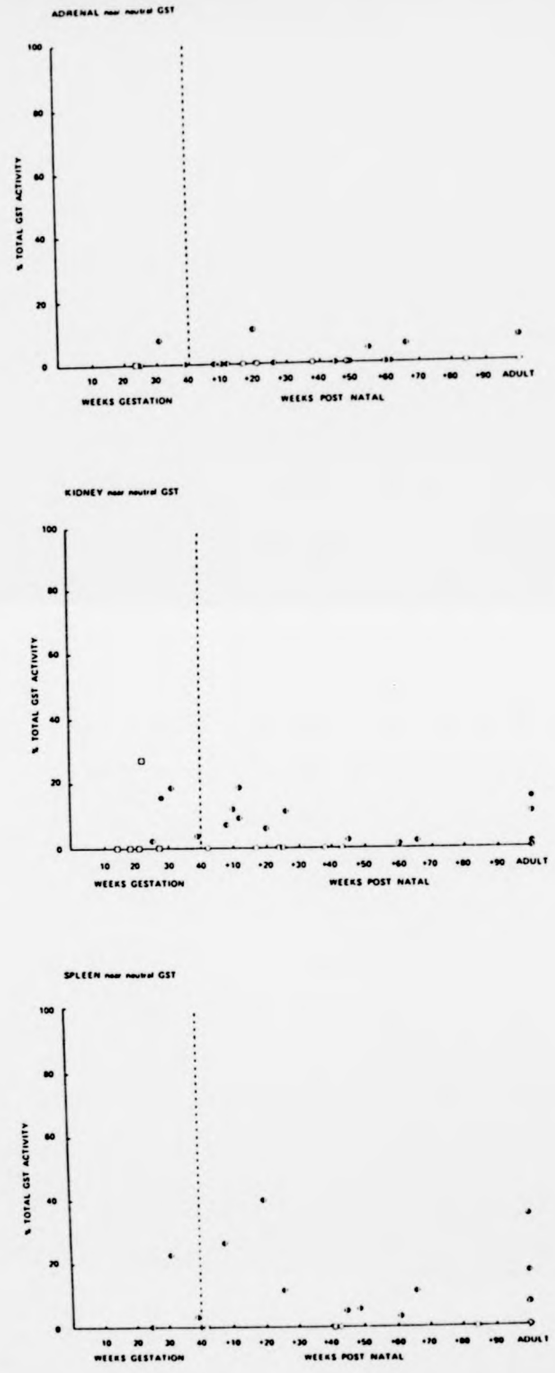


Figure 34.

Figure 34 Percentage contribution of near-neutral GST isoenzymes to total GST activity in adrenal, kidney and spleen cytosols throughout gestation and neonatal development. Typical adult near-neutral isoenzyme contributions are shown on the right for comparison.

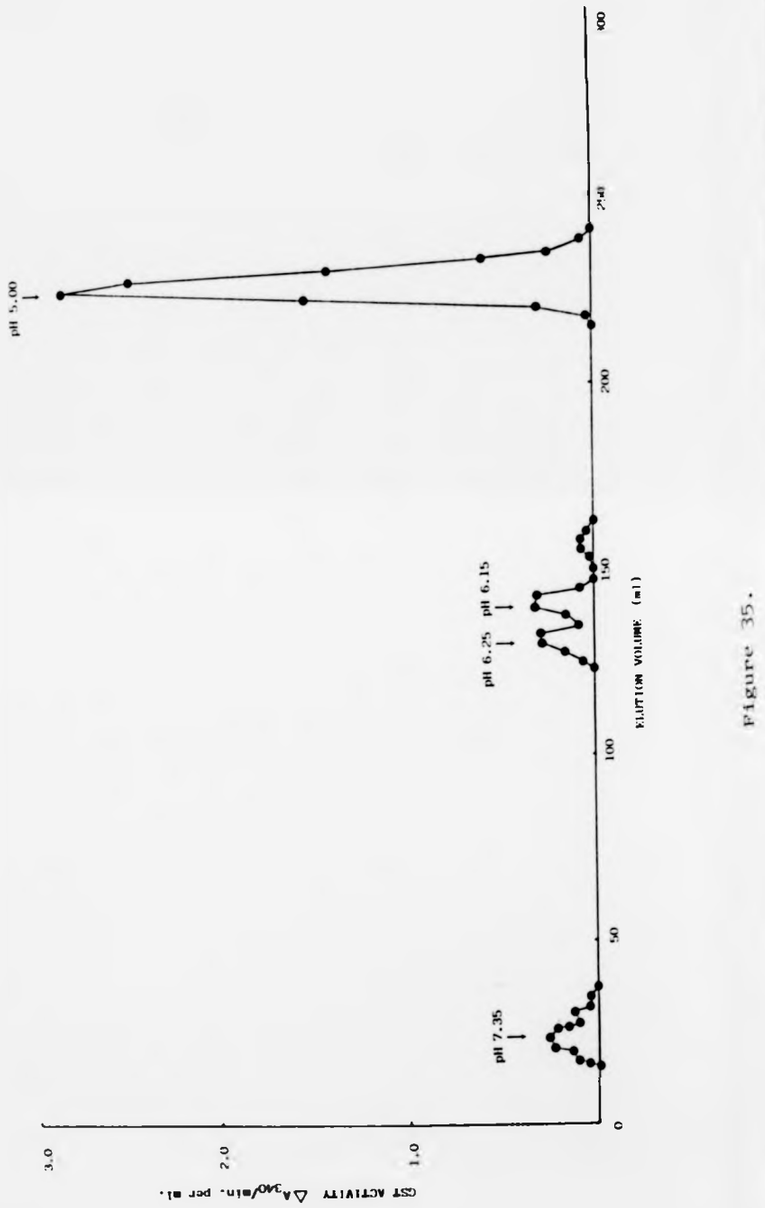


Figure 35.

Figure 35 Chromatofocusing profile of a kidney cytosol with a GST1 1 phenotype.

Post natal age 8 weeks.

eluted first (elution pH approximately 6.2) and that the most acidic isoenzyme of this group (elution pH approximately 5.8) was a minor product of GST3 (Strange et al., 1983). It is unclear whether the intermediate isoenzyme is a post-synthetic modification of the GST1 isoenzyme or a GST1/GST3 hybrid. Kidney samples with the GST1 2 phenotype also demonstrated activity that eluted between the GST1 2 (elution pH approximately 6.7) and GST3 isoenzymes (figure 36).

Development of the near-neutral GST in spleen was similar to that in the kidney (figure 34). Activity was demonstrated earlier in gestation than for the corresponding liver cytosol from the same subject (figure 31 (i) compared with figure 26 (i)). No developmental trends in expression were observed. The chromatofocusing profiles of these isoenzymes were similar to those described for kidney cytosol from the same subjects (figure 31).

3.4.5. Development of the Acidic Isoenzymes, ρ, π, ψ, ω; GST3.

Acidic isoenzymes have been identified in all human tissues studied so far. Board (1981a) originally suggested that the acidic isoenzymes in liver were the products of the GST3 locus but it is not clear whether the corresponding isoenzymes in other tissues are also products of this locus. There was considerable variety in the electrophoretic patterns of these isoenzymes, some of which resulted from quantitative changes in expression. Both starch-gel electrophoresis and

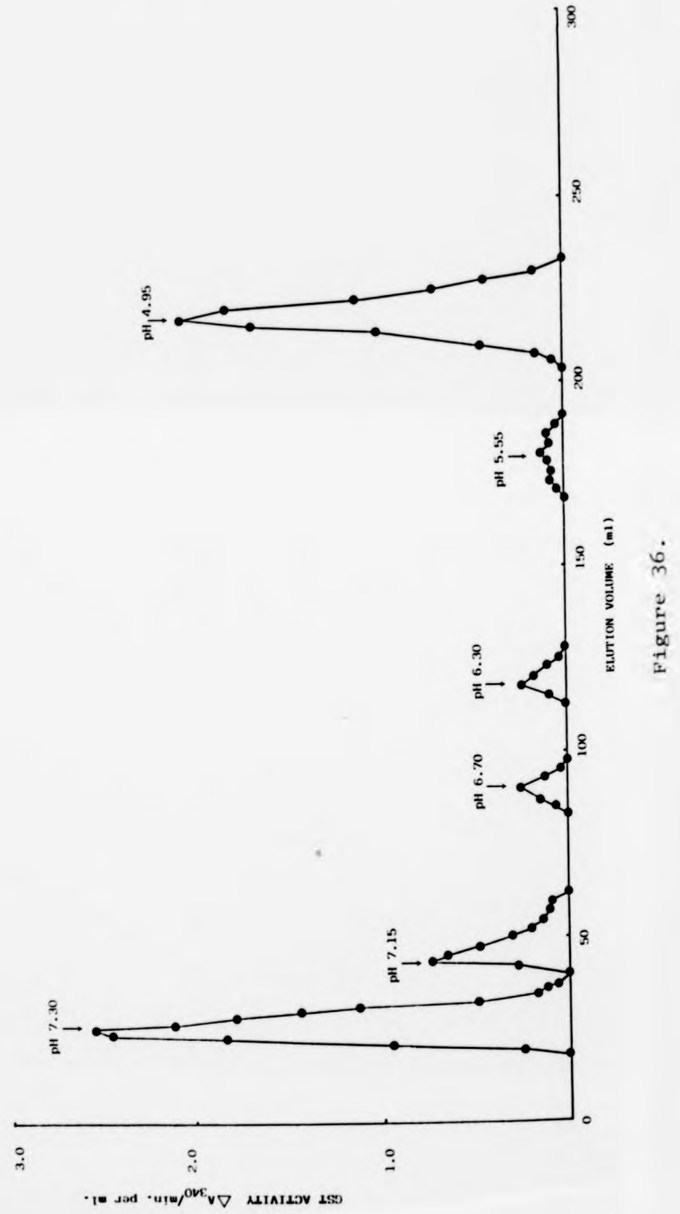


Figure 36.

Figure 36 Chromatofocusing profile of a kidney cytosol from a subject with a GST1 2 phenotype. Post natal age of 45 weeks.

chromatofocusing showed that the GST3 isoenzyme was predominant in livers obtained in early gestation and that the percentage contribution of this locus gradually declined so that little activity could be detected in infants and adults (figure 37). The chromatofocusing profiles of liver cytosols obtained during development are shown in figure 26 and demonstrate the decline in expression of the acidic isoenzyme.

The percentage contribution of the acidic isoenzyme in adrenal to total cytosol activity during development is shown in figure 38. This contribution was variable and no developmental trend was identified. Activity eluted from the chromatofocusing column as a single peak in all but three samples, in these cytosols a small amount of activity eluted between the main acidic and near-neutral isoenzymes but there was no evidence of the GST1/GST3 hybrid.

Examples of the elution of acidic GST isoenzymes in kidney cytosols are shown in figure 30. They eluted as a main peak of activity at pH values of approximately 5.0 and as described previously small amounts of activity also eluted before this main peak. The expression of acidic isoenzymes in kidney cytosol was constant until about 40 weeks post-natal age. After this time expression declined to the levels observed in adult samples (figure 38).

Elution of acidic isoenzymes in spleen cytosol was similar to that in kidney. The enzymes eluted as a main

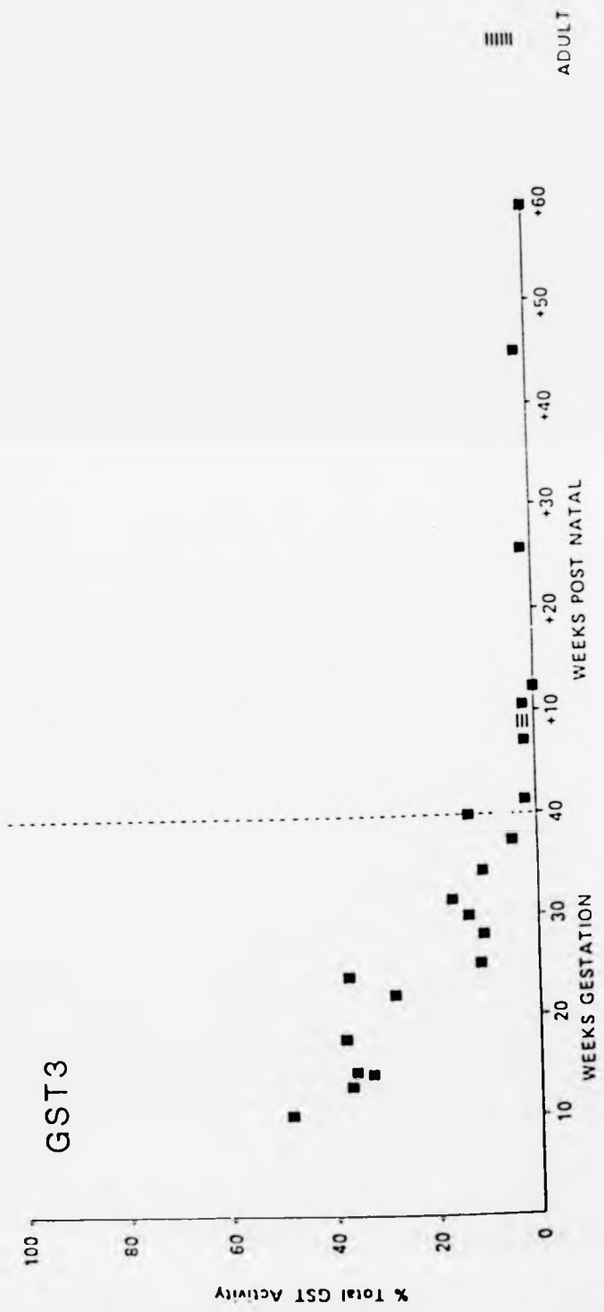


Figure 37.

Figure 37 Percentage contribution of acidic GST isoenzymes to total GST expression in liver cytosol throughout gestation and neonatal development. Adult contributions are shown on the right for comparison.

