GLUTATHIONE S-TRANSFERASES OF RAT KIDNEY

by

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ABSTRACT

The glutathione S-transferases are a family of proteins involved in detoxification. The different transferases in rat kidney were studied by isoelectricfocusing on polyacrylamide gels. Five forms of transferase, all of molecular weight ca. 45,000, were found in renal cytosol. Two correspond to hepatic transferases B and M. The relative proportion of the various forms is sex-dependent. Renal transferases exhibit narrower substrate specificity than do hepatic transferases. The renal transferases are individually regulated in that a) response to inducing agents is selective and b) the maturational pattern of each transferase is distinct even though all activities reach adult values by age 30 days. Transferase activity is inducible as early as the first week of life and the percent induction over basal levels is not age-dependent. The renal glutathione S-transferases are particularly suitable for studying the nature and regulation of the many heterogeneous enzymes involved in xenobiotic metabolism.

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Glutathion S-transférases du rein de rat

CONDENSE

Les glutathion S-transférases sont une famille de protéines impliquée dans la détoxification. Les différentes transférases présentes dans le rein du rat ont été étudiées par séparation isoélectrique sur gels de polyacrylamide. Cinq formes de transférase, toutes de poids moléculaire ca. 45,000, ont été trouvées dans le cytosol rénal. Deux de celles-ci correspondent aux transférases hépatiques B et M. La proportion relative des diverses formes dépend du sexe. Les transférases rénales font voir une spécificité plus grande pour le substrat que les transférases hépatiques. Les transférases rénales sont controllées individuellement en ce que a) la réponse aux agents inducteurs est sélective et b) le processus de maturation de chaque transférase est distinct, même si toutes les activités atteignent leurs valeurs adultes à l'âge de 30 jours. L'activité transférase est induite aussi prématurement qu'après la première semaine de vie et le pourcentage d'induction qui a lieu en sus du niveau de base est indépendent de l'âge. Les glutathion S-transférases rénales sont particulièrement appropriées pour l'étude de la nature et le contrôle des nombreuses enzymes héterogènes impliquées dans le métabolisme xénobiotique.

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DEDICATION

To my father and mother, Dr. and Mrs. L. G. Jaeger who first fostered my interest in science and who provided encouragement and support for all my studies.

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CHAPTER 1

INTRODUCTION

A. GENERAL INTRODUCTION

The investigations presented in this thesis were intended to contribute to understanding the nature, control and significance of multiplicity in the drug-metabolizing enzymes. Long-range comprehension of this issue is likely to bear directly on many important biomedical problems including the tissue selectivity of carcinogens and other toxins, the influence of genetic composition and environment on drug disposition and toxicity, details of drug interaction, and on more general problems of the process of enzymic and tissue differentiation.

ROLE OF DRUG-METABOLIZING ENZYMES IN DETOXIFICATION WITH SPECIAL REFERENCE TO THE GLUTATHIONE S-TRANSFERASES

Throughout phylogeny and ontogeny organisms have been and are exposed to a wide variety of exogenous compounds, the number and variety of which have been increased by the modern chemist. At certain concentrations many of these compounds, or xenobiotics, are not necessarily toxic and may even be beneficial. However, all will be toxic if their concentration in certain tissues is high enough. Concentration in any tissue will reflect the balance between intake into the body, uptake into the tissue, and elimination. Tissue content of the xenobiotic may increase if the amount of the xenobiotic ingested exceeds the body's ability to excrete the compound. Many hydrophilic compounds are readily excreted in bile and urine but lipophilic compounds must be metabolized to a

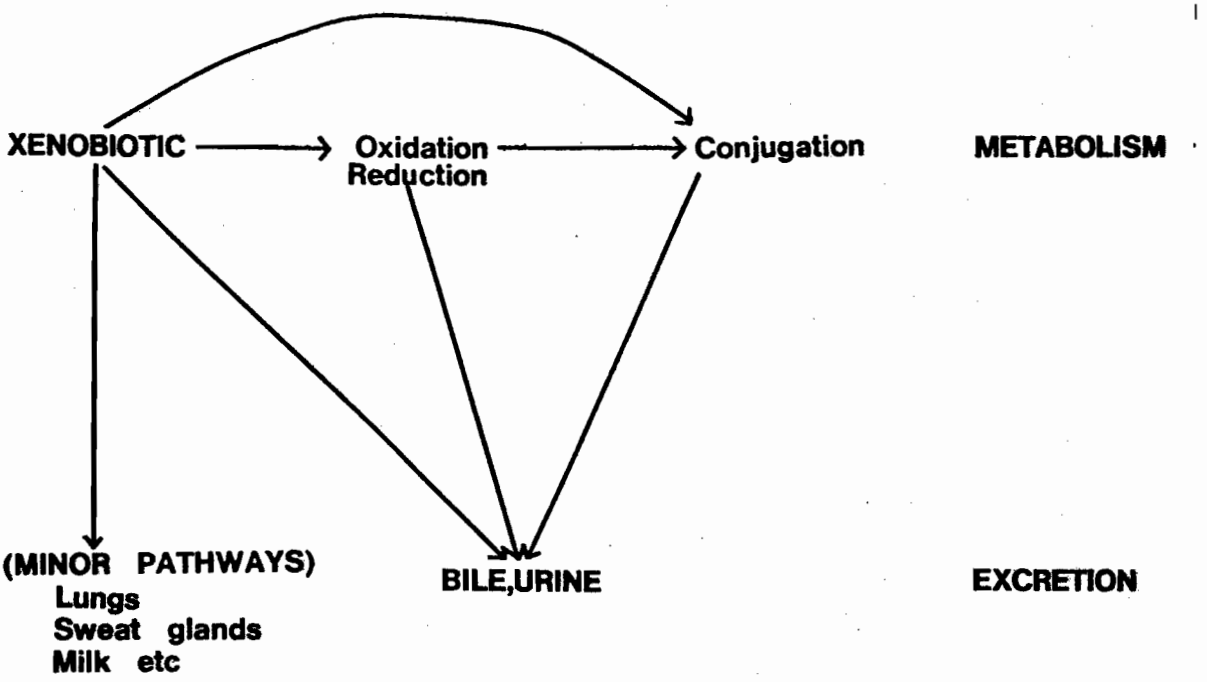
more water soluble form before excretion can be efficient. Enzymes involved in the metabolism of xenobiotics have existed for many thousands of years. The enzyme systems that metabolize drugs are the same as those that have evolved to handle endogenous lipophilic substances, e.g. cholesterol, bilirubin and thyroxin. The appearance of 'developed' forms of the glutathione S-transferases, the enzyme system with which this thesis deals, seems to coincide with the transition of amphibia from water to land (Levine et al., 1971). Some reports indicate that primitive forms of the glutathione S-transferases are present in several marine species (Bend and Fouts, 1973; Clark and Smith, 1975). In all mammalian species examined and in more highly evolved invertebrates such as insects, multiple forms of the glutathione S-transferases have been found (Clark et al., 1973).

The most common reactions catalyzed by xenobiotic-metabolizing enzymes are oxidation, reduction, hydrolysis and conjugation. With the exception of certain hydrolyses most such enzymatic activity is found in the liver. Smaller but significant amounts are found in kidney, lung, small intestine, placenta, skin and other tissues. The traditional description of drug metabolism involves a sequence of phases (for review, see Dutton 1978). According to this scheme in Phase I the administered molecule is hydroxylated, dealkylated or otherwise oxidized through reactions catalyzed by the cytochromes P-450 or it may be reduced by enzymes such as nitroreductase. In Phase II, endogenous polar moieties such as glutathione (GSH), sulfate or glucuronate can be conjugated to the product of the Phase I reactions or to the original drug molecule

if it already contains a suitable acceptor group (Figure 1). Conjugative enzymes include the glutathione S-transferases, glucuronyl transferases, acetyl transferases and sulfo-transferases. The hydrolysis of certain products of Phase I reactions may be catalyzed by epoxide hydrase. There are also enzymes, such as catechol-O-methyl transferase, whose main function is to handle endogenous compounds but which can also be considered as "drug-metabolizing enzymes". In general, the reactions catalyzed by Phase I enzymes had come to be considered as more likely to yield toxic products than those catalyzed by Phase II enzymes. However, there are exceptions to this generalization and the description of drug metabolism based on these two phases, one toxifying, the other detoxifying is too simplistic. It seems more appropriate to think in terms of a number of enzymatic pathways which can function both in parallel and in sequence and which compete for the drug molecule and its metabolites as substrates. The products of one of the pathways could go through the next pathway in line or they may recycle through the same type of reaction. Those pathways which involve and lead to the formation of the non-toxic intermediates of a particular drug molecule may be considered detoxifying with respect to that agent. Thus one pathway may be considered detoxifying with respect to drug A but toxifying with respect to drug B. The chemistry of the xenobiotic and the distribution of the enzyme systems are among the prime factors which determine the metabolic fate and biologic effect of the compound.

Many of the intermediates produced by the cytochrome P-450 mixed

FIGURE 1. COMMON PATHWAYS OF XENOBIOTIC DISPOSITION

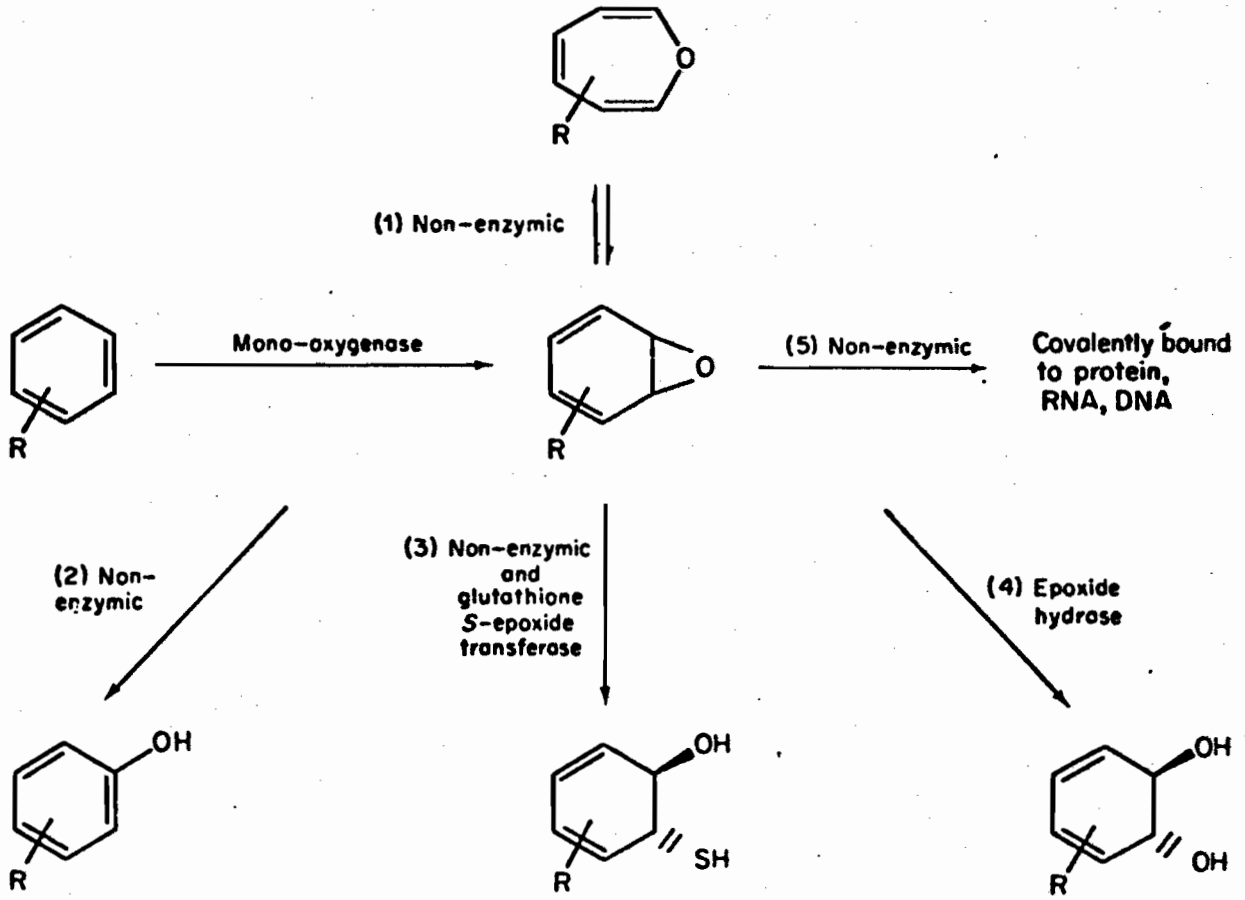

XENOBIOTIC
Oxidation
Reduction
Conjugation
METABOLISM
(MINOR PATHWAYS)
Lungs
Sweat glands
Milk etc
BILE,URINE
EXCRETION

function oxidase system are known to be toxic but the exact chemical structure of some of them remains unclear because of their intrinsic instability (which also relates to their toxicity). One example of the toxifying effects of mammalian mixed function oxidase is the conversion of aromatic and olefinic compounds into arene and alkene oxides (Jerina et al., 1970; Oesch et al., 1972). Most epoxides are highly reactive electrophiles which can not only rearrange non-enzymatically to phenols (Jerina and Daly, 1974), but which can also bind covalently to tissue macromolecules such as protein, DNA and RNA (Grover and Sims, 1970), and so have the potential to modify the function of these macromolecules. However, such reactive intermediates are also subject to further enzymatic metabolism, often to less toxic molecules (Jerina and Daly, 1974). Epoxide hydrase and the glutathione S-transferases catalyze the transformation of the epoxide intermediates into the corresponding dihydrodiols and glutathione conjugates respectively. These routes for the metabolism and disposition of arene oxides are outlined in Figure 2.

One of the most extensively studied examples of the role of glutathione and the glutathione S-transferases in detoxification involves their protective action against acetaminophen-induced hepatotoxicity. Although considered one of the safest of all non-narcotic analgesics, acetaminophen in large doses can cause fatal hepatic necrosis in human beings and experimental animals (Prescott et al., 1971; Mitchell et al., 1973; Davis et al., 1974). Recent reports (Medical Letter, 1978) state that prolonged administration of high

FIGURE 2. METABOLISM OF ARENE OXIDES

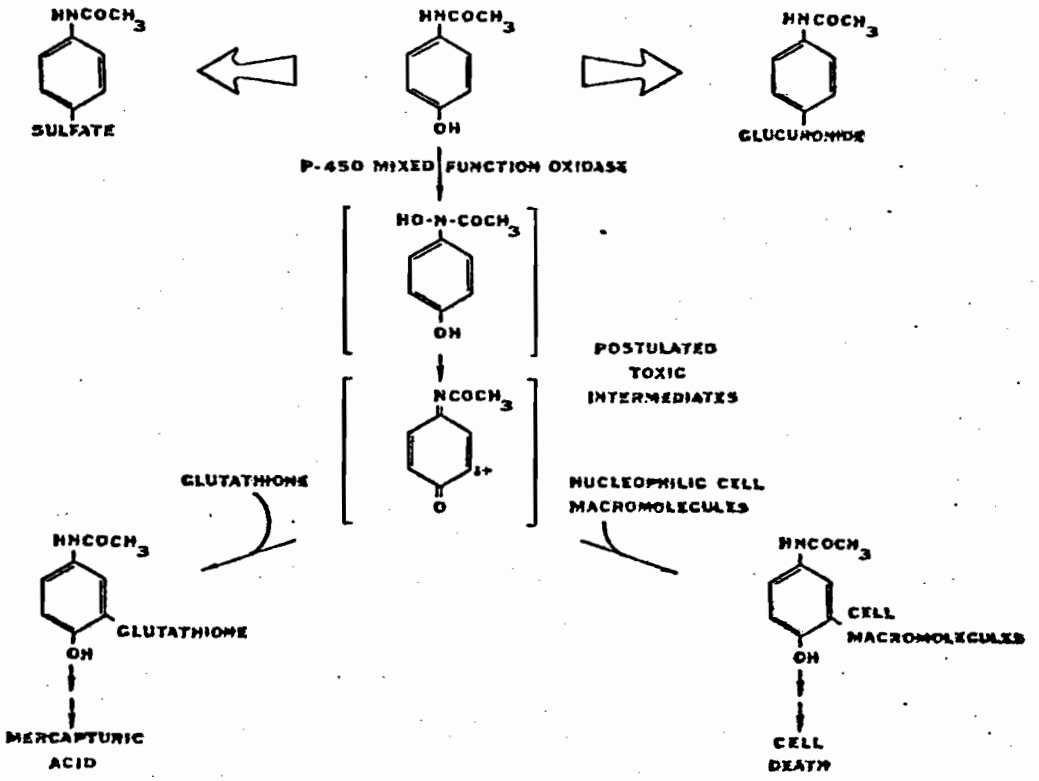
after F. Oesch (1975).


O
R
(1) Non-enzymic
Mono-oxygenase
O
R
(5) Non-enzymic
Covalently bound to protein, RNA, DNA
(2) Non-enzymic
(3) Non-enzymic and glutathione S-epoxide transferase
(4) Epoxide hydrase
OH
R
OH
SH
R
OH
OH
R

therapeutic doses may be associated with hepatotoxicity. The classical pathway of acetaminophen metabolism is illustrated in Figure 3. The essence and implications of this pathway are likely valid although some of the intermediate chemical structures may not be fully defined. Acetaminophen is converted to a chemically reactive intermediate capable of binding covalently to liver protein (Jollow et al., 1973). Sulfydryl containing compounds, particularly glutathione, are proposed to have a role in protecting tissues from the effects of such reactions (Mitchell et al., 1976). Specifically, Mitchell et al demonstrated that: (a) administration of acetaminophen caused a dose-dependent depletion of hepatic glutathione; (b) covalent binding by reactive metabolites of acetaminophen increased rapidly when supplies of available glutathione were depleted; and (c) pretreatment with diethyl maleate which depletes glutathione resulted in increased acetaminophen-induced hepatic necrosis while pretreatment with cysteine, a precursor of glutathione prevented liver damage. The glutathione S-transferases are capable of catalyzing the conjugation of glutathione with the acetaminophen reactive metabolite although a significant non-enzymatic rate does exist for this reaction (Potter et al., 1973). Therefore, it has been suggested that the contribution of the transferases to the overall process may vary as a function of the amount of glutathione left in the cell. For many compounds conjugation with glutathione represents the first enzymatic step leading to biosynthsis of the corresponding mercapturic acid derivative, prior to excretion (Boyland and Chasseaud, 1969a).

FIGURE 3. CLASSICAL PATHWAY OF ACETAMINOPHEN METABOLISM

after Mitchell and Jollow (1975).


HNCOCH3
SULFATE
HNCOCH3
OH
HNCOCH3
GLUCURONIDE
P-450 MIXED FUNCTION OXIDASE
HO-N-COCH3
OH
POSTULATED
TOXIC
INTERMEDIATES
NCOCH3
O
GLUTATHIONE
NUCLEOPHILIC CELL
MACROMOLECULES
HNCOCH3
GLUTATHIONE
OH
MERCAPTURIC
ACID
HNCOCH3
CELL
MACROMOLECULES
OH
CELL
DEATH

The amount of reactive intermediates in the cell depends not on the rate of the oxidative reactions per se but on the relative rates of the reactions which contribute to the toxic intermediate pool and those of the reactions which deplete it. Whether the toxicological effect associated with a particular xenobiotic is a threshold or a non-threshold event the size of the reactive intermediate pool would seem critical. Important factors which must be considered when evaluating pharmacologic and toxicologic effects of chemically reactive intermediates on individual tissues are presented in Table I. In work based on a similar rationale, Bend et al (1976) emphasized that the pattern of drug metabolism varies with age, tissue, species and previous exposure to xenobiotics. In any one tissue, the developmental patterns of the different enzyme systems involved in drug metabolism are varied; also, within one enzyme system the rates of development of catalytic activities toward different substrates may not be the same (Neims et al., 1976). Therefore the size and quality of the reactive intermediate pool may change as a function of age according to the rates of the various pathways filling and emptying the pool.

MULTIPLICITY OF THE DRUG-METABOLIZING ENZYMES

Except for perhaps epoxide hydrase, most of the enzyme systems normally considered to be primarily responsible for drug metabolism, that is the cytochromes P-450, glucuronyl transferases, glutathione S-transferases, sulfo-transferases and acetyltransferases have each been shown to be a family of related proteins. Evidence for the functional

Table 1. FACTORS AFFECTING THE TISSUE TOXICITY OF METABOLICALLY ACTIVATED XENOBIOTICS

A. Do inducers or inhibitors of drug metabolizing enzymes alter the relative proportion of the dose that is converted to the reactive metabolite?

B. Do inducers or inhibitors alter the relative proportion of the reactive metabolite that becomes covalently bound?

C. Do other substrates change the tissue level of cosubstrates used in conjugation reactions with the reactive metabolite?

D. Do high doses of the hepatotoxin itself lead to depletion of cosubstrates for conjugation reactions with the reactive metabolite?

E. Are chemically reactive metabolites formed in different tissues?

F. Do chemically reactive metabolites leave the tissues in which they are formed?

after Mitchell et al (1976).

heterogeneity of the glutathione S-transferases, glucuronyl transferases, sulfo-transferases and cytochrome P-450 systems comes from observations of selective induction and inhibition of activities toward some substrates and not toward others (Yaffe et al., 1968; Nebert et al., 1973; Hales and Neims, 1976a; Dutton, 1978) and from observations of the different developmental patterns of activity to the various substrates (Hales and Neims, 1976b). Direct biochemical evidence for the heterogeneity of these systems also exists for the glutathione S-transferases (Habig et al., 1974b), glucuronyl transferases (Dutton and Burchell, 1976), sulfo-transferases (Singer et al., 1976), acetyltransferases (Cohen et al., 1973) and cytochromes P-450 (Thomas et al., 1976).

Teleologically, such heterogeneity allows an efficient solution to a complex problem facing the organism in the disposition of xenobiotics; vis, the creation of an enzyme system with substrate specificity broad enough to handle the many different types of chemical groupings presented to it and yet specific enough to be regulated differentially and to exclude most cellular components from being substrates. This is not likely to be accomplished by one active site on a single enzyme. What seems to have evolved instead of single enzymes are groups of proteins such as the multiple forms of the glutathione S-transferases, the sum of whose catalytic activities is broad enough to handle the majority of encountered xenobiotics and metabolites, but whose individual members have substrate specificites narrow enough to allow the requisite fine control of the objects of metabolism.

The mechanism of the regulation of the various multiple forms is

not known but is an area of active research and cannot be separated from research into enzyme regulation in general. One approach to the problem has been to measure the total catalytic activity e.g. of the glutathione S-transferases, in various tissues and in response to various perturbations, to gain insight into the existence and response of the individual proteins. The rationale underlying this approach is that measurement of whole cytosolic activity provides information on the activity of the component proteins. Although it can be said that the whole reflects the sum of the parts, or for example total cytosolic transferase activity reflects the summation of the activities of the individual transferases, I wish to emphasize that the parts are not adequately described by the whole. It is necessary to examine the individual members of a family of enzymes in various tissues in control situations and in response to a variety of perturbations.

Another approach to the investigation of the nature and regulation of multiple forms has been to study the genetics of the enzymes involved. The working hypothesis of many investigators is that formulated by Paigen et al (1975) as a model for the genetic factors involved in the final realization of β-glucuronidase activity in mice. In this model four sets of genes are involved in the control of β-glucuronidase activity: 1) structural genes which code for the active sites of enzyme molecules as well as for the recognition sites which identify the enzyme for future processing by the cell; 2) processing genes which code for the proteins that will be involved

in the subsequent modifications of the enzyme; 3) regulatory genes which alter the synthesis of the enzyme in response to physiological and metabolic signals; and 4) temporal genes which determine the expression of the aforementioned three gene types in different cells at different stages of development. This model has been applied to the interpretation of the multiple forms of cytochrome P-450 in the rabbit (Atlas et al., 1977). The latter study provides evidence for at least three different tissue-specific forms of regulation of inducible mono-oxygenase activities and their associated P-450 subunits. The authors suggest that regulatory genes and temporal genes may play a role in determining susceptibility to chemical carcinogenesis in various tissues as a function of age and tissue localization. Like all proteins, the drug-metabolizing enzymes are synthesized according to information coded in DNA, so it may not be surprising to find corresponding 'multiplicity' in the functional activity of the genome. The above studies have not yet answered the question of 'What controls multiplicity of enzymes?' but have only reposed it as 'What controls multiplicity of gene expression?'. Genetic studies alone cannot solve the problem of the nature and control of the multiplicity of drug-metabolizing enzymes.

Studies of the response of multiple forms of drug-metabolizing enzymes to inducing agents, their developmental pattern and their presence in various tissues are needed to provide information on the regulation of these proteins. Studies of amino acid sequences and posttranslational modifications are needed to provide insight into the degree of homology between the proteins and into the problem of whether or not

they represent the product of one gene. The glutathione S-transferases are good enzymes to use in this approach to the problem. They are abundant proteins found in the cytosol of many tissues including liver, kidney, lung and small intestine from many species including man. In rat liver they comprise up to 10% of cytosol protein (Fleischner et al., 1972). The multiplicity of the hepatic transferases is well documented (for review see Jakoby, 1978) and it is likely that other tissues also possess multiple forms of these enzymes. The transferases respond selectively to inducing agents and, at least in liver, have individual patterns of development (Hales and Neims , 1976b). All the transferases have similar physical properties e.g., they are all dimers of approximate molecular weight 45,000 (Jakoby et al., 1976b). Some members of the glutathione S-transferase family are more closely related on the basis of amino acid composition and immunological reactivity than are others. From the above considerations it can be seen that the glutathione S-transferase system has characteristics which make it suitable for the study of the multiple forms of drug-metabolizing enzymes. The work leading to the present state of knowledge of the hepatic and renal glutathione S-transferases is described in the next section.

B. HISTORICAL BACKGROUND

Information leading to the present state of knowledge of the glutathione S-transferases comes from a number of distinct lines of research including primarily (a) that into the enzymes involved in mercapturic acid biosynthesis and (b) that into the proteins involved

in uptake and storage of organic anions by various tissues; and to a lesser extent (c) that into the protein(s) responsible for the binding of azo-dye carcinogens and (d) that into proteins involved in the binding of cortisol metabolites. It is now established that investigators in these four areas were unknowingly dealing with the same group of proteins. The above studies can basically be divided into those of ligand-binding proteins (ligandins) and those of proteins with catalytic activity. Evidence for the ligandin and catalytic roles of the glutathione S-transferases are presented in sections B1 and B2 respectively. A third role proposed for the glutathione S-transferases involves their function as a pool of proteins that sacrificially bind covalently to toxic intermediates which may otherwise react with vital cell molecules. Evidence for the importance of their function as covalent binders is based mainly on the fact that 1-chloro-2,4-dinitrobenzene, a substrate towards which most transferases are active, and the carcinogen 'butter yellow' bind covalently to transferases A and B (Pabst et al., 1974). In addition, h-protein quantitatively the most important protein which binds covalently to carcinogens in mouse liver, skin and lung, has been shown to retain glutathione S-transferase activity after extensive purification (Sarrif and Heidelberger, 1976). In my opinion, the evidence for this sacrificial role of the transferases is weak.

1. HEPATIC LIGANDIN

In their role as intrahepatic carriers of organic anions the

glutathione S-transferases or ligandins are thought to act in much the same way as albumin does in the circulation. The evidence which led to this concept is summarized below.

Bernstein et al (1966) injected radioactive bilirubin into rats and found that approximately 80% of the intrahepatic bilirubin was associated with the 100,000g supernatant fraction. After gel filtration of adult rat liver cytosol, bilirubin administered in vivo and in vitro could be recovered in three protein fractions labelled X, Y and Z (Levi et al., 1969). The presence of bilirubin binding in X fraction was found to be due to albumin contamination (Arias, 1972). Bromosulfophthalein, indocyanine green, flavispidic acid (Levi et al., 1969), iopanoic acid, iodipamide (Sokoloff et al., 1973) and fatty acids (Mishkin et al., 1972) also bound to proteins within the Y and Z fractions. The major binding proteins within the Y and Z fractions were called Y and Z protein respectively. Y protein was purified from rat liver and kidney by TEAE ion exchange chromatography, Sephadex G-100 gel filtration and QAE-A-50 chromatography (Kirsch et al., 1975; Fleischner et al., 1976). Identity between Y-protein, azocarcinogen binding protein (Ketterer et al., 1967), and cortisol metabolite binder I (Morey and Litwack, 1969) was established on the basis of physical, chemical and immunological characteristics of the proteins and the term ligandin was adopted in place of the individual classifications. Classical ligandin preparations from rat liver and kidney give identical structural patterns and cross-immunologic responses.

Arias et al (1976) hypothesized that ligandin influences the

net uptake and storage of organic anions in liver specifically by reducing the efflux from the liver into the plasma. Evidence for this is as follows: phylogenetic and ontogenetic studies show that absence of ligandin is associated with delayed plasma disappearance of organic anions and that the appearance of ligandin parallels the development of selective hepatic uptake (Levine et al., 1971). Administration of phenobarbital or DDT causes induction of hepatic ligandin with a concomitant increase in rates of plasma disappearance of bilirubin, bromosulfophthalein and indocyanine green (Reyes et al., 1971). Since phenobarbital and DDT also have other effects in the liver e.g. phenobarbital increases its size and blood flow, I don't feel that a cause and effect relationship can be drawn between the induction of ligandin and the increase in the rate of plasma disappearance of these organic anions. It is also known that thyroidectomy decreases hepatic bromosulfophthalein content and increases ligandin concentration indicating that factors other than ligandin influence the hepatic uptake and storage of organic anions (Reyes et al., 1971).