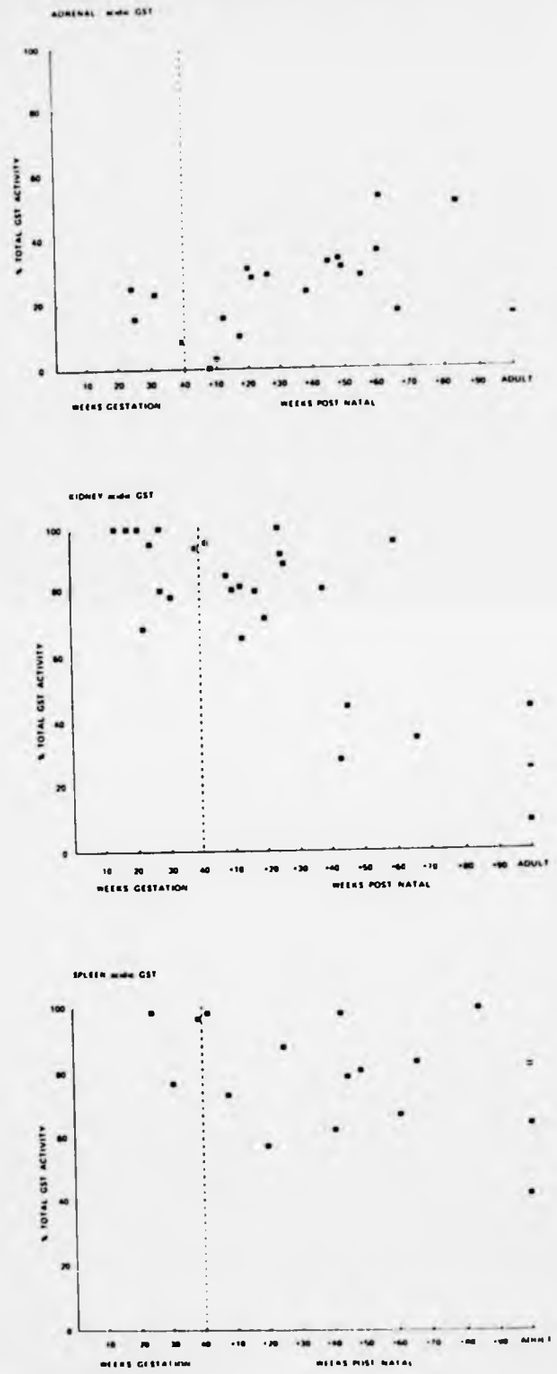


Figure 38.

Figure 38 Percentage contribution of acidic GST isoenzymes to total GST activity in adrenal, kidney and spleen cytosol during development. Adult acidic isoenzyme contributions are shown on the right for comparison.

peak of activity at pH values between 5.2 and 4.8. The level of expression of these isoenzymes remained high during development and no trends were observed although expression did appear to be lower in the adult samples examined.

3.4.6. Interlocus Hybrids.

The chromatofocusing experiments had indicated that interlocus hybrids (GST1/GST3) could be formed in kidney and spleen but not in liver or adrenal. Further support for this proposal would require *in vitro* hybridisation of GST1 and GST3 isoenzymes. It is interesting to note that in skeletal muscle obtained from an adult, a large amount of the putative hybrid was present. Figure 39 shows the chromatofocusing profile obtained. The GST1 isoenzyme eluted at pH 6.15 and the minor acidic isoenzyme at a pH of 5.35. The major acidic isoenzyme is also shown at an elution pH of 4.95. A major peak of GST activity eluted at pH 5.65 between the GST1 and the minor GST3 isoenzymes. A formal study of the development of GST3 in skeletal muscle together with appropriate hybridisation studies would help to identify the origins of this intermediate peak of activity.

3.5. ION-EXCHANGE STUDIES OF THE DEVELOPMENT OF THE BASIC (GST2) ISOENZYMES.

Starch-gel electrophoresis and chromatofocusing demonstrated that basic GST isoenzymes are consistently

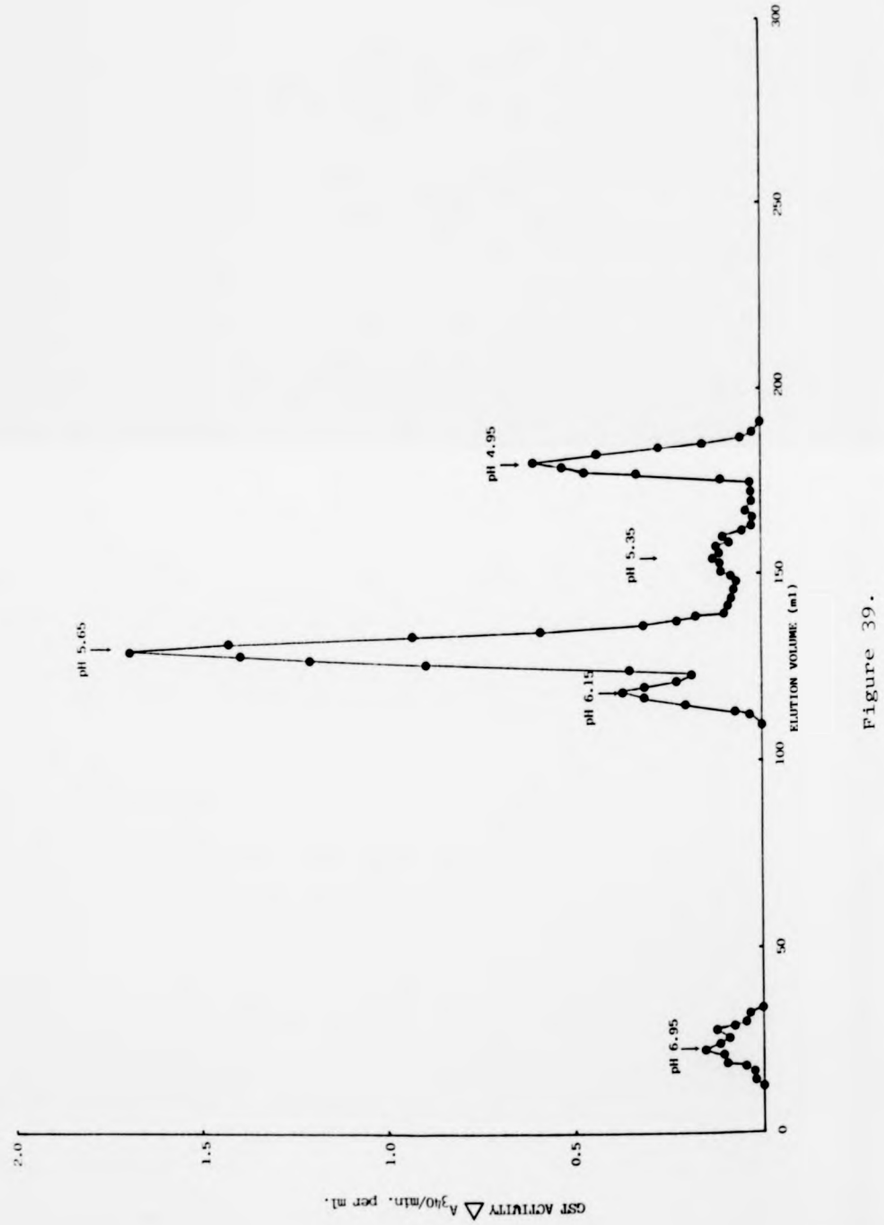


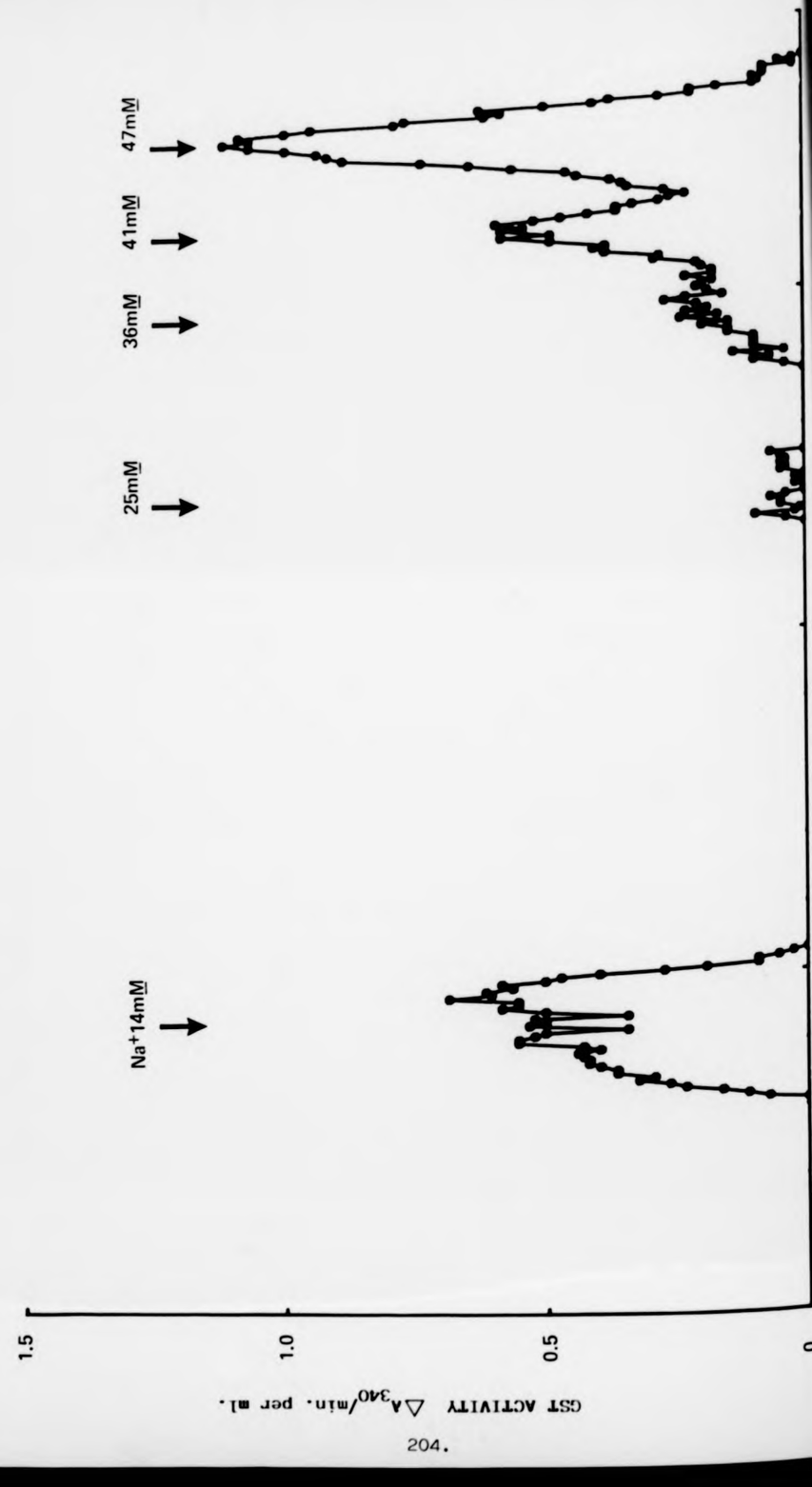
Figure 39.

Figure 39 Chromatofocusing profile of an adult skeletal muscle cytosol.

expressed in liver after 10 weeks gestation (Strange et al. 1985). However, these techniques, under the conditions used, resolved the basic isoenzymes into only two or three components. Since Kamisaka et al. (1975) had demonstrated five adult basic GST using CM-cellulose, ion-exchange chromatography was used to resolve the different isoenzyme forms during development.

Adult liver cytosols were eluted from DEAE-cellulose and the 'flow-through' fractions resolved using CM-cellulose. The elution pattern and the Na^+ concentration at which peaks eluted shown in figure 40 is similar to that of Hayes et al. (1983). Peaks of activity corresponding in order of elution to α, β, γ, δ and ϵ were identified and this profile was used as the reference for the identification of the various basic GST isoenzymes. A different elution profile was seen in some subjects and figure 41 shows that it resembled that described by Kamisaka et al. (1975).