Although ligandin was originally considered to be an organic anion binding protein it is now known that its specificity is directed towards compounds possessing an electrophilic centre whether or not they carry a formal negative charge (Jakoby, 1978). Limited work has been done on quantitative binding to ligandin. Kamisaka et al (1973) used circular dichroism to study the competition of a number of ligands with bilirubin for ligandin. Bilirubin binds at a single high affinity site with a K_D of 2×10^{-8} M. Dissociation constants for the organic

anions tested ranged from 10^{-6} M for indocyanine green and bromosulfophthalein to 10^{-4} M for thyroxine. Meuwissen and Heirwegh (1970) report a value of 2.5×10^{-7} M for the dissociation constant of haem and ligandin.

2. HEPATIC GLUTATHIONE S-TRANSFERASES

One of the earliest functions recognized for the glutathione S-transferases was the participation in the first enzymatic step of mercapturic acid biosynthesis (Figure 4). This pathway has four stages: the first step involves the conjugation of a compound possessing an electrophilic centre with the endogenous nucleophile glutathione whose structure is shown in Figure 5. This is followed by successive hydrolyses of the γ-glutamyl and cysteine moieties and finally by N-acetylation of the cysteinyl conjugate (Barnes et al., 1959; Bray et al., 1959).

Although the presence of mercapturic acids in urine was described almost a century ago (Baumann and Preusse, 1879) it was not until the 1940's and 1950's that evidence was collected to show that glutathione was the major if not the only source of the cysteine moiety. Several authors including Yamamoto (1941) and Barnes et al (1959) showed a parallel between decrease in liver glutathione following the injection of a variety of mercapturic acid precursors such as bromobenzene and the excretion of the corresponding mercapturic acid. It was also shown that the turnover rate for glutathione in liver was sufficient to maintain mercapturic acid biosynthesis and that rats dosed with S-alkylglutathione excrete alkyl mercapturic acids into the urine (Foxwell and Young, 1964). An enzyme catalyzing the conjugation of

FIGURE 4. MERCAPTURIC ACID BIOSYNTHESIS

after L. F. Chasseaud (1976).

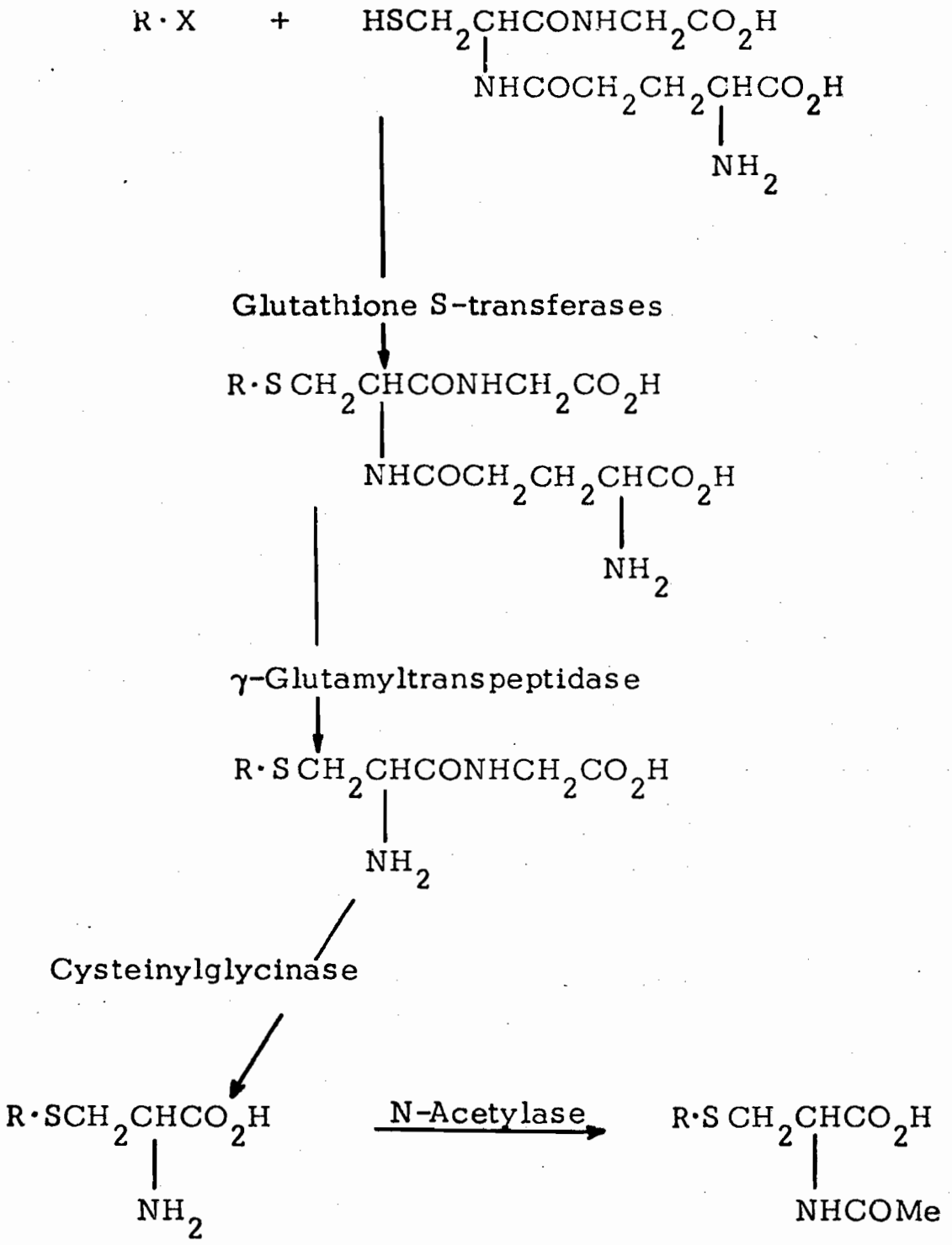

R·X + HSCH2CHCONHCH2CO2H
NHCOCH2CH2CHCO2H
NH2
Glutathione S-transferases
R·S CH2CHCONHCH2CO2H
NHCOCH2CH2CHCO2H
NH2
γ-Glutamyltranspeptidase
R·S CH2CHCONHCH2CO2H
NH2
Cysteinylglycinase
R·SCH2CHCO2H
NH2
N-Acetylase
R·S CH2CHCO2H
NHCOMe

FIGURE 5. GLUTATHIONE

after E. M. Kosower (1976).

GLUTATHIONE

GSH with several foreign compounds, including some that are metabolized to mercapturic acids, was found in the supernatant fraction of rat liver (Booth et al., 1961). This enzyme was found to be specific for glutathione as nucleophile but was active toward a number of electrophilic substrates. It was named glutathione S-aryltransferase after the chemical nature of the electrophilic substrate with which it was active (Grover and Sims, 1964).

Following the discovery of glutathione S-aryltransferase, glutathione S-transferases with activity toward a wide variety of electrophilic substrates were described. An attempt was made to classify these enzymes on the basis of the chemical structure of the electrophilic substrates to which they showed activity. Thus the names alkyltransferase (Johnson, 1966), aralklytransferase, aryltransferase (Suga et al., 1967; Boyland and Chasseaud, 1969b,; Gilham, 1971) alkene transferase (Boyland and Chasseaud, 1967; Chasseaud, 1973) and epoxide transferase (Boyland and Williams, 1965) arose. A summary of the tissue locations and characteristics of these enzymes is shown in Table 2 as well as those of some other enzymes then known to use GSH as a substrate. As work was undertaken in a number of laboratories to purify the enzymes involved it slowly became clear that categorization on the basis of 'aryl', 'alkyl' etc. was not rigorous, i.e. 'aryl' transferases also had activity towards 'alkyl' substrates.

Separation procedures for the glutathione S-transferases were based on the high isoelectric points of most but not all of these

Table 2. MAMMALIAN ENZYMES USING GLUTATHIONE AS SUBSTRATE

	Enzyme	Organs of high activity	Characteristics
1.	GSH peroxidase	Liver and erythrocytes of many species	
2.	GSH-homocystine transhydrogenase	Ox liver	
3.	GSH-oxidized CoA transhydrogenase	Ox liver	Identical with no. 2
4.	GSH-insulin reductase	Rat liver	Stable to water dialysis and to 40% (v/v) ethanol
5.	Nitroglycerine reductase	Rabbit liver, kidney stomach and uterus, and 'several organs of rat'	Pig-liver enzyme: (a) stable to water dialysis (b) is precipitated in active form by ethanol (54-65%, v/v) at pH5; (c) has optimum pH7-8.
6.	γ-Glutamyltransferase (glutathionase)	Predominantly kidney and pancreas of many species (also sheep brain and calf liver)	
7.	GSH S-aryltransferase	Liver of many species Little in rat kidney	(a) Stable to water dialysis; (b) optimum pH8
8.	GSH S-epoxidetransferase	Rat liver	(a) Stable to water dialysis; (b) optimum pH6-5
9.	GSH S-alkyltransferase	Liver and kidney of several species	(a) Inactivated by water dialysis; (b) inactivate by 30% (v/v) acetone or ethanol at 0^{o}; (c) optimum pH8-9

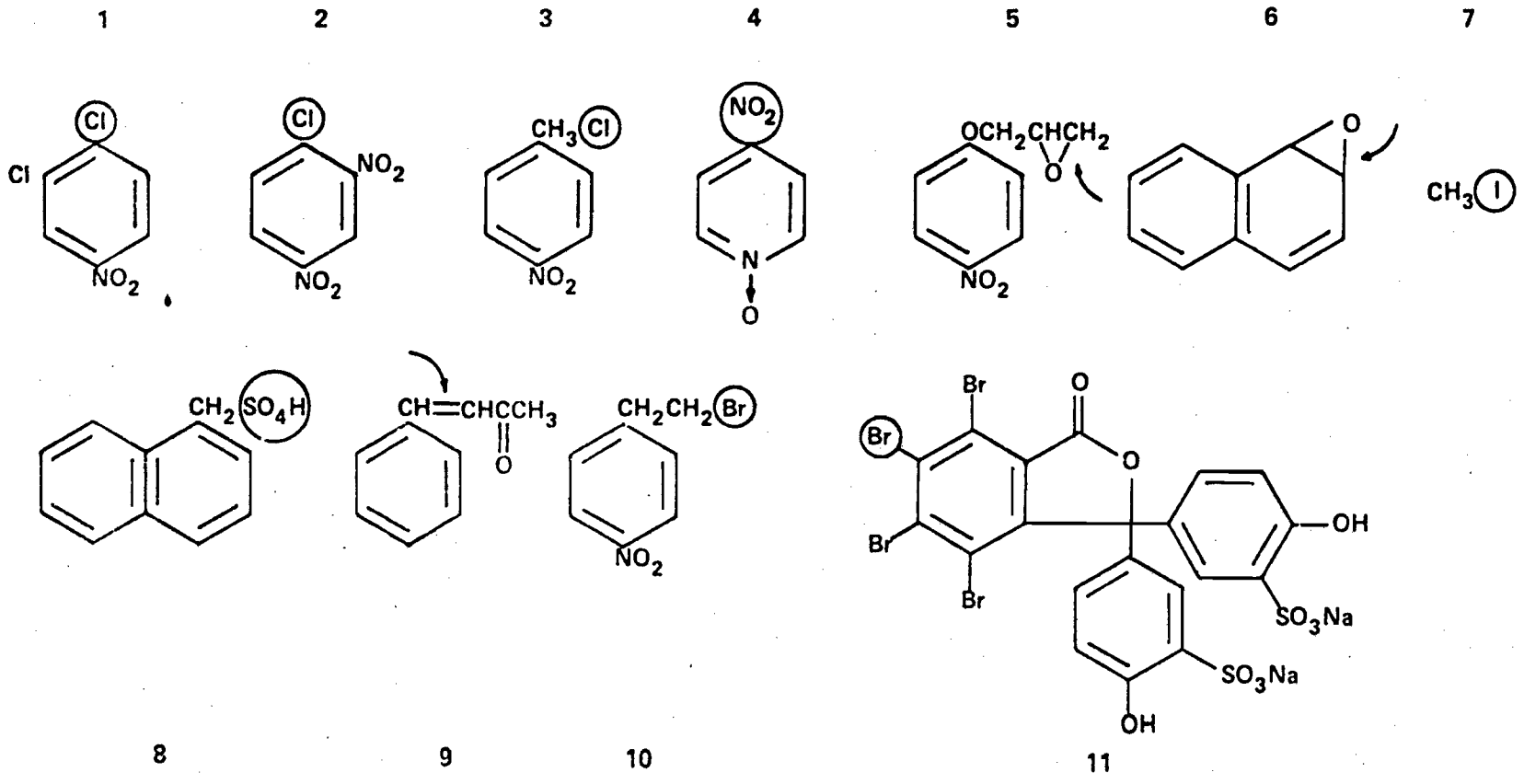
after Johnson (1966).

enzymes. At least seven distinct glutathione S-transferases, identified by the letters, AA, A, B, C, D, E and M have been found in rat liver (for review see Jakoby et al., 1976a). Transferase AA through E were named on the basis of their reverse elution from a carboxy-methylcellulose column: transferase E eluted first at low salt concentration while transferase AA eluted last at higher ionic strength. Glutathione S-transferase M, which has a slightly acidic isoelectric point, was isolated in relatively high purity from rat liver on the basis of its activity towards 1-menaphthyl sulfate (Gillham, 1973).

Some of the most common substrates used to assay glutathione S-transferase activity are shown in Figure 6. Other substrates of interest include pesticides such as malathion and parathion (Dauterman, 1971). Glutathione S-transferases are active toward a wide variety of exogenous compounds. Other compounds found to be substrates include Δ^5-androstene-3,17-dione and prostaglandin A_1 (Benson et al., 1977; Cagen et al., 1975; Christ-Hazelhof et al., 1976).

The hepatic transferases exhibit broad and overlapping substrate specificities with respect to electrophilic substrates e.g. all the transferases are active towards 1-chloro-2,4-dinitrobenzene and p-nitrobenzylchloride. The transferases can be distinguished from each other on the basis of the ratios of their activities toward various substrates rather than on presence or absence of activity toward any one substrate. The specific activities of the glutathione S-transferases from rat liver toward a number of substrates are shown

FIGURE 6. SELECTED SUBSTRATES OF THE GLUTATHIONE S-TRANSFERASES Leaving groups or points of addition are indicated. after Jakoby et al., (1976a). 1: 1,2-Dichloro-4-nitrobenzene. 2: 1-Chloro-2,4-dinitrobenzene. 3: p-Nitrobenzyl chloride. 4: 4-Nitropyridine-N-oxide. 5: 1,2-Epoxy-3-(p-nitrophenoxy)propane. 6: 1,2-Naphthalene oxide. 7: Iodomethane. 8: 1-Menaphthyl sulfate. 9: trans-4-Phenyl-3-buten-2-one. 10: p-Nitrophenethyl bromide. 11. BSP.


1
2
3
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7
Cl
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NO2
CH3
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N
O
OCH2CHCH2
O
NO2
O
CH3
I
CH2
SO4H
CH=CHCCH3
O
CH2CH2
Br
NO2
8
9
10
Br
O
Br
Br
Br
O
OH
SO3Na
SO3Na
OH
11

in Table 3. Glutathione appears to be an almost obligate nucleophile in the reactions catalyzed by these enzymes. Only homoglutathione, in which β-alanine replaces glycine, was active in place of glutathione: L-cysteine, 2-mercaptoethylamine, dithiothreitol and N-acetyl-L-cysteine could not replace glutathione as nucleophile (Keen et al., 1976).

A number of laboratories have contributed to the purification of the hepatic glutathione S-transferases. All the transferases except D and M have been purified to homogeneity in the laboratory of Dr. W. Jakoby and coworkers (Fjellstedt et al., 1973; Habig et al., 1974b; Pabst et al., 1974; Habig et al., 1976). Glutathione S-transferase E was first isolated from rat liver based on its activity toward 1,2-epoxy-3-(-4-nitrophenoxy)propane as a substrate (Fjellstedt et al., 1973). In a subsequent study (Habig et al., 1974b) three additional transferases A, B, and C were isolated in homogeneous form from rat liver and were characterized as regards physical properties and substrate specificity. Concurrently, Askelof et al isolated two glutathione S-transferases from rat liver which they named Form I and Form II (Askelof et al., 1975). On the basis of substrate specificity and isoelectric point these were determined to be transferases C and A respectively and the Jakoby nomenclature has been adopted. Transferase D has not yet been purified and transferase M was isolated by Gillham (1973).

Glutathione S-transferase B is the most abundant transferase in rat liver cytosol comprising about 5% of the protein in that

Table 3. SPECIFIC ACTIVITIES[a] OF THE GLUTATHIONE TRANSFERASES WITH SELECTED SUBSTRATES

Substrate	Rat					
	A	B	C	E	AA	M
Benzo[α]pyrene-4,5-oxide	0.087	0.011	0.098	0.069	0.004	
BSP	0.53	0.006	0.18	0[b]	0.004	
1-Chloro-2,4-dinitrobenzene	62	11	10	0.01	14	
1,2-Dichloro-4-nitrobenzene	4.3	0.003	2.0	0[b]	0.008	0.004
1,2-Epoxy-3-(p-nitrophenoxy)propane	0.1	0[b]	0	6.7		0[b]
Ethacrynic acid	0[b]	0.26	0.11	0[b]	0.3	
Iodomethane	0[b]	0.59	0	8.9	1.4	0[b]
1-Iodopropane	0.39	0.32		1.0		
Menaphthyl sulfate	0	0.004		0[b]		0.1
Naphthylene-1,2-oxide	0.04	0[b]		0.16		
p-Nitrobenzyl chloride	11.4	0.1	10.2	4.1	0.09	0.5
p-Nitrophenethyl bromide	0.1			6.1		
2-Nitropropane	0.012	0.008	0.014	0	0.01	
4-Nitropyridine N-oxide	1.7	0[b]		0[b]		0[b]
trans-4-Phenyl-3-buten-2-one	0.02	0.001	0.40	0[b]		
Prostaglandin A_1	0.013	0.005	0.021			
2,3,5,6 Tetrachloronitrobenzene	3.9	0[b]		0.001		0[b]

[a] μmoles per minute per milligram.
[b] A value of zero does not necessarily mean that the enzyme is incapable of catalyzing the reaction but rather that the reaction rate was no greater than the spontaneous rate in several trials with reasonable amounts of enzyme.

after Jakoby et al (1976a)

subcellular fraction. Altogether the transferases make up about 10% of cytosol protein in rat liver. Of all the hepatic transferases glutathione S-transferase B has been the most extensively studied with regard to chemical structure, response to inducing agents and its developmental pattern.

Like all of the glutathione S-transferases, transferase B has a molecular weight of approximately 45,000 and is comprised of two subunits of similar size. Until recently it was thought that these two subunits were identical with a molecular weight of 25,000 (Habig et al., 1974b). Recently, experiments using more discriminatory buffer and gel systems suggested that glutathione S-transferase B has two different subunits of 25,000 and 23,000 molecular weight (Bhargava et al., 1977; Jakoby, 1978). The only one of the transferases examined for its secondary structure is transferase B. This protein has a highly ordered secondary structure with 40% α-helical, 15% β-pleated sheet and 45% random coil character (Kamisaka et al., 1973). Some of the physical characteristics of this and the other glutathione S-transferases are described in Table 4.

All of the glutathione S-transferases can be considered 'ligandins' in that they have the ability to bind organic anions. Glutathione S-transferase B has been shown to be identical to ligandin (Y-protein) on the basis of substrate specificity, immunological cross-reactivity, ligand binding ability, molecular weight, pI, and amino acid composition (Habig et al., 1974a). Transferase B has also been shown recently to catalyze the Δ5-3-ketosteroid isomerase reaction

Table 4. PROPERTIES OF THE RAT GLUTATHIONE S-TRANSFERASES

Property	Transferase					
	AA	A	B	C	D	E
Relative concentration	0.14	0.22	1.0	0.52	0.02	0.10
MW (10^3)						
Sedimentation equilibrium		45	47			40[a]
Gel filtration	45	46	47	47		40[a]
Subunits						
Number	2	2	2	2		2
MW (10^3)	25	25	25	25		25
Isoelectric point[b]	10	8.9	9.8	8		7.1,7.3
$E_{280}^{1\%}$	8.3	12.5	8.1	11.8		

[a]Not corrected for partial specific volume, which is unknown.

[b]Data obtained by preparative isoelectric focusing except for transferase C where data was obtained by gel isoelectric focusing with elution with water of 2 mm gel slices.

after Jakoby et al (1976b)

(Benson et al., 1977) and to have glutathione peroxidase activity (Burk et al., 1977; Prohaska and Ganther, 1977).

There is an interaction between the binding of some ligands by glutathione S-transferase B and its catalytic activity. For example, the cholecystographic agent, cholografin, although not itself a substrate, binds to glutathione S-transferase B and inhibits activity towards 1-chloro-2,4-dinitrobenzene (Goldstein and Arias, 1975). Other ligands such as ethacrynic acid and bromosulfophthalein can also serve as catalytic substrates. Bhargava et al (1978) showed that only when bilirubin binds to a lesser affinity site on ligandin does this binding affect catalytic action. These authors state that the binding of bilirubin to the high affinity site and the catalytic activity of the protein take place at different sites on the protein and perhaps on different subunits.

An appreciation of the closeness of the structural relationship between the different hepatic transferases is critical for understanding the nature of the multiplicity of these enzymes. One indication of a close relationship between proteins is cross-reactivity with antibodies. The reactivity of the glutathione S-transferases with antibodies prepared against purified forms of each of them are presented in Table 5. Only transferases A and C, the two transferases with the most similar amino acid composition, cross-react immunologically (Habig et al., 1974b).

The human liver transferases also display multiplicity with respect to substrate specificity and isoelectric point (Habig et al., 1976a; Kamisaka et al., 1975a). They can be separated on CM-cellulose

Table 5. REACTION OF THE GLUTATHIONE S-TRANSFERASES WITH ANTIBODIES TO TRANSFERASES A^a, B^b, and E^b

	Transferase				
	AA	A	B	C	E
Reaction with antibody to					
A^a	-	+	-	+	-
B^b	-	-	+	-	-
E^b	-	-	-	-	+

[a]Rabbit Ig G

[b]Sheep Ig G

after Jakoby et al (1976b)

and have been assigned Greek letters in direct order of increasing isoelectric point: transferase α has the lowest pI while transferase ε has the highest. These proteins are thought to be more closely structurally related than are the rat liver transferases. All five human transferases cross-react with an antibody prepared against one of them (Fleischner et al., 1976) and their amino acid compositions are practically identical. Simons and Vander Jagt (1977) have recently purified the human transferases by affinity chromatography on a matrix prepared from epoxide-activated Sepharose 6B and glutathione following an initial work up of liver extracts.

RENAL LIGANDIN AND GLUTATHIONE S-TRANSFERASES

In the kidney, just as in the liver, organic anion secretion is an important means of eliminating metabolites of endogenous and exogenous compounds. The presence in the kidney of various forms of a protein which bound bromosulfophthalein was described by Combes and Stakelum in 1961. A protein immunologically similar to hepatic ligandin was later identified in rat kidney by immunodiffusion and localized to the proximal tubule by immunofluorescent microscopy (Fleischner et al., 1972). Indeed, the presence of ligandin in urine has been suggested to indicate acute injury to the proximal tubule (Feinfeld et al., 1977). Kirsch et al (1975) showed that renal and hepatic ligandin are identical on the basis of their pI 8.9, MW 45,000, subunit structure, reaction to monospecific antibodies and percent helical structure.

The importance of renal ligandins or glutathione S-transferases to organic anion transport is controversial. Some authors have proposed a role for renal ligandin in organic anion transport (Kirsch et al., 1975). Evidence in favour of this role is as follows: a) renal ligandin is very similar to hepatic ligandin which has been thought to function as an organic anion binding recepor; b) renal ligandin is a soluble protein capable of interacting reversibly with organic anions; and c) competitive binding between probenecid, bromosulfophthalein and penicillin correlated with their renal uptake from plasma. On the other hand it has been shown that a) there is no correlation between induction of p-aminohippuric acid transport and stimulation of glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene in rabbits (Kluwe et al., 1978) and b) that rat transferase activities reach adult levels prior to the maturation of the transport system (Pegg and Hook, 1977) suggesting that the glutathione S-transferase reaction is likely not rate limiting in renal organic anion transport.

Glutathione S-transferases have also been studied in kidney on the basis of catalytic activity towards aryl, aralkyl, epoxide, alkyl and alkene substrates (Booth et al., 1961; Boyland and Williams, 1965; Johnson, 1966; Boyland and Chasseaud, 1969b; Kaplowitz et al., 1976a). This catalytic activity, at least in the rabbit, is confined to the proximal convoluted and proximal straight tubules (Fine et al., 1978). All five activities eluted from Sephadex G-100 in a fashion which indicated that the renal transferases

had approximately the same molecular weight as the hepatic transferases.

There have been some studies of the regulation of glutathione S-transferases in kidney but only to the extent of measuring the catalytic activity of cytosol in control situations, in response to inducing agents and at various stages of development (Kirsch et al., 1975; Kaplowitz and Clifton, 1976b). The separation and examination of the renal transferases as discrete proteins, similar to that done in Dr. W. Jakoby's laboratory with the hepatic transferases, has not been done for the renal transferases. Experiments key to an understanding of the regulation of the multiple forms of glutathione S-transferases, that is examination of the individual proteins present and their response to various perturbations such as xenobiotic induction, hormonal changes, development or a combination of these factors, had not been reported prior to this thesis.

C. FORMULATION OF THE PROJECT

The experimental work presented in this thesis involves the glutathione S-transferases of rat kidney: the forms present, their response to inducing agents and their developmental patterns. The broader intention of this work was to develop methods by which one can measure not just total catalytic activity of cytosol but also the individual proteins' activities under a variety of perturbing conditions. By perturbing the system one can determine whether the individual activities revealed by the separation technique adequately describe the changes observed in cytosolic activity. The information

gained from these studies therefore should be viewed as a prelude to continuing work on the nature and regulation of the multiple forms of these enzymes.

1) To initially characterize the renal enzymes the subcellular distribution and approximate molecular weight of the renal glutathione S-transferases are determined. Overall glutathione S-transferase catalytic activities of male and female rat liver and kidney cytosols are compared.

2) The examination of individual members of a group of closely related enzymes required the development of a technique which would separate the different forms fairly rapidly and allow reasonable recovery and quantitation. Such a technique using preparative electrofocusing was developed in collaboration with Dr. B. Hales in our laboratories (Hales et al., 1978). I have applied this technique to the study of rat kidney transferases. Electrofocusing profiles of male and female renal and male hepatic glutathione S-transferase activities are presented.

3) The effects of pretreatment with phenobarbital, β-naphthoflavone and Arochlor 1254 on the glutathione S-transferases of male rat kidney are studied. Both overall catalytic activity of cytosol and the electrofocusing profiles of the transferases are examined. Female rats are used to study the effect of chronic alcohol pretreatment on

the renal glutathione S-transferases.

4) The developmental profile of the renal glutathione S-transferases in male rats is investigated. Absolute levels of glutathione S-transferase activities present at various ages are compared with adult values. The distribution of catalytic activities between the individual transferase peaks is examined using the electrofocusing technique and compared with the adult distribution.

5) The inducibility of the renal transferases as a function of age is studied and the time course of return to control levels after induction is followed in immature and adult rats.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

Animals

Sprague-Dawley rats weighing 200-250 g were obtained from Canadian Breeding Farms, St. Constant, Quebec. They were housed in the McIntyre Animal Centre, McGill University on pine shavings and given Purina rat chow and water ad libitum. The temperature of the rooms was 21-22° C and a light-dark cycle of 12 hr - 12 hr was maintained. For the developmental study, mothers each with ten one-day-old male pups were obtained from the same supplier. Pups were selected for experimental study in such a way as to leave each mother with approximately the same number of pups. The pups were kept with the mothers in the McIntyre Animal Center until they were 21 days old.

Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP) were obtained from Eastman Kodak, Co., Rochester, NY, USA. Sources of other chemicals were: p-nitrobenzylchloride (NBC) and β-naphthoflavone (BNF), K & K Laboratories Inc., Plainview, NY, USA; trans-4-phenylbut-3-en-2-one (TPBO) and p-nitrophenethyl bromide (NPB), Aldrich Chemical Co., Milwaukee, Wis, USA; 1-menaphthylsulfate (MNS), Calbiochem, San Diego, Calif., USA; glutathione (reduced form), cytochrome c and bovine serum albumin, Sigma Chemical Corp., St. Louis, MO, USA; ampholines in the pH ranges of 2-11 (40% w/v in 1% w/v sucrose), 8-10 (40% w/v in 1% w/v sucrose) and 9-11 (20% w/v in 1% w/v sucrose),

Brinkmann Instruments Inc., Westbury, NY, USA: glycerol, Fisher Scientific Co., Montreal, Canada; acrylamide and N,N'-methylene-bis acrylamide both of electrophoresis purity grade, Bio-Rad Laboratories, Mississauga, Ont., Canada; N,N,N',N'-tetramethylenediamine (TEMED), J. T. Baker Chem. Co., Phillipsburg, NJ, USA: Sephadex G-75 superfine (10-40 μ), Sephadex G-100, blue dextran, aldolase and ovalbumin, Pharmacia Fine Chemicals, Uppsala, Sweden; and agar for the immunodiffusion studies was special Agar-Noble, low ash, Difco Laboratories, Detroit, Michigan, USA.

Phenobarbitone sodium and Aroclor 1254 were gifts from Dr. D. Ecobichon, Dept. Pharmacology and Therapeutics, McGill University.

All other chemicals were at least of reagent grade.

Ampholine Mix

For all the electrofocusing experiments, on columns and on gels, the following ampholine mix was used: Brinkmann ampholines in the pH ranges of 2-11, 8-10 and 9-11 were mixed in a volume ratio of 1:1:2. This resulted in a final weight ratio of the ampholine components of 1:1:1 since the 2-11 and 8-10 ampholines are 40% w/v in 1% (w/v) sucrose while the 9-11 ampholines are only 20% w/v in 1% w/v sucrose.