In an attempt to investigate the relationship between the various GST2 isoenzymes a series of hybridisation experiments was carried out. Material from the profile shown in figure 41 was used. Each peak was subjected to guanidinium chloride dissociation as described in the Materials and Methods section. The results after hybridisation and subsequent CM-cellulose chromatography are shown in figures 42 to 45. Additionally equal activities of peaks Na^+ 36mM and Na^+ 45mM were taken, mixed and



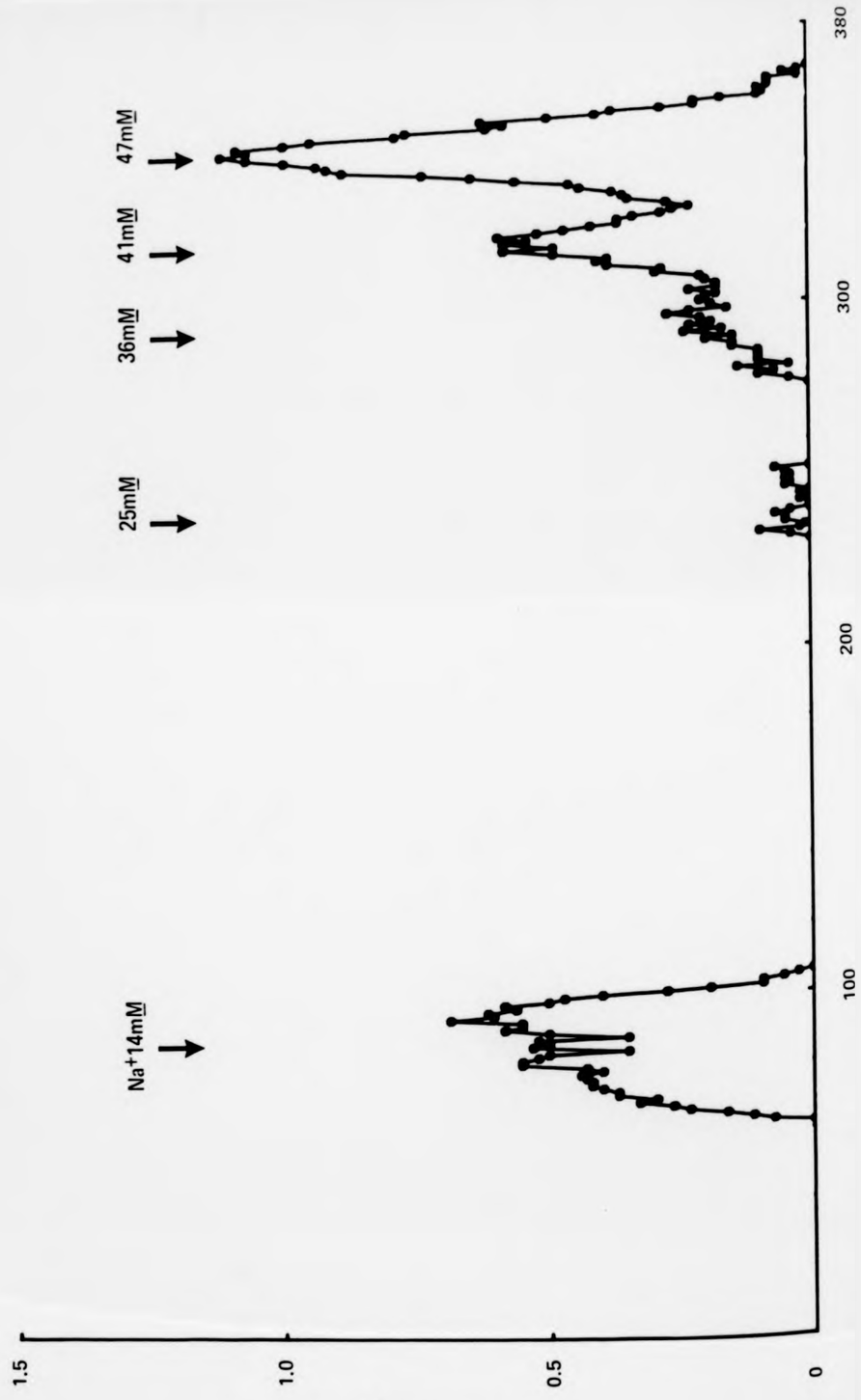


Figure 40 Profile of basic GST from adult liver after elution from a CM-cellulose column. The liver cytosol was first applied to a DEAE-cellulose column, the 'flow-through' fractions collected and then eluted from CM-cellulose.

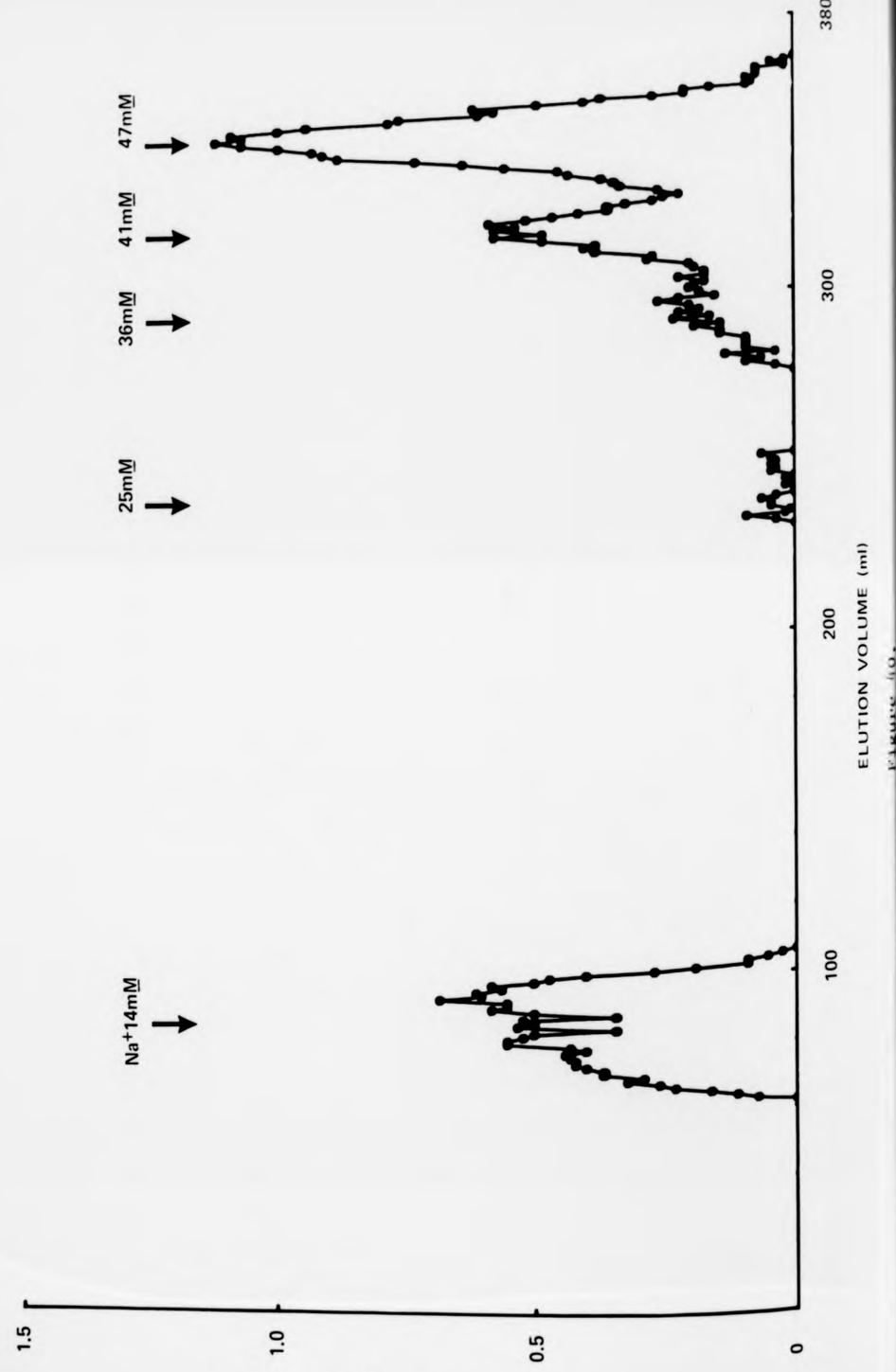


Figure 40 Profile of basic GST from adult liver after elution from a CM-cellulose column. The liver cytosol was first applied to a DEAE-cellulose column, the 'flow-through' fractions collected and then eluted from CM-cellulose.

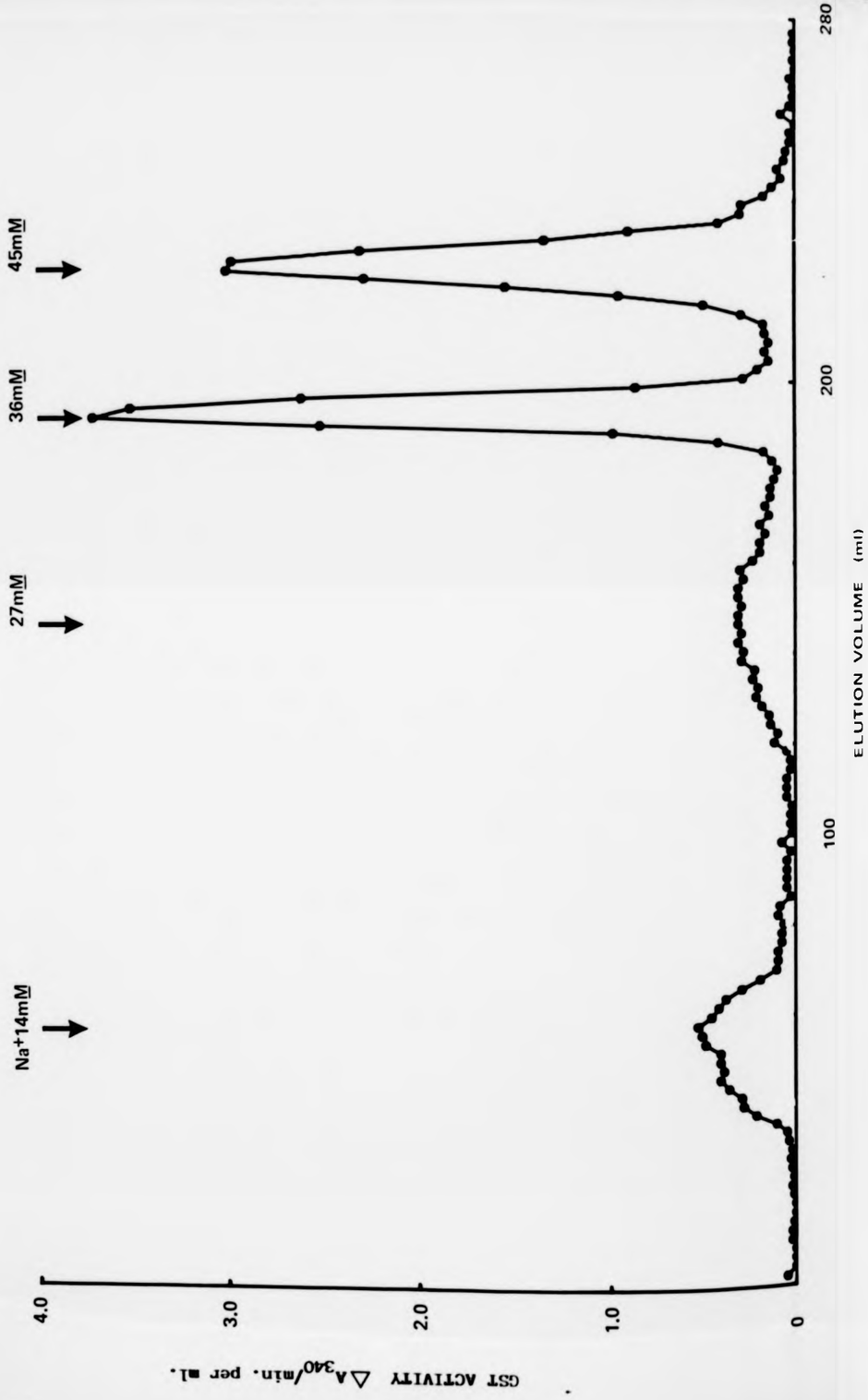


Figure 41 Profile of basic GST from adult liver after elution from a CM-cellulose column. The liver cytosol was first applied to DEAE-cellulose the 'flow-through' fractions collected and eluted from the CM-cellulose column.

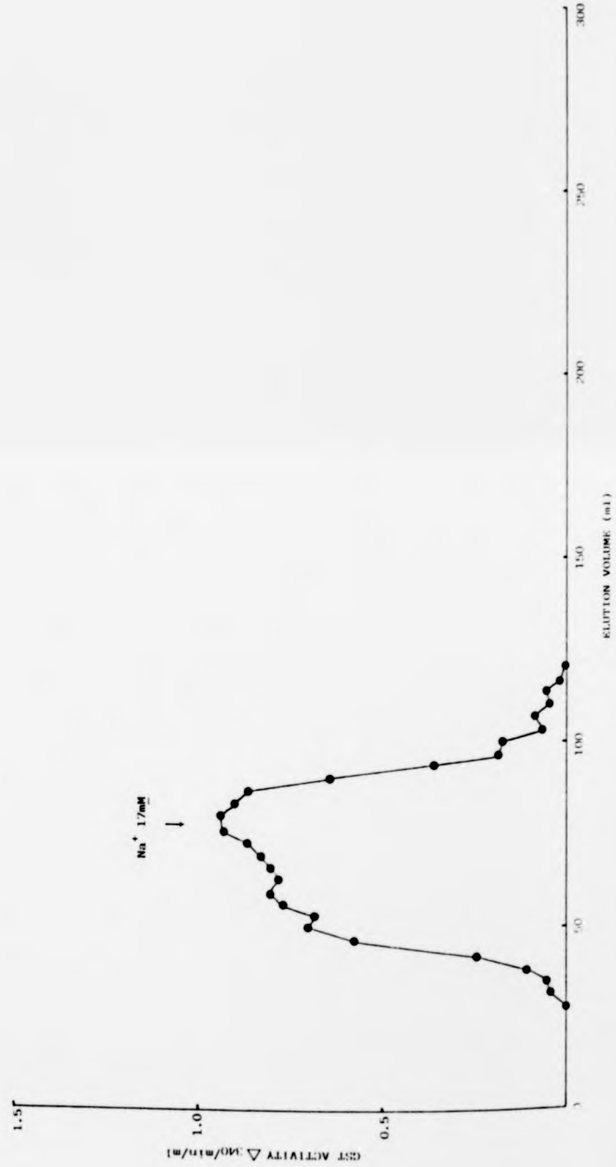


Figure 42.

Figure 42 CM-cellulose chromatography profile of pooled 'flow-through' fractions shown in figure 41 following dissociation with guanidinium chloride and subsequent reassociation.