Glutathione S-Transferase Assays

Assays were performed at room temperature, 21-23° C, according to the method of Habig et al (1974). All measurements were done in quartz cuvettes using a Beckman Model 35 double beam spectrophotometer. Glutathione S-transferase activities of the samples towards four

substrates were routinely determined and a total of seven substrates were tested. Each assay included the following ingredients (final concentrations within parentheses).

1) 1-Chloro-2,4-dinitrobenzene: CDNB, 10 ul of a 50 mM solution in ethanol (0.96 mM); glutathione, 5 ul of a 100 mM solution in deionized water (0.96 mM); enzyme sample in 20% v/v glycerol to a maximal volume of 50 ul; and sufficient 100 mM potassium phosphate buffer, pH 6.5 to give a final volume of 520 ul.

2) 1,2-Dichloro-4-nitrobenzene: DCNB, 10 ul of a 50 mM solution in ethanol (0.90 mM); glutathione, 25 ul of a 100 mM solution in deionized water (4.48 mM); enzyme sample in 20% v/v glycerol to a maximal volume of 250 ul; and sufficient 100 mM potassium phosphate buffer, pH 7.5 to give a final volume of 560 ul.

3) Trans-4-phenylbut-3-en-2-one: TPBO, 10 ul of a 2.5 mM solution in ethanol (0.044 mM); glutathione, 5 ul of a 2.5 mM solution in deionized water (0.22 mM); enzyme sample in 20% v/v glycerol to a maximal volume of 250 ul; and sufficient 100 mM potassium phosphate buffer, pH 6.5 to give a final volume of 515 ul.

4) p-Nitrobenzylchloride: NBC, 10 ul of a 50 mM solution in ethanol (0.93 mM); glutathione, 25 ul of a 100 mM solution in deionized water (4.65 mM); enzyme sample in 20% v/v glycerol to a maximal volume of 250 ul; and sufficient 100 mM potassium phosphate buffer, pH 6.5 to give a final volume of 535 ul.

5) p-Nitrophenethylbromide: NPB, 10 ul of a 5 mM solution in ethanol (0.09 mM); glutathione, 25 ul of a 100 mM solution in deionized water

(4.65 mM); enzyme sample in 20% v/v glycerol to a maximum volume of 250 ul and sufficient 100 mM potassium phosphate buffer, pH 6.5 to give a final volume of 535 ul.

6) 1,2-Epoxy-3-(p-nitrophenoxy)propane: ENPP, 10 ul of a 25 mM solution in ethanol (0.46 mM); glutathione, 25 ul of a 100 mM solution in deionized water (4.65 mM); enzyme sample in 20% v/v glycerol to a maximal volume of 250 ul and sufficient 100 mM potassium phosphate buffer, pH 6.5 to give a final volume of 535 ul.

7) 1-Menaphthyl sulfate: MNS, 10 ul of a 25 mM solution in deionized water (0.45 mM); enzyme sample in 20% v/v glycerol to a maximum volume of 250 ul; and sufficient 100 mM potassium phosphate buffer, pH 7.5, to give a final volume of 560 ul.

The glutathione solution was prepared daily. All the other solutions were prepared about once a month and stored at 4° C.

The activities of each enzyme sample toward CDNB, DCNB, TPBO, NBC, NPB, ENPP, and MNS were followed at 340 nm, 345 nm, 290 nm, 310 nm, 310 nm, 360 nm, and 298 nm respectively. These wavelengths correspond to a maxima in difference spectra between starting assay mixtures and assay mixtures after complete enzymatic conversion to product. Assays using CDNB, DCNB, TPBO, NBC, and MNS were linear with protein concentration and time for at least two minutes. Assays with NPB and ENPP had too much background noise to be really useful. In measuring glutathione S-transferase activity towards CDNB, DCNB, NBC, and MNS the appearance of the corresponding glutathione conjugate

is followed (see Figure 4) while in measuring activity toward TPBO, disappearance of substrate is monitored. Habig et al (1974) state that the difference in the molar extinction coefficients ($\Delta\varepsilon$) for the CDNB, DCNB, NBC, and TPBO assays are 9.6 $mM^{-1}cm^{-1}$, 8.5 $mM^{-1}cm^{-1}$, 1.9 $mM^{-1}cm^{-1}$, and -24.8 $mM^{-1}cm^{-1}$ respectively. Gillham (1971) determined the difference in the molar extinction coefficient ($\Delta\varepsilon$) for the MNS assay to be 2.5 $mM^{-1}cm^{-1}$. These values were used in all computations. Calculations and statistical analyses (means, standard errors and 'students' t-test) were performed on a Hewlett-Packard SR-52.

Protein concentration was measured by the method of Lowry et al (1951) with bovine serum albumin as standard.

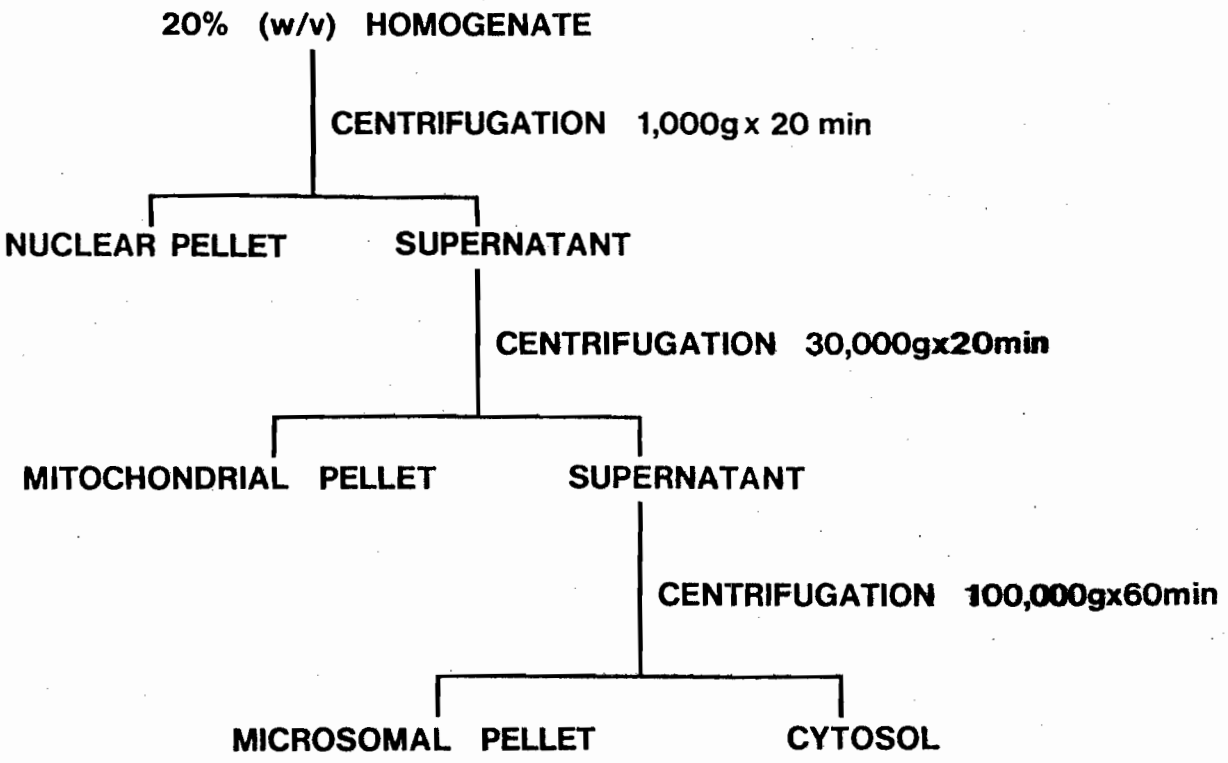
Preparation of Subcellular Fractions

The rats were decapitated, and both kidneys were quickly excised and homogenized in ice-cold 20% v/v glycerol using a Kontes glass homogenizer with Teflon pestle. A 20% w/v homogenate in the 20% v/v glycerol was prepared, which was then routinely centrifuged at 105,000 g x 60 minutes. The supernatant was removed by pipet and filtered through four layers of cheesecloth to remove lipid fragments. For the subcellular fractionation studies a modificiation of the method of de Duve et al (1955) was used (Figure 7). The 20% w/v homogenate was centrifuged at 1000 g x 20 minutes to prepare the nuclear fraction containing nuclei, unruptured cells and cell debri. The resulting supernatant was centrifuged at 30,000 g x 20 minutes to yield a mitochondrial pellet and a supernatant which was further

FIGURE 7. SUBCELLULAR FRACTIONATION OF RENAL HOMOGENATES

Rat kidneys were weighed and a 20% w/v homogenate in 20% v/v glycerol was prepared. Subcellular fractionation was carried out as illustrated.

SUBCELLULAR FRACTIONATION OF KIDNEY HOMOGENATE



centrifuged 105,000 g x 60 minutes. This yielded a microsomal pellet and a post-microsomal supernatant or cytosol. The first two centrifugations were carried out in a Sorvall RC2-B refrigerated centrifuge using a SS-34 rotor, r = 4.25 ins. The final centrifugation was carried out in a Beckman Model L ultracentrifuge in a Type 40 rotor. All operations were carried out at 4°C. Each pellet fraction was resuspended in cold 20% v/v glycerol to the volume of the original homogenate.

Electrofocusing on Columns

The method of O'Brien et al (1976) for electrofocusing on granulated gels was adapted for use in focusing the renal transferases. Whole cytosol (6-10 ml) prepared as described above was applied to the top of a 1.9 x 40 cm water-jacketed glass column packed with Sephadex G-75 superfine (10-40 u) already equilibrated with 20% v/v glycerol, 2% v/v ampholine mix. The dimensions of the bed itself were 33 cm x 1.5 cm (diameter) and the column was kept cool by water circulating at 4°C. The sample, which contained 4% v/v ampholine mix, was washed into the column with approximately 20 ml of a solution containing 20% v/v glycerol and 2% v/v ampholine mix. A millipore filter and plastic grid were then placed on top of the Sephadex bed. The top of the column was plugged with 5 ml of an acrylamide solution which contained 14% w/v acrylamide, 0.3% w/v N,N'-methylene bis acrylamide, 0.07% w/v ammonium persulfate and 0.5% v/v TEMED. After the acrylamide had polymerized the column was inverted and this procedure, namely the placing of

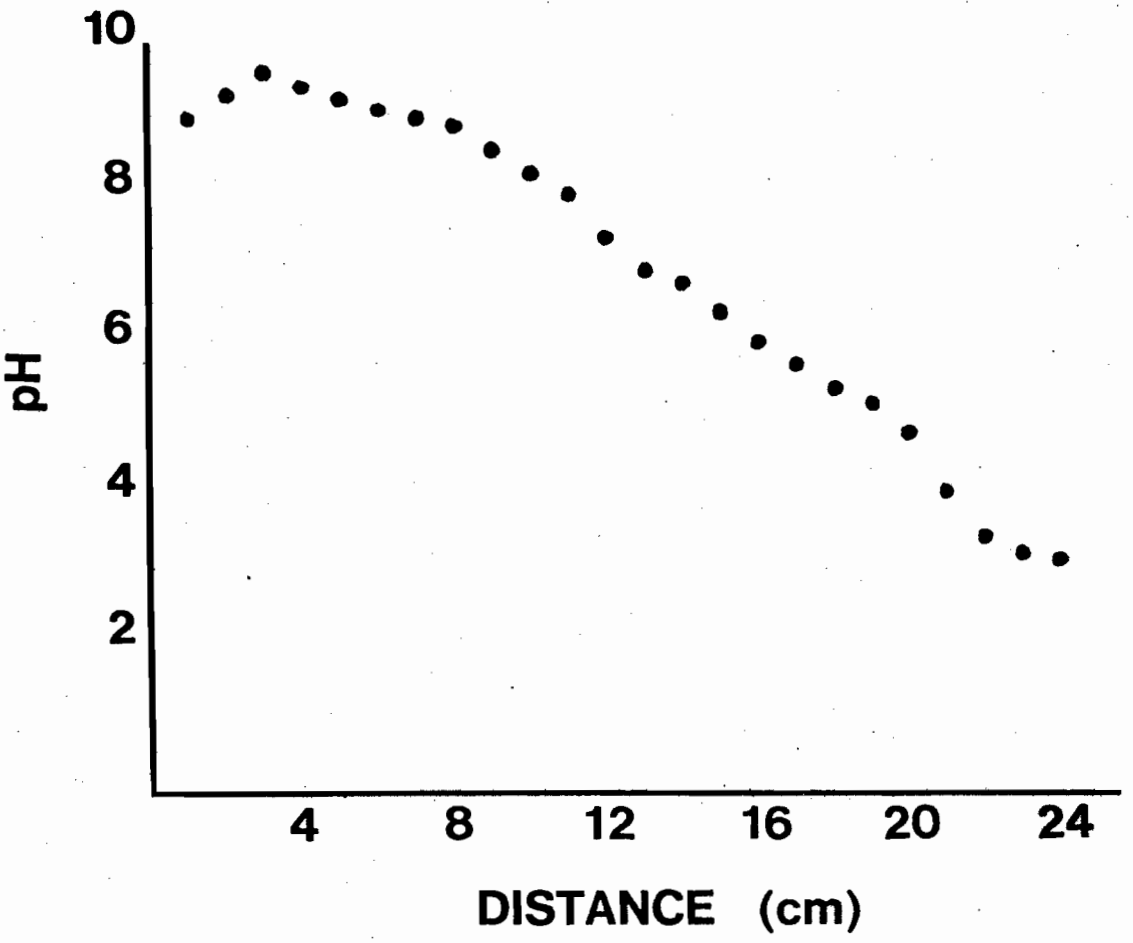
filters and formation of an acrylamide plug, was repeated at the other end. The column was returned to its original position and the lower end was immersed in 1% v/v phosphoric acid, the anode buffer. A reservoir filled with the cathode buffer, 0.4% v/v diethanolamine, was attached to the top end of the column. Platinum wire cathode and anode electrodes were immersed in the upper and lower buffers respectively and connected to a LKB Model 2103 power supply. Electrofocusing was carried out at 800 V for 40 hours by which time the current had fallen to zero. When focusing was complete, the acrylamide plugs were removed, and the Sephadex extruded from the column and sliced into 0.5 cm sections. The pH of each section was measured without the addition of liquid using the microelectrode of the Radiometer PH M62 Standard pH meter. A typical gradient is shown in Figure 8. The ampholine concentrations were specifically chosen to generate a shallow gradient in the pH range corresponding to the expected pI's of the transferases. Each 0.5 cm section of the column was then extracted with 3 ml of 20% v/v glycerol, and the Sephadex removed by centrifugation at 2000 g for 10 minutes. The supernatant was assayed for transferase activity toward each of the five substrates indicated above.

Electrofocusing on Polyacrylamide Gels

The method of Wrigley (1972) was used with chemical polymerization of the gels. Glass tubes, 120 mm, were marked 10 mm from the top and filled to this mark with approximately

Figure 8. pH GRADIENT OBTAINED AFTER ELECTROFOCUSING ON SEPHADEX G-75

Sample, which contained 4% ampholine mix, was applied to a Sephadex G-75 column already equilibrated with 20% v/v glycerol, 2% ampholine mix. Electrofocusing was carried out for 40 hours as described in the text. The Sephadex was extruded, sliced into 0.5 cm sections and the pH of each section was measured without the addition of liquid.


10
8
6
4
2
pH
4
8
12
16
20
24
DISTANCE (cm)

1 ml of a mixture composed of: 25 ul of 1% v/v TEMED; 1.0 ml of a combination of 30% w/v acrylamide and 0.8% w/v bis-acrylamide; 100 ul of ampholine mix; 2 ml of water; and 660 ul of 0.4% w/v ammonium persulfate. Deionized water was carefully layered on top of each gel. When polymerization was complete about 30 minutes later, the water was removed and 300 ul of sample in a combination of 20% v/v glycerol and 2% v/v ampholine mix was applied to the top of the gel. On top of this was layered a 10% v/v glycerol: 1% v/v ampholine mix solution to protect the sample from alkali at the beginning of the run. The upper reservoir held the cathode buffer, 0.4% diethanolamine, while the lower reservoir contained the anode buffer, 0.2% phosphoric acid. Focusing was carried out at an initial current of 1 mA per tube and at a constant voltage of 100 V for 16 hours in a Buchler electrophoresis apparatus. When focusing was complete the gels were extruded and sliced manually into 0.3 cm sections. After adding 0.5 ml of 10 mM potassium phosphate buffer pH 6.5 to each section, the samples of gel were vortexed and left to extract in the cold for at least 24 hours. Each was vortexed again just before assay for glutathione S-transferase activity. A blank gel containing ampholine mix but no sample was included in each run to determine the pH gradient for the gels. This gel was sectioned like the others but instead of buffer, 0.5 ml of deionized water was added to each section prior to vortexing and pH measurement.

Immunodiffusion Studies

The ability of various samples to cross react with an antibody raised in rabbit against purified rat liver glutathione S-transferase B (Hales and Neims, 1976) was tested by the method of Ouchterlony (1958). Five ml of a mixture of 1% w/v agar, 0.1% w/v sodium azide, 2.0% w/v polyethylene glycol 6000 and 0.9% w/v NaCl were pipetted into each circular plate, diameter 60 mm, height 15 mm. The centre well contained 40 ul of antiserum. The outside wells were punched so as to contain approximately 25 ul of sample. The precipitin line, if present, was usually visible within one day.

Determination of Apparent Molecular Weight

Molecular weight can be estimated by molecular sieve chromatography (Whitaker, 1963). Elution positions of samples separated by this technique correlate better with molecular Stokes radii or diffusion coefficients than with molecular weights (Polson 1961). The value for apparent molecular weight which is obtained is actually an upper limit for the hydrated molecular weight of the protein (Ackers 1964) but does approximate the actual molecular weight for globular proteins.

A Pharmacia K26/100 column was packed to a bed volume of approximately 500 ml with Sephadex G-100 swollen in 10 mM potassium phosphate, pH 6.5 containing 20% v/v glycerol. This buffer was used for both the sample and calibration standards which were all run at an upward flow rate of 20 ml per hour. The volume of each

fraction collected was 7.2 ml. The following molecular weight calibration standards were used: Blue dextran, 2,000,000; aldolase, 158,000; bovine serum albumin, 66,500; ovalbumin, 45,000; and cytochrome c, 12,700. Blue dextran concentration was monitored at 254 nm, cytochrome c at 520 nm and ovalbumin, bovine serum albumin and aldolase concentrations at 280 nm. Three ml of each standard were applied to the column as 1% w/v solutions in 10 mM potassium phosphate, pH 6.5, containing 20% v/v glycerol. Five ml of whole cytosol were applied to the column. Each 7.2 ml- fraction was monitored for protein concentration at 280 nm and assayed for glutathione S-transferase activity toward each of the substrates.

Induction Studies: Adult Rats

All injections were given intraperitoneally. The following pretreatment regimens were used for induction experiments:

Phenobarbital: Male rats were injected with phenobarbitone sodium 75 mg/kg/day as a 75 mg/ml solution in bacteriostatic normal saline for 7 days. Control animals were injected with an equivalent volume of vehicle alone for 7 days. The rats were sacrificed 24 hours after the last dose.

β-Naphthoflavone: Male rats were injected with β-naphthoflavone 40 mg/kg/day as a 20 mg/ml solution in corn oil for 4 days. Control animals received an equivalent volume of corn oil alone for the 4 days. The rats were sacrificed 24 hours after the final dose.

Aroclor 1254: Two dosing schedules were used. In the first,

male rats were injected with Aroclor 1254, 50 mg/kg/day dissolved in peanut oil to a concentration of 50 mg/ml, for 3 days. Control animals received an equivalent volume of peanut oil alone for 3 days. The rats were killed 72 hours after the last dose. In the second, the experimental animals received a single dose of Aroclor 1254, 500 mg/kg in 0.5 ml corn oil. Their controls had a single injection of 0.5 ml corn oil. Rats in this group were killed 5 days after the injection.

Alcohol: Kidneys from female Sprague-Dawley rats were obtained courtesy of Dr. J. G. Joly, Hôpital St-Luc, Montreal, Canada. These rats had been fed a liquid diet containing ethanol (De Carli and Lieber, 1967) for 24 days and were matched with controls according to weight and caloric intake. Each member of the experimental group consumed an average of 2 g alcohol/day. Control rats had a similar liquid diet in which the alcohol was replaced by a calorically equivalent amount of carbohydrate.

Inducibility with Development

Twenty-four male rat pups were injected with β-naphthoflavone 25 mg/kg/day as a 20 mg/ml solution in corn oil on days 2, 3, and 4 after birth. Four groups of two pups were killed on each of days 5, 9 and 17. Control animals received an equivalent volume of corn oil on days 2, 3 and 4 and were killed on the same days as the experimental animals.

A second group of animals was injected with β-naphthoflavone 25 mg/kg/day as a 20 mg/ml solution in corn oil on days 13, 14 and 15. These rats were killed in groups of eight on days 16, 22 and 30. Control animals were injected with equivalent volume of corn oil for days 13, 14 and 15 and were killed on the same days as the experimental animals.

RESULTS

SECTION I

INITIAL CHARACTERIZATION OF THE RENAL GLUTATHIONE S-TRANSFERASES

Subcellular localization of the renal glutathione S-transferases

The hepatic glutathione S-transferases are located primarily in the 100,000 g supernatant. To determine if this was also the case for the renal transferases each subcellular fraction was assayed for glutathione S-transferase activity toward CDNB, DCNB, NBC and TPBO. The results are presented in Table 6. More than 80% of the original activity toward each substrate tested was recovered in the 100,000 g supernatant. This corroborates and extends the finding of Fleischner et al (1972) that Y-protein (ligandin) activity in rat kidney is cytosolic. More than 9% of each of the original glutathione S-transferase activities were recovered in the 1,000 g pellet. These activities were probably due to the presence of unbroken cells.

Determination of apparent molecular weight

The elution profiles of the standards Blue Dextran, aldolase, bovine serum albumin, ovalbumin and cytochrome c from Sephadex G-100 are shown in Figure 9. All the resulting peaks were symmetrical. Renal cytosol was then applied to the column, and the effluent fractions were assayed for protein concentration, OD 280 nm, and glutathione S-transferase activity toward CDNB, NBC, and TPBO (Figure 10). The three activities showed basically the same elution profiles including fraction number of the peak tube. Peak fractions of renal glutathione S-transferase activities did not coincide with any

Table 6. SUBCELLULAR LOCALIZATION OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES

Subcellular Fraction	Glutathione S-Transferase activity[a] toward CDNB	DCNB	NBC	TPBO
1,000g x 20 min. pellet Nuclear pellet	9%	10%	12%	23%
30,000g x 20 min. pellet 'Mitochondria'	5%	4%	4%	-
105,000g x 60 min. pellet 'Microsomal'	3%	2%	-	-
105,000g x 60 min. supernatant "Cytosol'	85%	87%	85%	82%

[a]Activities are expressed as per cent of total catalytic activity found in whole homogenate and represent the means of two separate determinations.

Figure 9. ELUTION PROFILE OF PROTEIN STANDARDS CHROMATOGRAPHED ON SEPHADEX G-100

Protein standards, in 3 ml potassium phosphate buffer pH 6.5, were applied to a Sephadex G-100 column and run at an upward flow rate of 20 ml per hour as described in the Methods section. The volume of each fraction collected was 7.2 ml.
(o) Blue Dextran was monitored at 254 nm; (•) aldolase, (▵) bovine serum albumin and (▲) ovalbumin were all monitored at 280 nm and (■) cytochrome C at 520 nm.

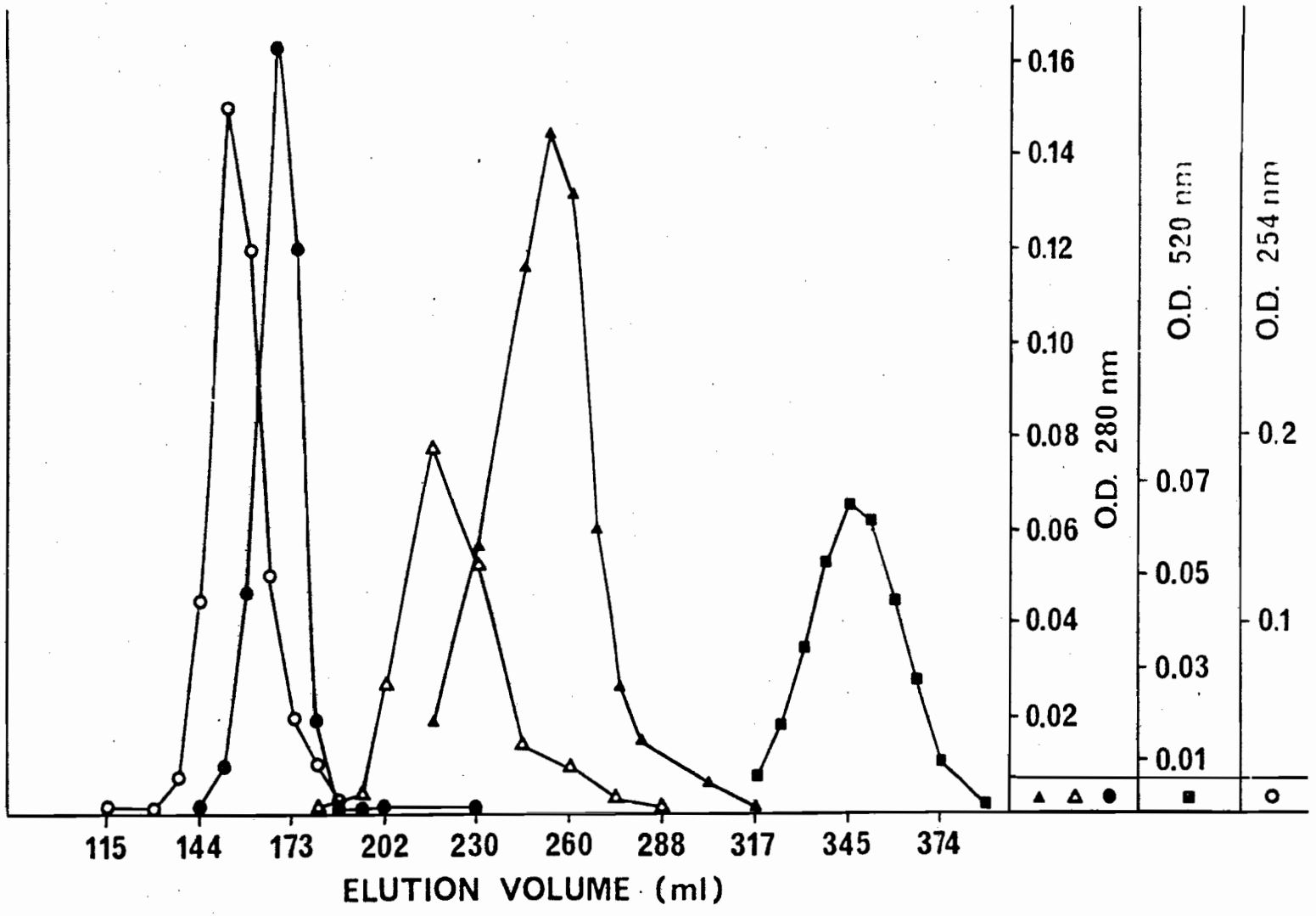
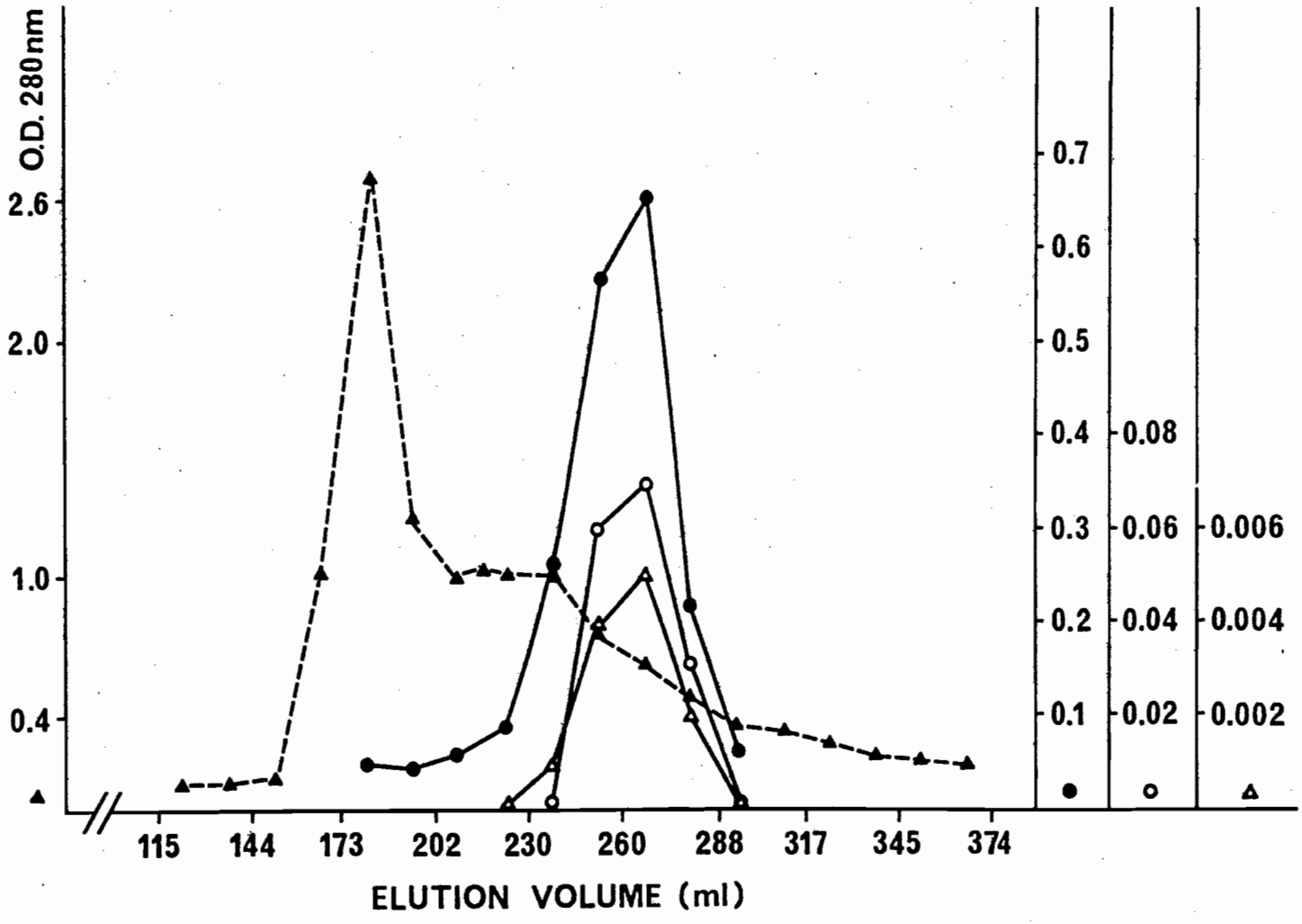

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
O.D. 280 nm
0.07
0.05
0.03
0.01
O.D. 520 nm
0.2
0.1
O.D. 254 nm
115
144
173
202
230
260
288
317
345
374
ELUTION VOLUME (ml)

Figure 10. ELUTION PROFILE OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM CYTOSOL CHROMATOGRAPHED ON SEPHADEX G-100

Five ml of renal cytosol prepared from male rats were applied to a Sephadex G-100 column and run at an upward flow rate of 20 ml/hour. The volume of the fractions collected was 7.2 ml. Each fraction collected was monitored for protein concentration at O.D. 280 nm and assayed for glutathione S-transferase activity towards CDNB (•), NBC (o) and TPBO (Δ). Glutathione S-transferase activities are expressed as μmoles min^{-1} per ml of the fraction assayed.