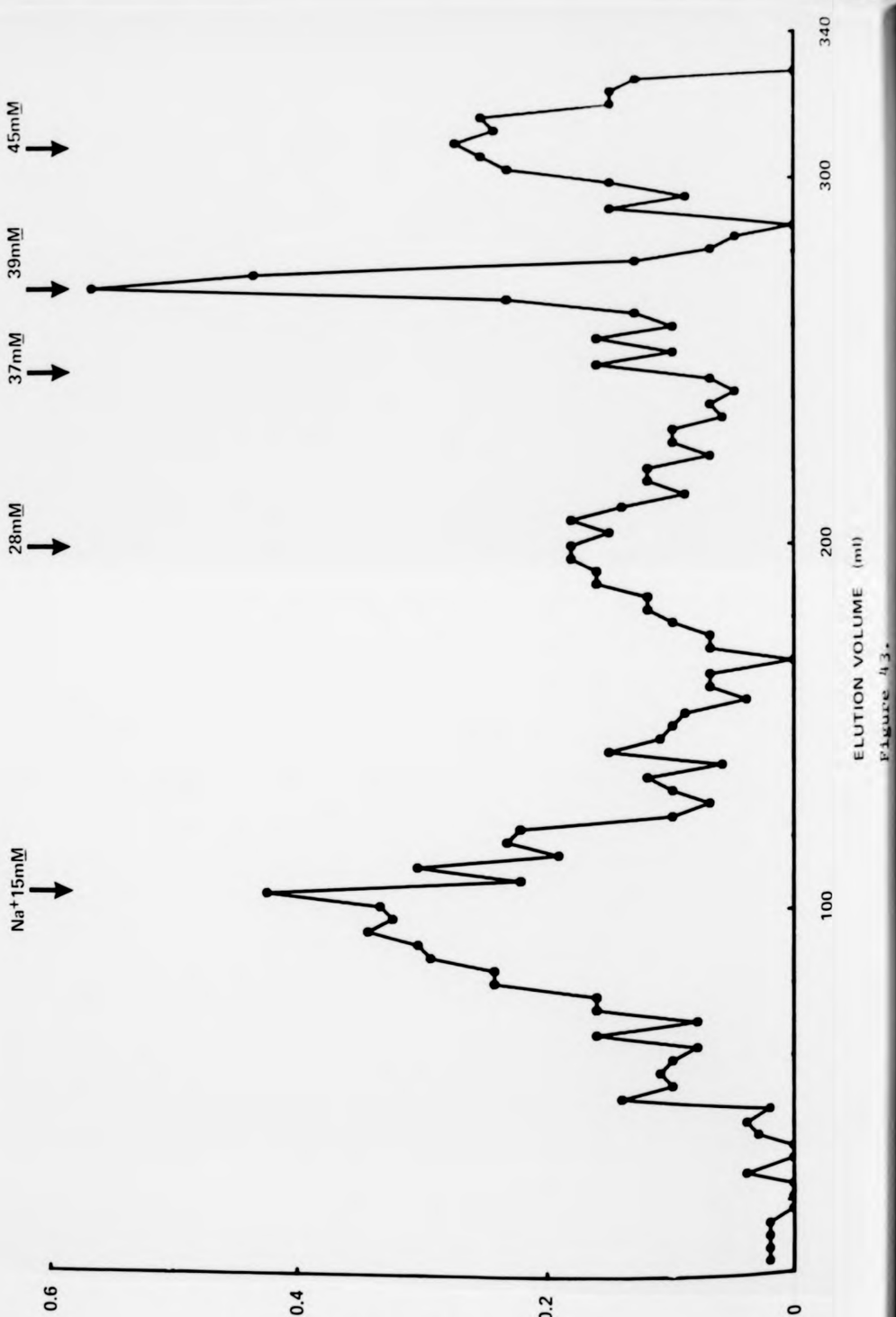


Figure 43.

Figure 43 CM-cellulose chromatography of Na^+ 27m<u>M</u> peak shown in figure 41 following dissociation with guanidinium chloride and subsequent reassociation.

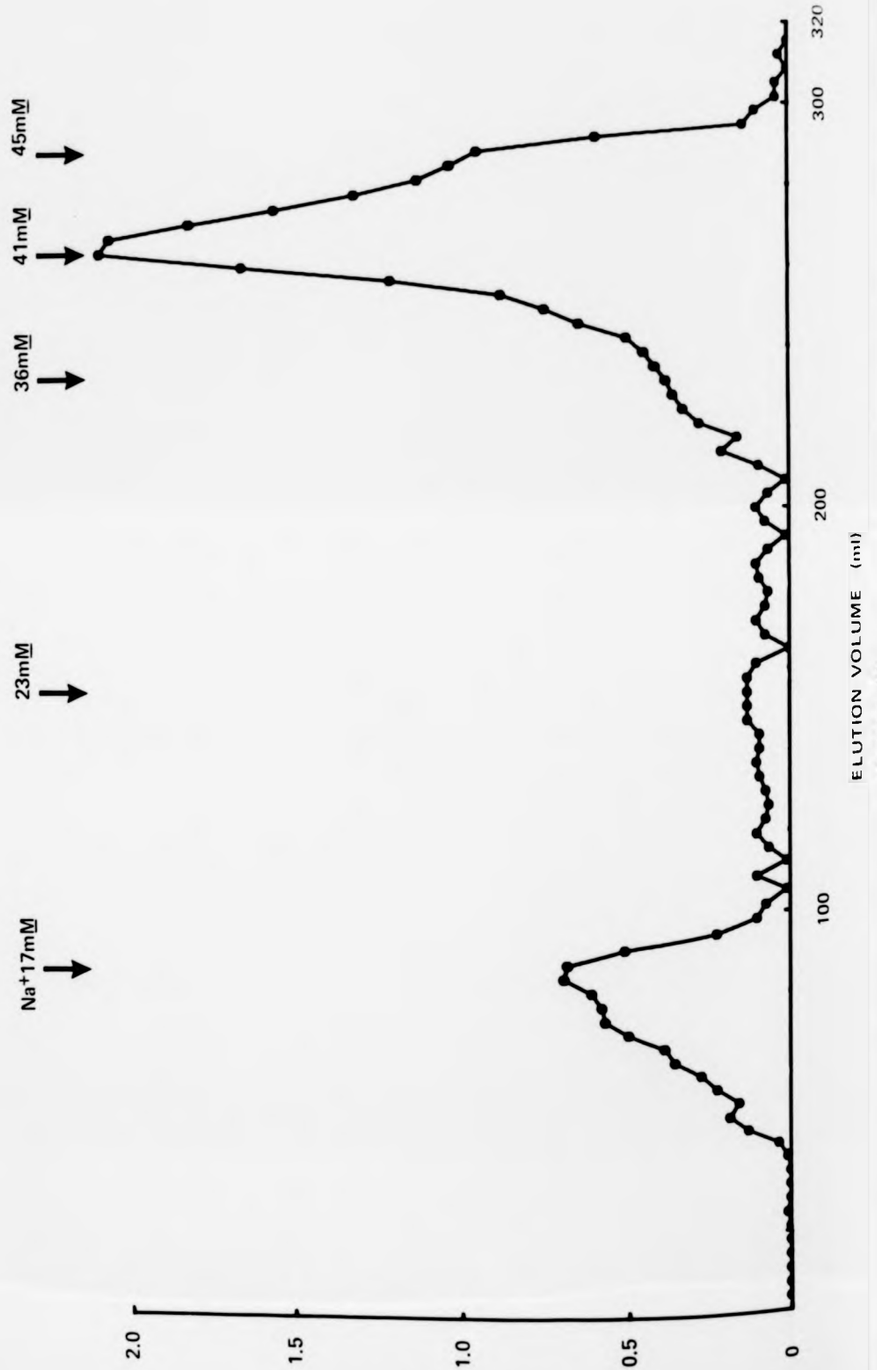


Figure 44 CM-cellulose chromatography of peak Na^+ 36mM shown in figure 41 following dissociation with guanidinium chloride and subsequent reassociation.

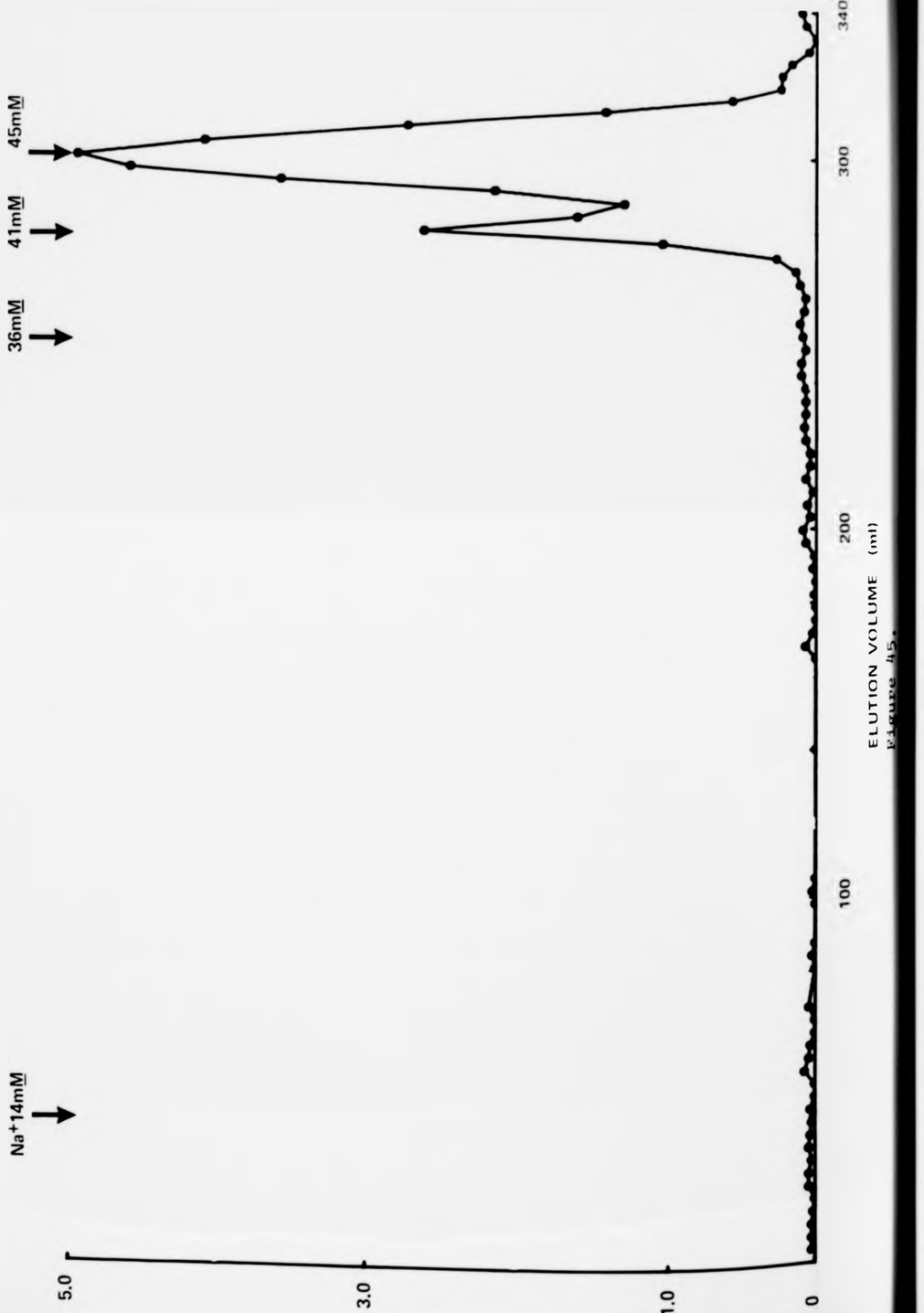


Figure 45 CM-cellulose chromatography of peak Na^+ 45m<u>M</u> shown in figure 41 following dissociation with guanidinium chloride and subsequent reassociation.

treated with guanidinium chloride. The result of this experiment is shown in figure 46.

Figure 42 shows that the Na^+ 14m*M* peak produced an almost identical profile to the original in that only a single 'flow-through' peak was seen. The Na^+ 27m*M* peak after hybridisation produced a completely different profile (figure 43). A series of peaks were generated that corresponded to those of the original profile shown in figure 40, although not in the same proportions. Hybridisation of the Na^+ 36m*M* peak generated further peaks that, although poorly resolved, represented elements of all peaks seen in the original profile (figure 41) with a major component at Na^+ 41m*M* (figure 44).

Although these profiles represent the result of a single hybridisation experiment on each peak, there is significant evidence for the interconvertibility of some enzyme forms with the generation, in two cases, of more basic components.

When the Na^+ 45m*M* peak was hybridised only the two peaks of activity resulted (figure 45). Significantly, no 'flow-through' activity was generated by the Na^+ 45m*M* peak on hybridisation.

When equal activities of Na^+ 36m*M* and Na^+ 45m*M* peaks were hybridised it was interesting to see that the Kamisaka *et al* (1975) type profile was converted into the Hayes *et al*. (1983) type profile (figure 46).

The observation from starch-gel electrophoresis

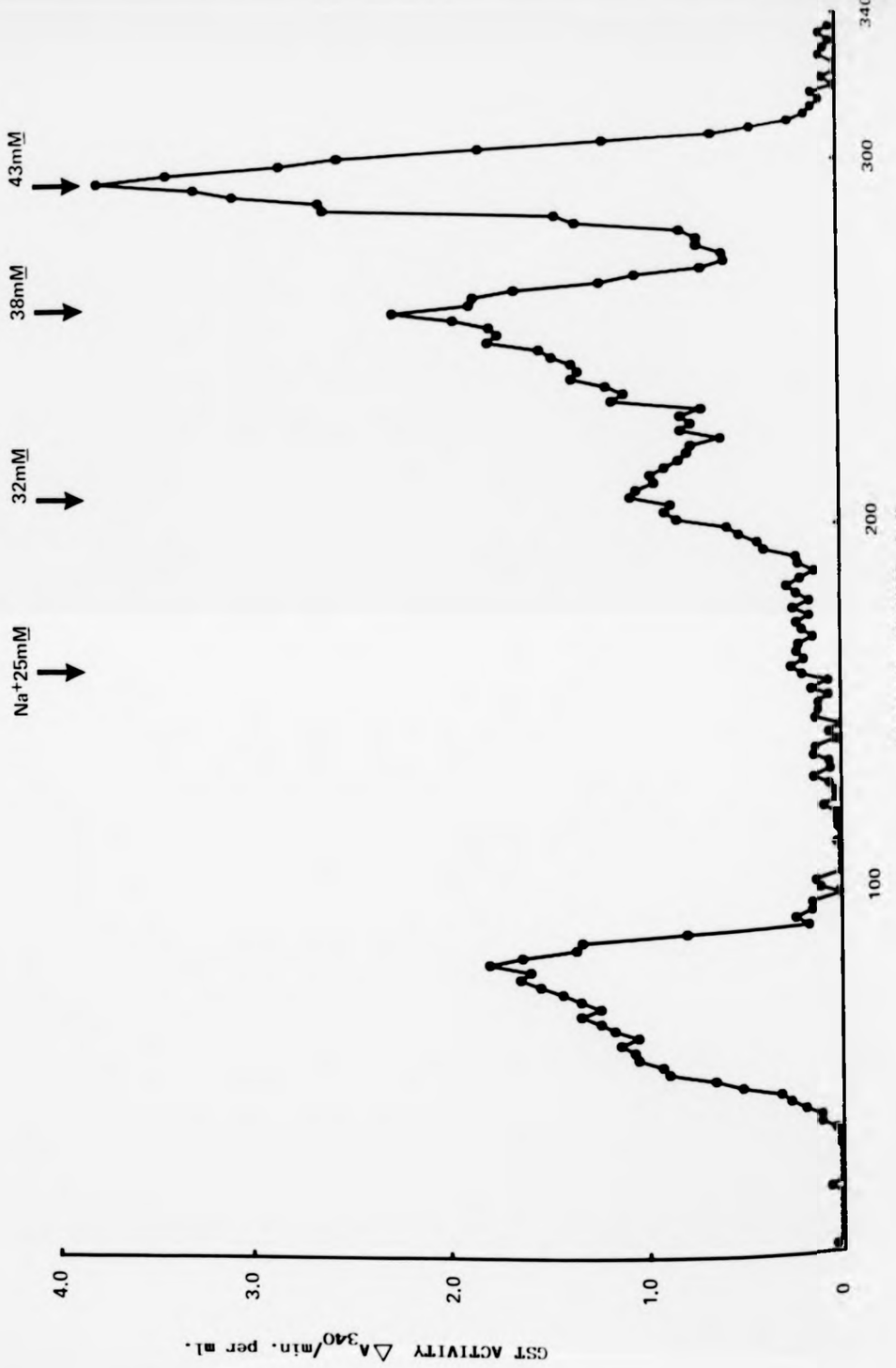


Figure 46.

Figure 46 CM-cellulose chromatography of peaks Na^+ 36m$\underline{M}$ and Na^+ 45m$\underline{M}$ shown in figure 41 following dissociation with guanidinium chloride and subsequent reassociation.

studies that GST2 expression was not a constant individual characteristic was confirmed when profiles of kidney and adrenal from the same individual were studied using CM-cellulose chromatography. Figures 47 and 48 respectively show that kidney and adrenal profiles of GST2 differ considerably from that of the corresponding liver (figure 41).

Having examined the adult patterns of expression of the GST2 locus, a series of liver cytosols from foetal and neonatal subjects were eluted from ion-exchangers to determine whether multiple basic forms of GST were present during development. The results of these studies are summarised in Table 6. The basic GST isoenzymes eluted at similar Na^+ concentrations to the adult isoenzymes and no specifically foetal forms were identified. Both types of elution profiles were seen throughout development. The α and β components were, however, virtually absent until about one year of age. The proportions corresponding to γ, δ and ϵ were not found to be age dependent.

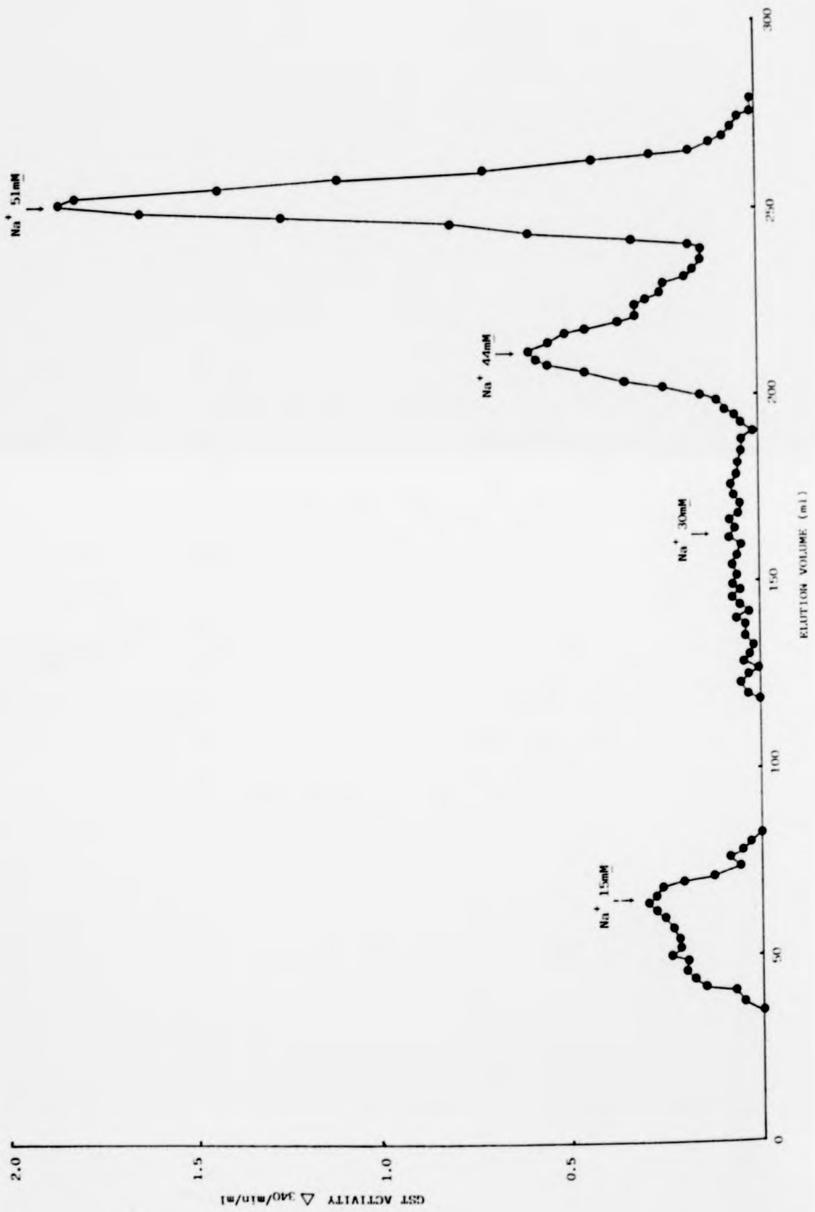


Figure 47.

Figure 47 CM-cellulose chromatography elution profile of adult kidney basic GST isoenzymes.

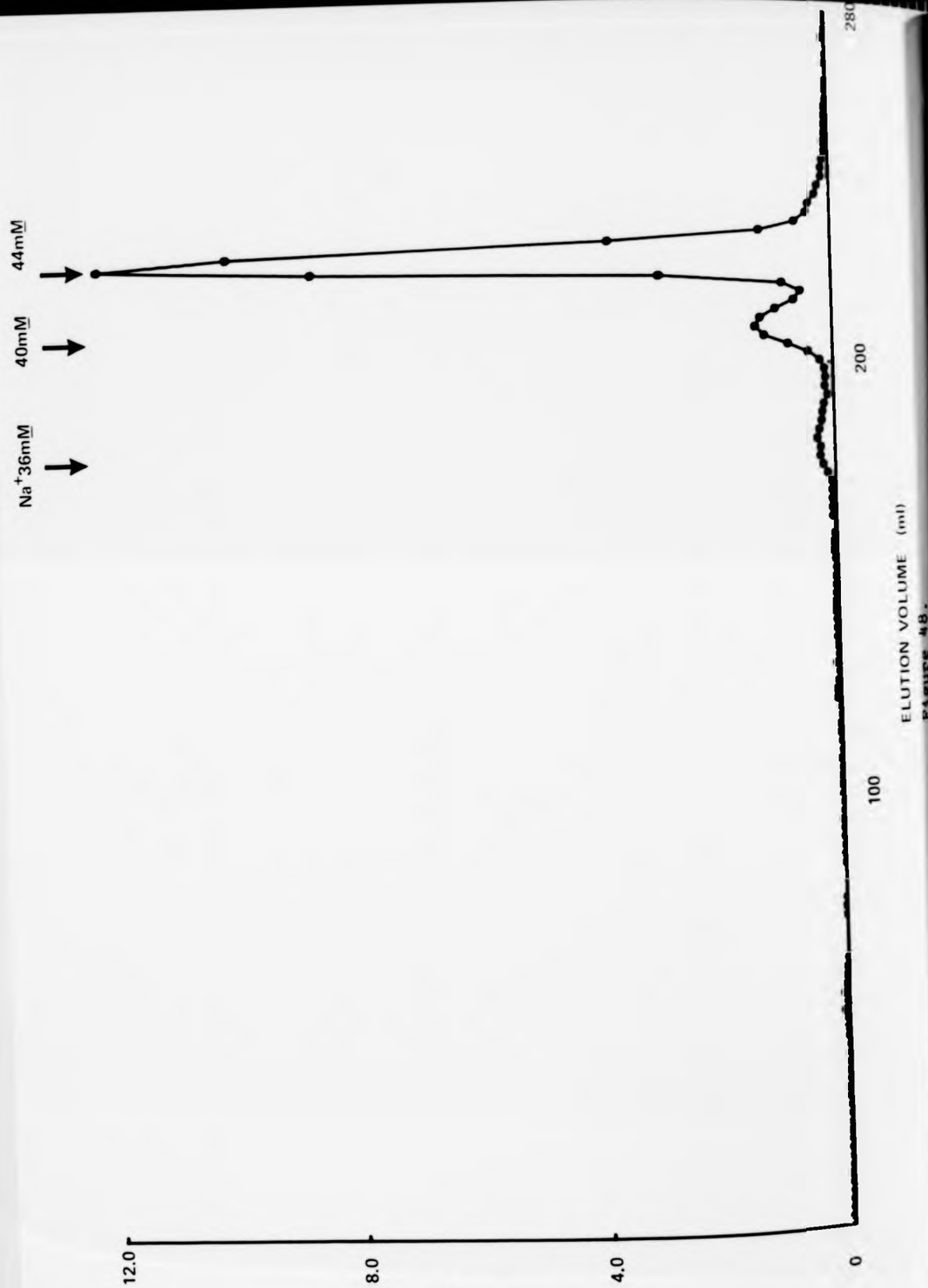


Figure 48 CM-cellulose chromatography elution profile of adult adrenal basic GST isoenzymes.

Separation of the basic GST isoenzymes in liver. Samples of liver cytosol were obtained during development and basic GST isoenzymes separated using DEAE- and CM-cellulose chromatography.

Weeks of gestation	Na^+ 14-16mM	Na^+ 27mM	Na^+ 34-36mM	Na^+ 39-41mM	Na^+ 43-47mM
Adult 1	+	+	Adult pattern		1
Adult 1	+	+ +	Adult pattern		2
21	-	-	Adult pattern		2
28	-	-	Adult pattern		1
30	trace	-	Adult pattern		1
39	trace	-	Adult pattern		1
40 + 12 weeks postnatal	+	-	Adult pattern		1
40 + 38 weeks postnatal	trace	-	Adult pattern		1
40 + 61 weeks postnatal	+	-	Adult pattern		2

Table 6.

DISCUSSION

The purpose of the work described in this thesis has been to investigate the extent of variation in the GST in several human tissues as well as to describe the ontogeny of the different isoenzyme sets. The basic isoenzyme set has been studied in most detail but since the techniques used also provided information about the other isoenzyme sets, data relating to their variation and ontogeny has also been included. The experiments described here as well as those of others (Baars et al., 1981) indicate that GST activity is present in all human cell types and probably at all stages of development. The isoenzyme composition in different human tissues varies considerably and it seems likely that further study of human tissues would reveal similarly complex isoenzyme patterns to those found in the rat. Study of GST in that animal have been complicated because of the large number of GST so far identified and because no coherent scheme has been suggested that allows classification on the basis of origin. Classification of the human isoenzymes however is simpler as a result of the three gene locus model proposed by Board (1981a) as well as the division of human GST isoenzymes into basic, near-neutral and acidic sets as proposed by Mannervik and his colleagues. The results presented in this thesis are described in terms either of the genetic model or of the Mannervik classification. The use of these systems does not indicate that the full extent of variation of these

isoenzymes in humans has been uncovered, indeed there is evidence to indicate that more than three gene loci are involved but if further forms are identified they could be included in these types of classification schemes.

A further feature of the various GST isoenzymes of the rat is their ability to catalyse the conjugation of GSH with a variety of hydrophobic substrates. Before the more recent use of SDS discontinuous polyacrylamide gel electrophoresis to identify the different subunits, use was made of substrate specificity to identify particular isoenzymes. The human isoenzymes appear to be simpler in this regard since CDNB is the only compound that can act as a universally effective second substrate. In experiments not presented in this thesis, the ability of tissue cytosols to catalyse the conjugation of GSH with ethacrynic acid and dichloro-nitrobenzene was found to be extremely low and consequently they were not used further. Perhaps the only important alternative substrate for the basic isoenzymes is cumene hydroperoxide since this isoenzyme set provides the non-selenium dependent peroxidase activity present in many tissues. Styrene oxide is a useful substrate when studying the μ group (Warholm *et al.*, 1983).