O.D. 280nm
2.6
2.0
1.0
0.4
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.08
0.06
0.04
0.02
0.006
0.004
0.002
115
144
173
202
230
260
288
317
345
374
ELUTION VOLUME (ml)

discernible peak in protein concentration. Peaks of hepatic transferase activity do coincide with a peak in protein concentration presumably because the hepatic transferases constitute a higher percentage (10%) of total cytosolic protein than do the renal transferases (Jakoby et al., 1976a).

K_{av} is defined as $\frac{V_e - V_o}{V_t - V_o}$ where

V_e = elution volume for the protein

V_o = elution volume for Blue Dextran 2000 and

V_t = total bed volume.

K_{av} is constant for a given protein when chromatographed on a specific Sephadex gel. When K_{av} for each standard was plotted against the log of mol. wt., a straight line, the selectivity curve, was obtained over the mol. wt. range of 12,700 to 65,000 (Figure 11). Aldolase with a mol. wt. of 158,000 is outside the linearity range, mol. wt. 5,500 - 100,000 for this gel (Sephadex, Gel Filtration in Theory and Practice; Pharmacia Fine Chemicals). The K_{av} for the peak of transferase activities was calculated to be 0.363 (see Figure 12). From the selectivity curve this corresponds to a mol. wt. of about 41,500. This is similar to the value of approximately 45,000 obtained by Pabst et al (1973) and Litwack et al (1971) for the hepatic transferases since using the procedure of obtaining molecular weight by gel filtration is associated with an error of at least 10%.

Glutathione S-transferase catalytic activity of hepatic and renal cytosols from male and female rats

The glutathione S-transferase activities toward CDNB, DCNB, NBC,

Figure 11. SELECTIVITY CURVE FOR PROTEIN STANDARDS CHROMATOGRAPHED ON SEPHADEX G-100

The Kav values for the protein standards were calculated as described in the text and plotted against the log of their corresponding molecular weights. The Kav for the transferase activities was calculated to be 0.363. This corresponds to an approximate molecular weight of 41,500.

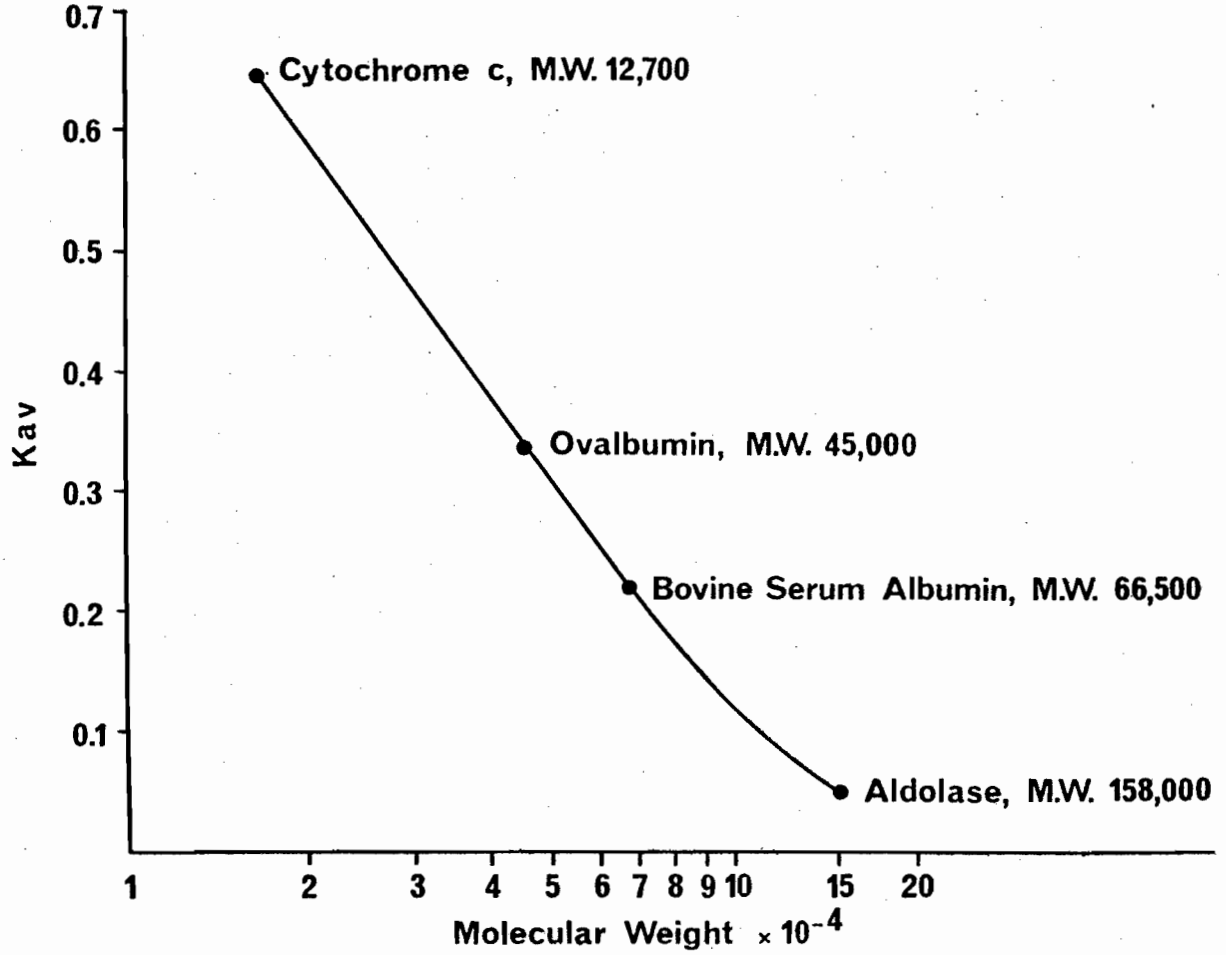

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Kav
Cytochrome c, M.W. 12,700
Ovalbumin, M.W. 45,000
Bovine Serum Albumin, M.W. 66,500
Aldolase, M.W. 158,000
1
2
3
4
5
6
7
8
9
10
15
20
Molecular Weight x 10-4

Figure 12. SAMPLE CALCULATION OF Kav

'r' column = 1.3 cm.

'l' column = 85 cm.

Volume of a cylinder = $\pi r^2 l$

Volume of column used = $\pi x(1.3cm)^2 x(85cm)$

V_t = 451.1 ml

Void volume V_o = 151.2 ml (see Figure 10)

$\therefore V_t - V_o$ = 299.9 ml

V_e, position of elution of transferase activity was 260 ml (see Figure 11)

$$\frac{V_e - V_o}{V_t - V_o} = \frac{260.0ml - 151.2ml}{451.1ml - 151.2 ml} = \frac{108.8ml}{299.9 ml} = 0.363$$

and TPBO in male and female rat liver and kidney cytosols are presented in Table 7. Activities toward NBC and TPBO did not differ significantly ($p < 0.05$) between male and female livers. Activity toward CDNB in female livers was somewhat higher ($p \approx 0.04$) than that in male livers while male rat livers had three times more DCNB activity than did female rat livers. Since the ratio of activity toward CDNB to activity toward DCNB is higher with glutathione S-transferase B, these results reflect the fact that female hepatic cytosol contains a higher percentage of transferase B relative to the other transferases than does male hepatic cytosol (Hales and Neims, 1976a). Sex differences were also apparent in the overall catalytic properties of the combined renal glutathione S-transferases. Male renal cytosol had significantly higher activity toward CDNB ($p<0.05$) than did female renal cytosol. Activity toward the other two tested substrates did not differ significantly between male and female renal cytosols.

All the results in this and the succeeding sections with the exception of Figures 25 and 30, are expressed as μmoles of product formed per gm of tissue wet weight. Expression of the results in terms of μmoles of product formed per ml cytosol or per mg protein does not alter the overall pattern or my interpretation of the data.

In male rats hepatic transferase activities toward the four substrates were 4 - 27 fold higher than the respective male renal activities (CDNB 4-fold, NBC 10-fold, TPBO and DCNB 27-fold). In the female the difference in activity between hepatic and renal cytosols was CDNB 8-fold, NBC 11-fold, DCNB 10-fold and TPBO 10-

Table 7. TISSUE AND SEX DIFFERENCES IN CATALYTIC ACTIVITY OF GLUTATHIONE S-TRANSFERASES

	MALE		FEMALE	
SUBSTRATE	LIVER	KIDNEY	LIVER	KIDNEY
CDNB	66.17 ± 5.08	13.90 ± 1.36	82.86 ± 1.72	10.15 ± 0.96
DCNB	3.00 ± 0.23	0.112 ± 0.05	1.09 ± 0.10	0.110 ± 0.04
NBC	20.66 ± 2.94	2.09 ± 0.14	24.49 ± 2.81	2.20 ± 0.19
TPBO	1.155 ± 0.165	0.063 ± 0.010	0.924 ± 0.132	0.0810± 0.012

Enzyme activities toward CDNB, DCNB, NBC and TPBO assayed as described in the Methods section are expressed in μmol/min per gm wet weight of tissue. Values are means ± S.E.M. for five to seven animals.

fold. These results suggest that not only absolute amounts of transferase activities but also the distribution of the various transferases is different in liver and kidney.

RESULTS

SECTION II

ELECTROFOCUSING PATTERNS OF HEPATIC AND RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES

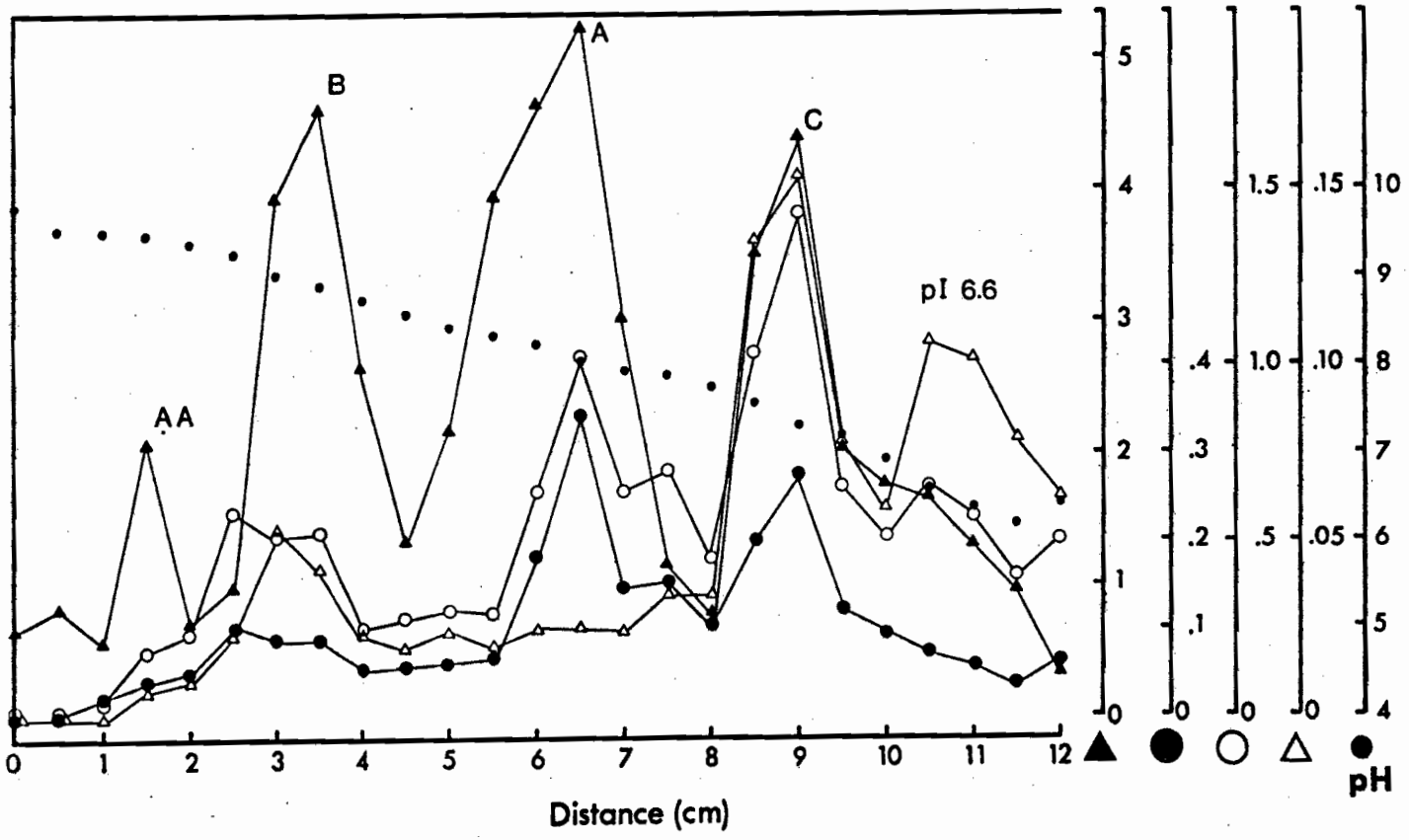
Electrofocusing pattern of hepatic glutathione S-transferase activities

The preparative electrofocusing technique for assessing the various forms of glutathione S-transferases was applied first to liver cytosol so that the technique could be evaluated in relation to published data. Individual transferases present in rat liver have been described (Habig et al., 1974b; Jakoby et al., 1976a) and so provide a basis for comparison of the forms observed with the electrofocusing technique.