The ability of the basic isoenzymes to catalyse the detoxication of organic peroxides raises the interesting question as to the true *in vivo* substrates for these isoenzymes. Meyer and Ketterer (1982) have shown GSH

conjugation of cholesterol α-epoxide and other workers have shown similar conjugation of oestradiol-17β. It has also been suggested that quinones might be endogenous substrates and more recently that leukotrienes may be endogenous substrates (Samuelsson, 1982). It is clear therefore that the true role of these isoenzymes remains unclear but since the ability of the different isoenzymes to catalyse the conjugation of glutathione with CDNB was high, the latter was used. It is, of course possible that unrecognised isoenzymes were present in the samples studied that had low activity towards this substrate but available evidence suggests that this is unlikely.

4.1. Variation of the Basic (GST2) Isoenzymes.

The basic GST isoenzymes are characterised by cathodal mobility on starch-gels and in the study described no difficulty was experienced in obtaining zymogram patterns similar to those originally described by Board (1981a). He interpreted his data as indicating that enzymes identified on the cathodal side of the origin were products of a single locus (GST2) and that variations in the patterns seen resulted from the presence of two alleles (GST2*1 and GST2*2). Board's model was based on experiments in liver only and his proposal that two alleles were present at the locus was largely based on agreement between the observed and expected distribution of phenotypes.

The starch-gel electrophoresis method used in this laboratory was a hybrid of that originally described by Scott and Wright and by Board. One important difference between the two methods was in the use of overlays to identify enzyme activity. Whilst Scott and Wright used overlays of agarose containing CDNB and glutathione, followed by a second agarose overlay containing I_2 and KI, Board used two filter paper overlays with the same substrates and chemicals. The use of filter paper overlays was found to give poor results compared with agarose overlays. Whereas our technique showed resolution of the basic isoenzymes, Board's data show essentially no resolution between the enzymes with fastest cathodal mobility and those with slowest cathodal mobility. The presence or absence of the enzyme bands has to be inferred from the intensity of the enzyme staining.

A further difference between the data presented here and those of Board concerns the relatively high incidence of samples in which no basic isoenzyme activity was detected. During the course of this thesis about 200 samples of liver cytosol were examined and in all but one sample the basic isoenzymes were strongly expressed. Board (1981a) however,found that 19 out of 198 liver samples studied demonstrated an absence of these isoenzymes. He suggested that these were "inactive, degraded" samples and they were excluded from his Hardy-Weinberg analysis. The explanation for this relatively high

incidence of apparent non-expression is not clear. The samples used in Board's (1981a)study were obtained from several racial groups and it is possible that the non-expression trait is genetical and has a higher incidence in Chinese and Indian subjects. Since Board does not state that he excluded samples on the basis of time since death it is also possible that these samples were obtained a relatively long time after death and that the GST2 isoenzymes are less resistant to post-mortem autolysis than are the near-neutral isoenzymes. This difference between my data and those of Board (1981a)is important since, apart from the non-expressors, he also found subjects who demonstrated enzyme activity that migrated on starch-gels with slow cathodal mobility. This electrophoretic pattern was not seen in any of the samples that I studied and it is not clear whether this represents a genuine genetical trait or is a consequence of post-mortem autolysis.

Since individuals with the putative GST2 2 phenotype were not seen in this present study our data do not support the proposal that two alleles are present at this locus and I have interpreted my results as supporting the original proposal of Kamisaka *et al*. (1975) that the various basic isoenzymes arise from the post-synthetic modification of a primary gene product.

The finding of a single individual who failed to express the basic isoenzymes is interesting because, as previously stated, it cannot be excluded on the grounds of

post-mortem effects. Unfortunately, adrenal, kidney and other tissues that express this locus in adults were not obtained from this individual, but nonetheless, the absence of B_1 and B_2 monomers in this sample suggests a failure of synthesis rather than degradation of active enzyme.

As already stated, examination of the zymogram patterns of liver cytosols did not support Board's proposal that the between-sample variation was a result of genetical differences. Further support for this conclusion was obtained by studying other tissues obtained from the same subject. These experiments showed that adrenal cytosol almost invariably demonstrated a single band with cathodal mobility even in individuals whose liver samples demonstrated three bands of activity. Interestingly, elution of adrenal cytosol from CM-cellulose columns revealed the presence of γ, δ and ϵ thereby indicating that the B_1 and B_2 monomers identified by Stockman *et al*. (1985) are present in the zymogram band with fastest cathodal mobility. The starch-gel patterns obtained from kidney cytosol were closest to those of liver but the other tissues examined such as spleen and stomach demonstrated either weak expression or, in the case of blood cells, no detectable expression at all. Since there appeared to be no relationship between the expression of these isoenzymes in liver and in the other tissues these observations support the

argument that the various basic isoenzymes arise by post-synthetic modification of the most basic enzyme.

The nature of the modification has not been identified. The zymogram patterns of the basic isoenzymes could not be altered by incubating cytosol at 4°C for up to 96h or by allowing them to react with a variety of sulphydryl agents. Kamisaka et al. (1975) have suggested that de-amidation of asparagine or glutamine might be the cause and this possibility remains to be tested.

4.2. Number of Basic (GST2) Isoenzymes.

One of the difficulties in studying the set of basic GST isoenzymes is the problem of defining exactly how many forms exist. Kamisaka et al. (1975) originally described five basic GST isoenzymes although subsequent studies by Hayes et al. (1983) showed that the activities of α and β were often low. Two series of experiments are described in this thesis that provide information concerning the number of isoenzymes. In the first scheme, hydrophobic interaction chromatography was used to resolve basic isoenzymes obtained from the "flow-through" fractions eluted from DEAE-Sephadex. This scheme was used to investigate the basic isoenzyme composition of three liver cytosol samples and in each case similar results to those described in the Results section of this thesis were obtained. These showed that five forms of the enzyme could be identified, a finding that is in agreement with Kamisaka et al. (1975).

This agreement is reassuring since the separation scheme used by Kamisaka et al. (1975) and Hayes et al. (1983) depends on the elution of the isoenzymes from the ion-exchanger using a sodium ion gradient. The differences in the elution concentration of sodium ions were extremely small and indeed it was difficult to identify particular isoenzymes on the basis of elution concentration. Hydrophobic interaction chromatography might have been expected to offer an effective way of separating the different isoenzymes since the basic set is able to bind a variety of hydrophobic ligands. One difficulty in using chromatographic techniques that depend on enzyme-ligand interaction is that in many cases the GST appear to be so tightly bound as to be removable only with great difficulty. For example, in preliminary experiments carried out in this laboratory, the basic GST was found to bind so avidly to columns of Affigel Blue (Biorad) that high concentrations of urea were required before they could be eluted. Similarly, Mannervik (1985) has reported that many GST bind so strongly to immobilised bromosulphthalein that this ligand cannot be used in a purification scheme. The GST also bound strongly to Phenyl Sepharose and gradients of ethanediol were required for elution. The scheme was cumbersome and since it provided no more resolution than the scheme originally proposed by Kamisaka et al. (1975) and that more recently presented by Vander Jagt et al. (1985) this method of preparing homogeneous basic isoenzymes

was not used in further studies of this isoenzyme set.

It is interesting to note that the immobilised GSH column failed to retain all of the GST isoenzymes before addition of the glutathione to the column. Other workers have also reported that relatively small amounts of GST often elute in this way. The origin of this material is not clear and it has been suggested that it represents partially denatured enzyme. More recently however, Vander Jagt *et al*. (1985) used GSH coupled to epoxy-activated Sepharose to separate a variety of GST isoenzymes present in human liver. They showed that about 25% of the GST in human liver was not retained by the column and this activity was termed the "low-affinity" set. The "low-affinity" and the retained "high-affinity" isoenzymes were subsequently further resolved using chromatofocusing. The "low-affinity" set from one liver sample was resolved into five components having isoelectric points of 8.68, 7.42, 7.09, 6.12 and 5.50 and the two basic isoenzymes were found to be present in four other liver samples. These two "low-affinity" basic isoenzymes presumably correspond with the two isoenzymes that were not retained by the affinity column described in the "Results" section of this thesis.

Vander Jagt *et al*. (1985) are the only group so far to claim to have isolated up to thirteen different hepatic GST that share similar characteristics. All were found to be identical immunologically, none were affected by

sulphydryl group reagents and all demonstrated GST peroxidase activity towards cumene hydroperoxide. All thirteen forms bound bilirubin with subsequent changes in conformation leading to loss of activity. The loss in activity was, however, prevented by the presence of other proteins. Vander Jagt et al. (1985) classified these isoenzymes using Roman numerals I - XIII. The majority of individuals were found to have between eight and ten of the possible thirteen isoenzymes found in liver. In addition to these I - XIII, they also found two other isoenzymes with higher molecular weight and demonstrating complete inactivation by sulphydryl group reagents. Interestingly, these isoenzymes were completely absent in some subjects. The characteristics of these two isoenzymes suggest that they correspond to those classified as GST1 by Board (1981a) and the isoenzyme μ described by Warholm et al. (1981). The group classified as I - XIII by Vander Jagt et al. (1985) almost certainly incorporate those isoenzymes described by other investigators as basic GST or GST2. It is interesting to note that several groups including Kamisaka et al. (1975), Hayes et al. (1983) and Stockman et al. (1985) have all failed to identify more than five basic GST isoenymes in human liver. My own work also did not detect more than five forms of basic hepatic GST isoenzymes even though a diverse range of separative techniques was employed.

The observation reported by Vander Jagt et al. (1985)

that most individuals have between eight and ten putative basic isoenzyme in liver (double the number reported by other investigators) is an expected consequence of the use of a GSH-affinity method. The separation of many GST isoenzymes using GSH-affinity chromatography results in the detection of a "low-affinity" GST, well before the main GST isoenzyme (the so called "high-affinity") is eluted with a high concentration of GSH. The relationship between the 'high' and 'low' affinity sets is not clear but they may represent conformational isomers.

The second separation scheme described in this thesis made use of the ion-exchange system described by Kamisaka et al. (1975), Hayes et al. (1983) and Stockman et al. (1985). This scheme utilises DEAE-cellulose and CM-cellulose and although these two ion-exchangers have been partly superceded by the introduction of alternative matrices such as Sephadex, agarose and polyacrylamide, the investigations of the basic GST have resulted in the use of these exchangers becoming the reference method for their study. It is important to note that the schemes used by other workers were designed to prepare purified protein but since the objective of this thesis was to determine whether isoenzymes were present at particular stages of development a more simple strategy was employed to include only the ion-exchange steps. Use of the complete published scheme would have been extremely time-consuming and would have greatly restricted the amount of data collected.

Until recently it was widely accepted that the basic isoenzymescomprised the products of a single gene locus that Board (1981a) termed GST2. The study described by Stockman et al. (1985) however, presented the unexpected finding that two monomers termed B_1 and B_2 were present in liver cytosols and that γ comprised a B_2B_2 homodimer, δ a B_1B_2 heterodimer and ε a B_1B_1 homodimer. α and β were also B_2B_2 and were assumed to arise by a process of post-synthetic modification.

So far the proposal that two alleles code for the basic isoenzyme set rests on the use of antiserum to the two monomers. The findings of Stockman et al. (1985) are contrary to those of Kamisaka et al. (1975). The latter group found that antiserum to human "ligandin" cross-reacted with α, β, γ and δ as well as with ε. These data are rather hard to interpret since the preparation of human "ligandin" is not given and is described in the text of the paper as "unpublished" results. It is possible that since the enzyme originally called "ligandin" that was isolated from rat liver was found to contain two enzymes, that the preparation termed human "ligandin" was a mixture of B_1 and B_2 containing enzymes that although homogeneous with respect to other proteins antiserum would be expected to cross-react with α, β, γ, δ and ε. Clearly further work is required before the origins of B_1 and B_2 can be identified. Peptide maps of B_1 and B_2 should indicate whether they are the products of the same

gene although such studies have not yet been reported.

Whatever the origins of B_1 and B_2 they would appear to be extremely similar structurally. The amino acid composition of α, β, γ, δ and ϵ are very close and although no analysis of difference was perfomed the results for the five isoenzymes appear within the expected errors of the method. A further indication of the closeness in their structures is the finding that both monomers migrate with fast cathodal mobility. Figure 40 shows an adult cytosol that demonstrated only one band with cathodal mobility. When eluted from DEAE-cellulose and CM-cellulose this cytosol was resolved into α, β, γ, δ and ϵ. It is clear therefore that apart from the similarity in their amino acid compositions, B_1 and B_2 must have similar net charges.

A further difficulty in accepting the proposal that B_1 and B_2 are the products of separate alleles is the finding of one cytosol that failed to express this isoenzyme set. The finding that the basic isoenzymes were absent is difficult to reconcile with the presence of two alleles since it is unlikely, but not impossible, that both alleles would simultaneously fail to express. Furthermore, if the two alleles are present at the same locus, the data so far obtained would suggest that all the subjects so far studied were heterozygotes. This is clearly not possible since, by any genetic model, one would expect homozygotes for either B_1 or B_2. It is

possible that B_1 and B_2 are the products of separate homozygous loci but if this was the case it might be expected that the differences between them would be more readily detected particularly by starch-gel electrophoresis.

A further interesting point to note concerning the basic isoenzymes is that the elution profile obtained from CM-cellulose was not a fixed individual characteristic. Thus, when the profiles from liver and adrenal, from the same individual were compared, patterns corresponding to the type 1 and type 2 profiles were seen.

Further evidence that the relative activities of α, β, γ, δ and ϵ were not a fundamental individual characteristic was indicated by the _in vitro_ hybridisation experiments. These showed that α could be generated from the reassociation of monomers derived from δ but not from ϵ. This observation was expected from the data of Stockman _et al_. (1985). Hybridisation of δ also appeared to result in the formation of γ and ϵ although the degree of resolution of these forms was relatively poor. The interesting finding was that hybridisation of a mixture of δ and ϵ resulted in the formation of α, β, γ, δ and ϵ in proportions that were different to those of the original untreated cytosol. These experiments support the suggestion that there are two basic monomers if one also assumes that α is produced _in vitro_ from δ by an unknown mechanism. However, the acceptance

that there are two separate monomers does not signify that they are the products of separate alleles since the hybridisation data would also be compatible with the suggestion that some of the basic isoenzymes are conformational isomers. A further suggestion is that the isoenzymes have bound different amounts of hydrophobic ligand and that differences in the extent of binding affect their chromatographic properties. This latter suggestion is supported by the finding that there was considerable absorption in the 260 nm region by the α-containing fractions whereas very little absorption was observed in this region when fractions from the other peaks were examined. This could indicate that the α fractions contained enzyme with a high negative charge because of the binding of RNA. This phenomenon has been reported for other proteins (Scopes, 1984).

4.3. The Relationship Between the Different Near-neutral (GST1) Isoenzymes.

GST activity in liver primarily results from the presence of near-neutral as well as basic isoenzymes. The possibility that the near-neutral set demonstrates genetic variation was suggested firstly from the observation that only 60% of livers appear to express the near-neutral isoenzyme μ and secondly from the results of starch-gel electrophoresis experiments originally described by Board (1981a).

Warholm et al. (1981) first described the μ isoenzyme and showed that its amino acid composition and immunological properties were different from those of the basic isoenzyme set. They inferred therefore that μ was the product of a separate gene locus. Traditional biochemical chromatographic techniques do not allow large numbers of samples to be studied and Warholm et al. (1981) do not appear to have studied sufficient samples to allow them to deduce whether different forms of μ exist. The results of Board (1981a) would indicate that separate forms do exist since he was able to identify four phenotypes by using starch-gel electrophoresis. Board's model is based on the hypothesis that the near-neutral isoenzymes are the products of three alleles whose frequencies are similar in Chinese, Indian and Caucasian Australians. One interesting finding was the high incidence of the null allele GST1*0. Board found that the frequency of this allele varied between 0.56 and 0.81 in the populations that he studied and results obtained in our laboratory have shown similar frequencies for the allele for the local English population (0.64) and a Scottish population (0.75). Subsequent work by Laisney et al. (1984) has shown a similar frequency in a French population. Because of the high frequency of this allele, many individuals with the GST1 1 and GST1 2 phenotypes will be heterozygous for the GST1*0 and GST1*1 or GST1*2 alleles. Unfortunately, at present, it is difficult to distinguish between homozygous and heterozygous

individuals who demonstrate GST1 1 and GST1 2 phenotypes. Hardy-Weinberg analysis indicates that only about one in eight individuals with these two phenotypes will be homozygotes (Strange et al., 1984). It may be possible to distinguish between homozygotes and heterozygotes by using chromatofocusing since the relative contribution of the near-neutral isoenzymes can be determined and compared with that in subjects with the GST1 2-1 phenotypes. These latter subjects can be used as a control group since although they are heterozygotes, both of their alleles are able to produce active protein. The use of chromatofocusing would however be rather cumbersome since at least 50 cytosols would need to be studied. A further difficulty may be the very wide interindividual variation seen in liver GST activity, a phenomenon that has also been reported in erythrocytes (Strange et al., 1982) and platelets (Rogerson et al. 1984).

While it is recognised that many enzymes in natural populations are polymorphic (Harris 1980) the biological consequences of the phenomenon are largely unknown. This is because it is usually not possible to relate a particular polymorphism to a physiological characteristic. In this case, the high frequency of the GST1 0 phenotype is of particular interest and both Board (1981a)and Silberstein and Shows (1982) have suggested that subjects with this phenotype may be disadvantaged either because they were less effective at detoxicating xenobiotics or because they

were less able to transport ligands across the hepatocyte. This hypothesis would be tenable if it was proven that the GST have an important role in detoxication or in hepatic ligand transport. Subjects with the GST1 0 phenotype do demonstrate reduced GST activity in liver cytosol and presumably also reduced concentrations of the enzyme but there is no evidence, so far, to suggest that either reduced activity or concentration has any effect. Studies in this laboratory have suggested that, at least in terms of bile acid transport, subjects with the GST1 0 phenotype are as able to bind these ligands as well as subjects with other GST phenotypes (Scriven et al., 1986). There is as yet no study confirming or disproving the suggestion that detoxication is less effective in subjects with the GST1 0 phenotype but in a small study of 30 subjects with cancer of the large bowel or stomach I found the incidence of the GST1 0 phenotype to be very similar to that in subjects who had died from acute myocardial infarction. Clearly much larger numbers of subjects will have to be studied before any definite conclusions can be drawn and since so many enzymes are involved in detoxication, it is likely that, at best, the relationship between susceptibility and phenotype will be similar to that between the ABO blood groups and stomach cancer.

A further point of interest concerning the near-neutral isoenzymes is the relationship between the isoenzymes found in subjects with GST1 1, GST1 2-1 and

GST1 2 phenotypes. On the basis of starch-gel electrophoresis experiments it was proposed that the GST1 1 isoenzyme is a homodimer (GST1*1/GST1*1), the GST1 2-1 a heterodimer (GST1*1/GST1*2) and the GST1 2 a homodimer (GST1*2/GST1*2). In order to investigate this proposal further, a series of hybridisation experiments was performed. They were facilitated by obtaining samples of the different isoenzymes from a subject with the GST1 2-1 phenotype. Hybridisation of the putative heterozygous isoenzyme resulted in the expected formation of the two homozygous isoenzymes as well as the original heterozygous isoenzyme. Similarly, hybridisation of a mixture of the two homozygous isoenzymes resulted in a mixture of the three expected isoenzymes. The mixture of isoenzymes resulting from each of the hybridisation experiments was in the appropriate ratio of 1:2:1. These data support the conclusions of the starch-gel electrophoresis studies and it was felt justified therefore to study the development of the GST1 locus in terms of two active monomer proteins. In addition to this, the observation that the two allelic products of the GST1 locus are present and able to hybridise *in vivo* and *in vitro* in the ratio 1:2:1 reveals four important features about this locus. Firstly, there is demonstration of the expression of two alleles. Secondly, there is codominance of these alleles at the GST1 locus. Thirdly, the monomers are synthesised at the same rate in the cell and fourthly, synthesis takes place simultaneously in the same cell compartment.

It should also be noted that whilst electrophoresis of different tissue cytosols from the same individual failed to support the hypothesis that there are two alleles at the GST2 locus, this approach provided evidence to support Board's proposal that the GST1 locus is polymorphic.

Although evidence is presented concerning the relationships between the different GST1 isoenzymes it is still not clear which one of them corresponds with μ. The term μ has therefore been used to encompass all the near-neutral GST1 isoenzymes.

4.4. The Acidic (GST3) Isoenzymes.

The acidic isoenzymes have been ascribed to the GST3 locus although there is some controversy as to whether the forms found in erythrocytes, placenta, eye and liver are all products of the same locus. The acidic isoenzymes are ubiquitous and appear to be strongly expressed in all tissues except adult liver. The low levels of activity found in adult liver did not result from contamination with erythrocyte GST but appear to be the result of down-regulation of the locus during development.

Determining whether the various acidic isoenzymes originate from different loci will require structural studies. However, the similarities of the zymograms of tissues studied in this thesis indicated that they are the products of the same locus that can conveniently be referred to as GST3.

4.5. Rare Alleles.

The starch-gel electrophoresis and chromatofocusing results have largely been interpreted in terms of the three locus model proposed by Board (1981a). In the several hundred samples examined, bands or peaks of activity were ascribed to one or other locus, labelled as post-synthetic modifications or were assumed to be inter-locus hybrids. In neither the foetal nor the adult tissues were enzymes encountered that could not be classified. This is partly a circular argument since the techniques used were chosen since they offered useful ways of studying the isoenzymes in terms of the three gene locus model and it is possible that, in some samples, unrecognised enzyme activity co-migrated or co-eluted with forms already recognised.

It is also possible that, although a relatively large number of samples were studied, further isoenzyme forms remain to be identified. Examples of the products of rare alleles have been described for many enzymes and whereas polymorphism can be detected in between 50-100 samples, identification of rare alleles may require many thousands of samples before a single heterozygote example is found. Examples of this phenomenon have been reviewed by Harris (1979) who surveyed 31 loci for the presence of rare alleles. Interestingly, both polymorphic and non-polymorphic loci appear to demonstrate similar incidences of rare alleles. It is interesting to speculate that the subject previously described who failed to express basic

isoenzyme activity was homozygous for a rare allele. Clearly identification of heterozygotes would be extremely difficult.

4.6. DEVELOPMENTAL STUDIES OF THE GST ISOENZYMES.

The second major part of this thesis was concerned with the ontogeny of the various GST isoenzymes already described in adult tissues. The study of enzyme development has important implications in terms of the switching on of gene loci particularly in the case of multilocus enzymes. A literature review undertaken before these developmental studies were begun revealed that although there were reports of GST activity in fetal tissues from the mouse, there was no systematic study of the ontogeny of these isoenzymes in human tissues. There were however, isolated reports of the presence of GST activity in human foetal tissue (Mukhtar *et al*. 1981, Pacifici *et al*. 1981, Warholm *et al*. 1981). These studies had been mainly limited to measuring enzyme activity in samples before 20 weeks of gestation although ion-exchange studies by Warholm *et al*. (1981) had indicated that the isoenzyme composition of tissues in early gestation was more simple than that in adults.

Studying enzyme ontogeny in humans is difficult because of the obvious ethical problems and these studies would not have been possible without the close co-operation of Dr. R. Hume in Edinburgh. He ensured that, wherever

possible, tissues were obtained shortly after death.

4.6.1. Development of the Basic (GST2) Isoenzymes.

The ontogeny of the basic isoenzyme set was studied in most detail since it was of interest to know whether the appearance of B_1 was different to that of B_2. Consequently a series of liver cytosols obtained during gestation were eluted from DEAE-cellulose and CM-cellulose. The elution profiles were examined for the presence of α, β, γ, δ and ε. In the series of samples studied there appeared to be no differences between subjects at different periods of development. The ε form was present at 21 weeks of gestation and in all of the samples obtained thereafter. The γ and δ isoenzymes were also identified indicating that both B_1 and B_2 are present by at least the second trimester. The α and β isoenzymes were not usually present in these samples, a finding that would be in keeping with the suggestion that they are post-synthetic modifications of a primary product. Fetal isoenzyme patterns are often more simple than those of corresponding adult tissues because proteolytic enzymes have yet to be expressed.

No changes in the expression of basic GST in liver during gestation were demonstrable by using chromatofocusing. In this tissue these isoenzymes consistently contributed about 80% of total cytosol activity.

Similarly expression of the basic isoenzymes in adrenal

also appeared to be a constant feature. Resolution of this set using the ion-exchangers also showed the presence of γ, δ and ϵ indicating the presence of B_1 and B_2.

Spleen and kidney tissues that were studied quantitatively however, did demonstrate changes in expression during development. This isoenzyme set did not appear to be expressed to any extent in either tissue before birth but in kidney a steady, progressive increase in expression was seen after this time. No clear pattern could be discerned in the spleen samples.

4.6.2. Development of the Near-neutral (GST1) Isoenzymes.

The observation that many adults failed to express the near-neutral isoenzymes complicated the study of their development. However, sufficient samples were studied during gestation to be reasonably certain that the absence of these isoenzymes during early gestation was due to the low level of expression of the locus rather than to a particularly high incidence of the null allele in the population studied. The conclusion is supported by the finding that the frequency of the GST1*0 allele was similar in these samples to other adult populations (Board, 1981a; Strange et al., 1984; Laisney et al., 1985). In liver these isoenzymes were usually first detected at about 30 weeks of gestation, a time at which the expression of the acidic isoenzymes was already at a low level. Since GST3 activity declined in subjects with the GST1 0 phenotype,

there was no evidence to suggest that the down-regulation of GST3 was a response to increased expression of GST1.

In the samples studied the development of the GST1 isoenzymes was similar in subjects with GST1 1 or GST1 2 phenotypes.

The GST1 isoenzyme can be detected in adult adrenal, kidney and spleen although their level of expression is lower than that in liver. When GST1 isoenzymes were identified in these tissues the phenotype was usually as predicted from investigations of liver samples from the same subject. This was not always the case in adrenal but this appears to be a consequence of the particularly low level of expression in this tissue. No developmental profile could be discerned in these three tissues.

4.6.3. Development of the Acidic (GST3) Isoenzymes.

These isoenzymes were identified in all the samples examined during development although in liver the level of expression declined markedly during the first and second trimesters so that at birth their level of activity was often at the detection limits of the starch-gel electrophoresis method. A decline in the level of expression was also observed in kidney although the time-course of the fall was rather different since expression appeared to be constant until about 40 weeks post-natal age. Expression in spleen and adrenal appeared to be more constant and no developmental trend was identified.

Interpretation of the starch-gel zymograms was complicated in the case of the acidic isoenzymes. Two bands that migrated with similar mobilities to the erythrocyte enzyme were seen in most samples and this was taken as support for the belief that these isoenzymes are products of the same locus. The origins of the two isoenzymes is not clear although it is likely that the form with the fastest anodal mobility is a modified form of the slower parent band (Strange et al., 1983). In some samples an isoenzyme with mobility slightly faster than the fast erythrocyte band was also seen. The origin of this band is not known but has been assumed to be a further example of post-synthetic modification (Strange et al., 1985). Another isoenzyme band was also seen in kidney and spleen samples and this has been tentatively classified as a GST1/GST3 interlocus hybrid.

4.6.4. Review of Developmental Data.

There are no definite rules concerning the order of expression of a multilocus group of isoenzymes and it is not easy to determine why particular loci should be first expressed or down-regulated at a particular time in development. Edwards and Hopkinson (1977) were perhaps the first to review the data available from electrophoretic studies of human enzyme development. At the end of their review they presented some general statements and it is useful to compare these with the results of the studies presented in

this thesis. Their initial comment was that human development is difficult to study for the reasons already stated in this 'Discussion' and also because tissues are not generally obtained until at least 10 weeks of gestation. This may already be too late to identify many important changes since at this stage organogenesis has been largely completed. Their second conclusion was that enzymes that are the product of only one locus generally demonstrate the least developmental change. Such enzymes are present in many foetal and adult tissues and since the level of their activity does not change they can be considered to be essential for cell function. These enzymes have been described as catalysing "household" reactions. It should be emphasised however that it is not usually possible to identify these enzymes as being more essential for life than those that demonstrate marked developmental patterns. The third conclusion of Edwards and Hopkinson was that changes in enzyme expression generally occur during the second half of gestation and in early infancy and that adult patterns of expression are achieved by one year of post-natal age. The fourth conclusion was that specifically fetal isoenzymes are rarely encountered. Fifthly, the isoenzyme patterns of multilocus enzymes demonstrate little between-tissue variation in early gestation and as development progresses tissue specific patterns begin to appear so that in early infancy there are marked differences between different organs. A sixth conclusion

was that changes in the level of expression are usually gradual and appear to be synchronised. The finding of interlocus hybrid enzymes indicates that there is little compartmentation of the products of the different loci. Lastly, it would appear that as development progresses, the proportion of acidic to basic isoenzymes declines.

The data presented in this thesis would support the proposal that the isoenzyme patterns of the multilocus GST appear to be similar to those of adults by about one year of post-natal age. Most of the changes in the expression of isoenzymes occurred during the second half of gestation (as in the case of GST1 and GST3 in the liver) or in infancy (as in the case of GST2 and GST3 in the kidney). The isoenzyme patterns of several tissues were similar in early gestation in that expression of GST3 was predominant whilst that of GST2 was weak. As development progressed the zymogram patterns of different tissues became easily recognisable. Furthermore, in cases where developmental trends could be identified, changes in expression did appear to be gradual although in many tissues between subject variation appeared to be so large as to obscure any trends. Lastly, the data presented here do support the proposal that the relative contribution of acidic isoenzymes to basic isoenzymes does fall as development progresses.

More general conclusions concerning enzyme development have been presented by Whitt (1983). These guidelines are

largely based on data obtained from studies on fish and therefore may not apply to the ontogeny of human isoenzymes. Whitt suggested that those isoenzyme loci that are most tissue restricted in their expression are not usually expressed until later in development. In the context of GST, the GST1 and GST2 isoenzymes would therefore be expected to be expressed later in gestation and this is frequently found to be the case. Whitt's second proposal was that enzyme loci that have widespread tissue expression are generally expressed continuously during development. This suggestion is generally supported by the data for the expression of the GST3 isoenzymes. Whitt lastly suggested that enzyme loci that are most recently derived tend to have restricted tissue expression and have late developmental appearance. Thus the ontogenic order of gene expression parallels the phylogenic order of loci. It is not clear which of the GST loci is the most recently derived although since acidic enzymes have been found in simple unicellular organisms it is tempting to speculate that GST3 is the parent locus and that GST2 and GST1 have arisen by a process of progressive gene duplication.

4.6.5. Embryological Origin and Tissue Expression of GST.

The expression of the three sets of GST isoenzymes varied markedly during development. Differential expression of the three loci was observed. Kidney and spleen are derived from the embryonic mesoderm and the

pattern of expression of the three sets of GST isoenzymes in these tissues was similar. For example, there was no significant expression of basic GST isoenzymes until after birth and both tissues demonstrated adult levels at about the same post-natal age of one year, although by differing rates. The expression of the near-neutral isoenzymes in kidney and spleen was extremely low before birth and attained the low levels seen in adults at about one year after birth. The acidic GST in both these tissues was expressed in high proportions throughout foetal development and well into early infancy.

In contrast to the mesodermal kidney and spleen tissues, liver, derived from the endoderm, demonstrated a completely different pattern of developmental expression for all three loci. Basic GST isoenzymes in liver were consistently strongly expressed in the earliest foetal subjects and throughout development. Expression of the near-neutral isoenzymes was also different. There was a progressive gradual increase in the expression of the near-neutral isoenzymes beginning at about 25 weeks gestation and reaching the high levels of expression of the adult GST1 1, GST1 2 and GST1 2-1 phenotypes at about one year of age. The acidic GST was strongly expressed in the early fetal period but demonstrated a rapid reduction in expression during the second half of gestation to reach low adult levels of expression at birth (Strange et al. 1985).

The adrenal is composed of two embryonically distinct

tissues. The cortex is derived from the mesoderm and the medulla from the ectoderm. Since the cortex and medulla were always present in the samples studied, interpretation of the data was difficult. Although there were similarities in the expression of the near-neutral and acidic isoenzymes in adult kidney and spleen and adrenal, this finding may reflect the expression of the mesodermal component of the adrenal since, as yet, there is no data on the ontogeny of the GST isoenzymes in a purely ectodermally derived tissue.

Although at present the data are too limited to establish a relationship between the developmental expression of the three GST loci and the embryological origin of the tissue, the similarities and differences are interesting and further experimentation may reveal that the expression of the GST loci is related to the embryological origin of the tissue.

Both endodermal liver and ectodermal adrenal medulla have investments of mesodermally derived tissue and one of the problems in studying the expression of the three GST loci is assessing how much mesodermal tissue is present at a particular stage of development. In adult liver there is only a small investment of mesodermal tissue and this may be reflected by the proportion of GST3 expression in the adult. No data are available for the proportion of mesodermal investment in the fetal and neonatal liver only that a large number of cells have an erythropoetic function. This mesodermal erythropoetic tissue in early fetal life retrogresses towards birth.

In this respect the decline in GST3 expression in fetal liver paralleles the decline in erythropoetic function. It is tempting to speculate therefore that the mass decline in erythropoetic tissue is responsible for the proportional decline in GST3 activity and that there is no down-regulation of the GST3 locus, merely fewer mesodermally derived cells expressing GST3 fully. If this is the case there may be no expression of the GST3 locus in hepatocytes. Observations in support of the idea that GST3 expression in liver is of mesodermal origin can be seen from starch-gel electrophoresis and chromatofocusing experiments. In kidney, spleen, skeletal muscle and heart muscle, all pure mesodermally derived tissues, there is evidence for GST1/GST3 interlocus hybrids. This phenomenon would only arise if GST1 and GST3 were simultaneously expressed in the same cell. These putative interlocus hybrids are not seen in liver. One explanation would be that there is no GST3 production in hepatocytes and therefore no GST3 available for *in vivo* hybridisation.

Cell culture experiments would help to reveal the true expression of GST at the cellular level. This area is now being developed by colleagues at Edinburgh University who have started work on lung tissue cell culture. Cell culture has the advantage over tissue studies in that pure cell lines can be examined.

4.7. CONCLUDING REMARKS.

It is interesting to compare the ontogeny of the GST with that of other enzymes. Because of the obvious ethical problems involved in obtaining suitable material, enzyme development in humans is difficult to study and not surprisingly, the enzyme systems that have been most intensively studied have been those of immediate clinical interest.

One area that has received much attention is the ontogeny of the enzyme systems responsible for the production of surfactants. Surfactant is necessary for proper lung functionand the failure of the premature neonate to express the enzymes required for its synthesis appears to be the cause of the complex lung disorders termed respiratory distress syndrome and bronchopulmonary dysplasia. Gluck (1973) has shown that full synthesis of phosphotidyl choline, a major constituent of surfactant, cannot take place until the gene that codes for phosphocholine transferase is expressed. This gene is switched-on at about 34 weeks gestation. Gluck (1973) demonstrated a surge in phosphocholine transferase activity a few weeks before birth. This sudden increase in activity of phosphocholine transferase prepares the foetus for pulmonary independance with a time margin in the event of premature birth. No such surge in activity was demonstrated in the expression of any of the GST in the tissues studied. The observation that at least one of the GST loci in each

tissue is fully expressed at birth is however important and it could be argued that the requirement for GST "cover" is always accommodated at birth.

The UDP glucuronyl transferases, a family of detoxicating enzymes, have also received much developmental attention because of their role in the etiology of neonatal jaundice. Recently, Burchell et al. (1986) have shown that the UDP glucuronyl transferases responsible for the conjugation of bilirubin are expressed soon after birth and reach full expression at about six months of age. This pattern is of course similar to that seen for GST in the kidney. At birth GST2 in kidney shows a steady progressive increase in activity reaching adult proportions of expression at about one year of age.

Foetal specific enzyme forms are uncommon and although no specifically f tal forms of GST were identified, studies on thymidine kinase (Bernard et al. 1977) have shown expression of a foetal form in early development. During the middle period of gestation a transition from the foetal to adult form of the enzyme occurs. A similar phenomenon has been demonstrated for lactate dehydrogenase in liver and heart muscle (Werthamer et al. 1973). An interesting observation emerging from studies demonstrating the disappearance of foetal enzyme forms early in gestation is that they often reappear in adult neoplastic tissue. This has been found to be the case with thymidine kinase. Other foetal enzymes also reappear in neoplastic liver

tissue and an account of this phenomenon has been given by Weinhouse (1982). In the context of GST, a comparative study between normal and tumour liver tissue from the same individual (Sherman et al., 1983) has demonstrated the appearance of an acid form of GST in the tumour tissue. It is possible that abnormally high levels of GST3 in human liver therefore may be a foetal characteristic typical of liver carcinoma.

Although no specifically foetal forms of GST were identified using starch-gel electrophoresis or chromatofocusing in any of the tissues obtained during development, there are however two ways in which foetal forms may have been missed. Firstly, the separative techniques used were initially employed to study adult tissue cytosols and it is possible that foetal forms may have been expressed at such low levels that the methods failed to detect them. Secondly, it is possible that isoenzymes ostensibly classified as adult GST may be foetal forms that are indistinguishable by the separative techniques used.

However, in spite of these reservations there is reasonable evidence to suggest that foetal forms of GST are not present at least after 10 weeks gestation. The first set of experiments that support this hypothesis are the immunological data recently published by Mannervik and his colleagues (1985). They found that basic GST isolated from foetal and adult human liver were extremely similar immunologically as were the acidic isoenzymes

obtained from foetal and adult tissues. As expected the basic and acidic sets were entirely immunologically different. Further evidence has been obtained in our laboratory by Mrs. Pamela Hirrell. She has examined the effect of inhibitors on the basic, near-neutral and acidic GST isoenzymes from foetal and adult tissues and has shown that the behaviour of each set is similar in different tissues at different stages of development. Combined, these experiments are all compatible with the view that the same loci are expressed during development and that the isoenzymes of a particular set in any tissue are the same as the isoenzymes in any other tissue. More detailed structural or sequencing studies would be needed to confirm this belief.

As a final comment it is interesting to relate GST expression to concepts of the immaturity of the human neonate. In one of a series of fascinating essays describing different aspects of human evolution, Gould (1980) refers to the work of Portmann (1941) in which the reproductive strategies of mammals are divided into two distinct groups. Mammals either have short gestational periods producing helpless, poorly developed young or they have long gestational periods giving rise to well developed young that are capable of self preservation from birth. So-called "primitive" mammals such as rodents fall into the first group and are described as altricial (literally meaning "of birds" and referring to

the helplessness of many species of young birds when they first emerge from the egg). Advanced mammals such as primates fall into the second group and are described as precocial.With so many anatomical and physiological features in common with primates, man would have been expected to be precocial. Since he is not, Portmann has described the helpless human baby as secondarily altricial. In man it has now been estimated that many anatomical features, such as the brain, are about nine months under-developed at birth compared to those of other primates. Much anatomical and physiological evidence has accumulated in support of the theory that human babies are born approximately nine months too early, and it is interesting to observe that some biochemical evidence is also accumulating. In this context the late development of GST in some human tissues is of interest. GST1 in liver, for example, does not reach adult levels of expression until well into infant life, GST2 in kidney and spleen is not expressed in adult proportions until about one year after birth. In this respect, my observations on the development of some GST may provide biochemical evidence in support of the secondary altricial human reproductive strategy proposed by Portmann (1941).

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PUBLICATIONS.

Experimental work described in this thesis has been published in the following papers:

1. Strange, R.C., Faulder, G.C., Davis, B.A., Hume, R., Brown, J.A.H., Cotton, W. and Hopkinson, D.A. (1984). The Human glutathione S-transferases: Studies on the tissue distribution and genetic variation of the GST1, GST2 and GST3 isoenzymes. Ann. Hum. Genet. 48, 11.

2. Strange, R.C., Davis, B.A. Faulder, G.C., Cotton, W., Bain, A.D. and Hume, R. (1985) The Human Glutathione S-Transferases: Developmental aspects of the GST1, GST2 and GST3 loci. Biochem. Genet. 23, 1011.

The following paper has been submitted for publication as a result of experiments described in this thesis:

1. Faulder, G.C., Hirrell, P.A., Hume, R. and Strange. R.C. (1986) Studies of the Development of Basic, Near-neutral and Acidic Isoenzymes of Glutathione S-Transferase in Human liver, Adrenal, Kidney and Spleen. Biochem.J.

The following presentations have been made at meetings listed below:

1. Biochemical Society Meeting, University of Cambridge (1983).

Strange, R.C., Faulder, G.C., Davis, B.A., Brown, J.A.H., Hopkinson, D.A and Cotton, W. (1984) The Human Glutathione S-Transferases: Studies on the distribution of the GST1, GST2 and GST3 isoenzymes. Biochem. Soc. Trans. 12, 285

2. Biochemical Society Meeting, University of Keele (1984).

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3. Biochemical Society Meeting, University of Leeds (1984).

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5. The Biochemical Society Meeting, University of Dundee (1986).

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