The cytosols prepared from six male rat livers were electrofocused individually on Sephadex columns. A representative profile of the focused glutathione S-transferase activities toward four substrates, CDNB, DCNB, NBC, and TPBO is shown in Figure 13. Five major peaks of transferase activity (pI 9.6, 9.1, 8.4, 7.7, and 6.6) were resolved, and each peak exhibited distinctive substrate specificity. To simplify discussion electrofocused peaks of glutathione S-transferase activity have been assigned letters designating the tissue of origin (l for liver, k for kidney) and numbers starting with number one for the most basic transferase peak. Subdesignations indicating male (m) or female (f) are included where necessary. The activity in each peak was specific for glutathione as nucleophile in so far as neither L-cysteine nor 2-mercaptoethanol (1-5 mM) were active nucleophilic substrates. Catalytic activity towards any one

Figure 13. ELECTROFOCUSING PROFILE OF GLUTATHIONE S-TRANSFERASE ACTIVITIES OF HEPATIC CYTOSOL FROM MALE RATS

Male rat hepatic cytosol (6 ml) was applied to a column of Sephadex G-75 Superfine and electrofocused as described in the text. Sections (0.5 cm) of the extruded Sephadex bed were eluted into 3 ml of 20% (v/v) glycerol and assayed for glutathione S-transferase activities toward (▲) CDNB, (o) NBC, (Δ) TPBO and (●) DCNB. Activities are expressed in μmoles min^{-1} ml^{-1}. (•) indicates the pH of each fraction.


A
B
C
AA
pI 6.6
0 1 2 3 4 5 6 7 8 9 10 11 12
Distance (cm)
5 4 3 2 1 0
.4 .3 .2 .1 0
1.5 1.0 .5 0
.15 .10 .05 0
10 9 8 7 6 5 4
pH

electrophilic substrate was divided between two or more transferase peaks. On the basis of substrate specificity and relative isoelectric point the first four peaks of transferase activity (in order of decreasing basicity) correspond to transferases AA, B, A and C (Habig et al., 1974b; Habig et al., 1976b; Pabst et al., 1974). On Ouchterlony immunodiffusion a line of identity between the second peak and purified transferase B (Hales and Neims, 1976a) was formed against rabbit anti-transferase B (Figure 14). The fifth peak, pI 6.6, does not correspond to transferase E. Transferase E is reported to have activity toward both 1,2-epoxy-3-(p-nitrophenoxy) propane (ENPP), and p-nitrophenethylbromide (NPB), but not toward TPBO (Fjellstedt et al., 1973). Peak L5 had activity toward TPBO but no detectable activity toward either ENPP or NPB although this lack of activity is not definitive because of the background noise in these assays. It is possible that peak L5 is identical to either transferase D or M, neither of which has been described in sufficient detail to allow definitive comparison. The average recovery of activity toward each substrate after electrofocusing was CDNB 83%, DCNB 74%, NBC 68%, TPBO 69%. Thus, this one step technique using four substrates of differing activities with the known hepatic transferases allows effective separation and some quantitation of the well characterized hepatic transferases. It has also revealed the presence of previously undescribed forms of glutathione S-transferase.

Certain complexities cannot be ignored, however. It has been

Figure 14. OUCHTERLONY IMMUNODIFFUSION OF PEAKS L1 and L2 FROM MALE AND FEMALE RATS

The ability of peaks L1 and L2 from male and female rat livers to cross react with rabbit antiserum to purified rat liver glutathione S-transferase B was tested.

The peak L2 fraction was diluted 1:10 and the peak L1 fraction was diluted so as to contain the same amount of activity toward CDNB per ml as the diluted peak L2 fraction.

The wells were filled as follows

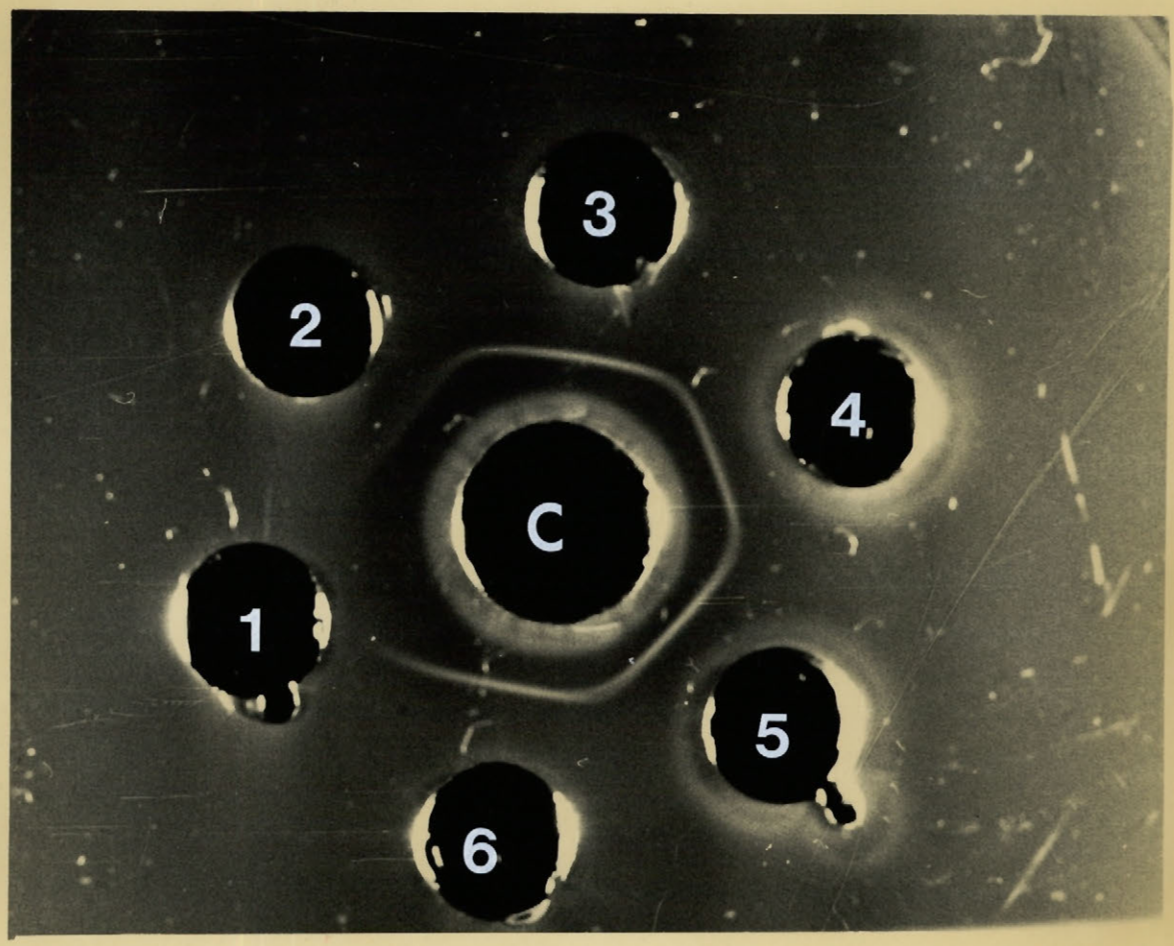
C - 40 µl of antiserum

1 - 25 µl diluted peak L1 from male rat liver

2 - 25 µl diluted peak L1 from female rat liver

3,4 - 25 µl diluted peak L2 from female rat liver

5,6 - 25 µl diluted peak L2 from male rat liver


3
2
4
C
1
5
6

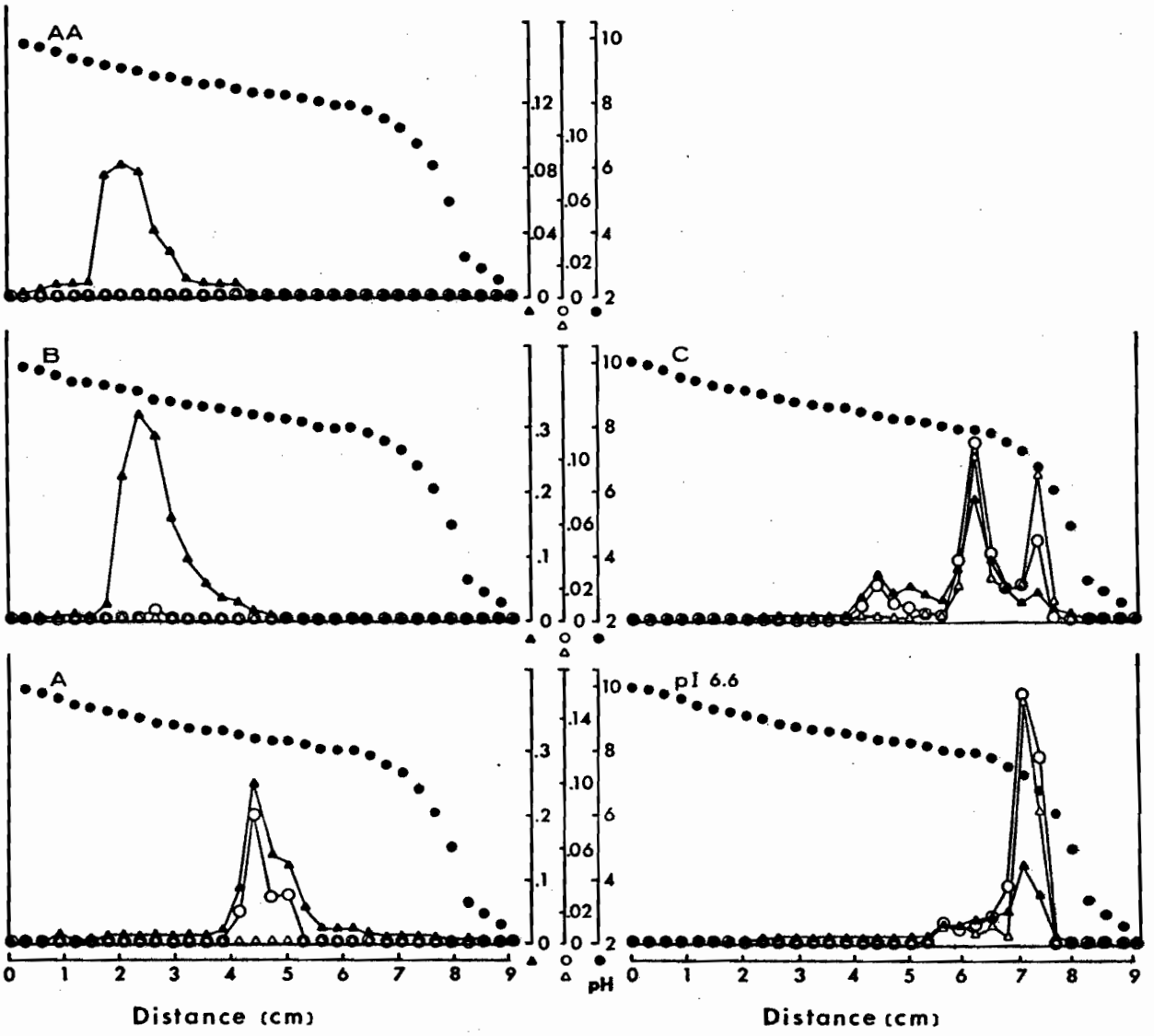
shown that ligandin is a dimer which exhibits two non-identical subunit species on discontinuous polyacrylamide gel electrophoresis in SDS (Listowsky et al., 1976). Bass et al (1977) pointed out that three combinations of the subunits are possible and suggested that in all likelihood two of these exist in vivo. In order to test the possibility that some forms of transferase activity might interconvert and to assess the extent of overlap between the various transferase peaks we refocused each of the five individual peaks on acrylamide gels after dialysis against 20% (v/v) glycerol to remove as much of the free ampholines as possible. Gels were used instead of the original columns because of a) the small size of the samples obtained after electrofocusing on columns, b) the greater reproducibility of the pH gradients on the acrylamide gels and c) the speed and ease with which many samples could be tested at once. Four of the five peaks of transferase activity refocused with the same isoelectric point and substrate specificity as the source peak (Figure 15). The fractions corresponding to peak L4, presumably mostly transferase C, refocused into three peaks of activity, the sum of which indicated significant contamination of the original peak fractions with adjacent peak L3 (transferase A) and peak L5.

Electrofocusing pattern of renal glutathione S-transferase activity

The preparative electrofocusing technique was then applied to the study of the renal glutathione S-transferases. Renal cytosols

Figure 15. REFOCUSING OF FIVE ELECTROFOCUSED PEAKS OF GLUTATHIONE S-TRANSFERASE ACTIVITY FROM HEPATIC CYTOSOL OF MALE RATS

The peak fractions with glutathione S-transferase activity from an isoelectric focusing column of male rat liver cytosol were pooled. Each peak was then dialyzed for 24 hrs. against 3 x 2 litres of 20% (v/v) glycerol. Ampholine mix was added to a final concentration of 2% (v/v) and the sample refocused overnight in acrylamide gels. Enzyme activity was eluted and assayed for activity towards (▲) CDNB, (o) NBC and (Δ) TPBO as described in the Methods section. Glutathione S-transferase activity is expressed as μmoles min^{-1} per ml of eluate.


AA
B
C
A
pI 6.6
pH
Distance (cm)
Distance (cm)

prepared from six male rats and six female rats were electrofocused individually. Representative profiles of the transferase activities of each sex are shown in Figures 16 and 17. In cytosols from each sex three major and two minor peaks of transferase activity were observed. 'Major' and 'minor' are used in reference to observed peak area and not in reference to potential biologic importance of these transferases to the animal. Average overall recoveries of activity toward each substrate in males were CDNB 74%, NBC 89% and TPBO 77% and in females were CDNB 77%, NBC 84% and TPBO 100%.

The isoelectric points and relative substrate specificities of the major electrofocused peaks of glutathione S-transferase activity from male and female rat kidney are shown in Table 8. In contrast to liver where transferase activity toward each substrate was diffusely distributed among two or more focused peaks, glutathione-S-transferase activity toward each substrate tended to be highly localized into one or another of the renal transferase peaks. Transferase activity toward CDNB focused predominantly in K1. This peak was identical to hepatic transferase B on the basis of pI and substrate specificity. It also reacted with an antibody directed against purified hepatic transferase B (Figure 24, wells 1 and 4). Activity toward NBC in males and females focused predominantly in K2m and K2f respectively and activity toward TPBO in K3. Activity toward DCNB was at the limit of detection. Smaller peaks of activity toward two of the substrates, CDNB and NBC, were often present. In both sexes a peak with a pI of 7.0 showed activity

Figure 16. ELECTROFOCUSING PROFILE OF GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOL FROM FEMALE RATS

Female kidney cytosol (6 ml) was applied to a column of Sephadex G-75 Superfine and electrofocused as described in the text. Sections (0.5 cm) of the extruded Sephadex bed were eluted into 3 ml of 20% (v/v) glycerol and assayed for glutathione S-transferase activity toward (▲) CDNB, (●) DCNB, (o) NBC and (Δ) TPBO. (•) indicates pH of each section. Activity is expressed in μmoles min^{-1} per ml of eluate.

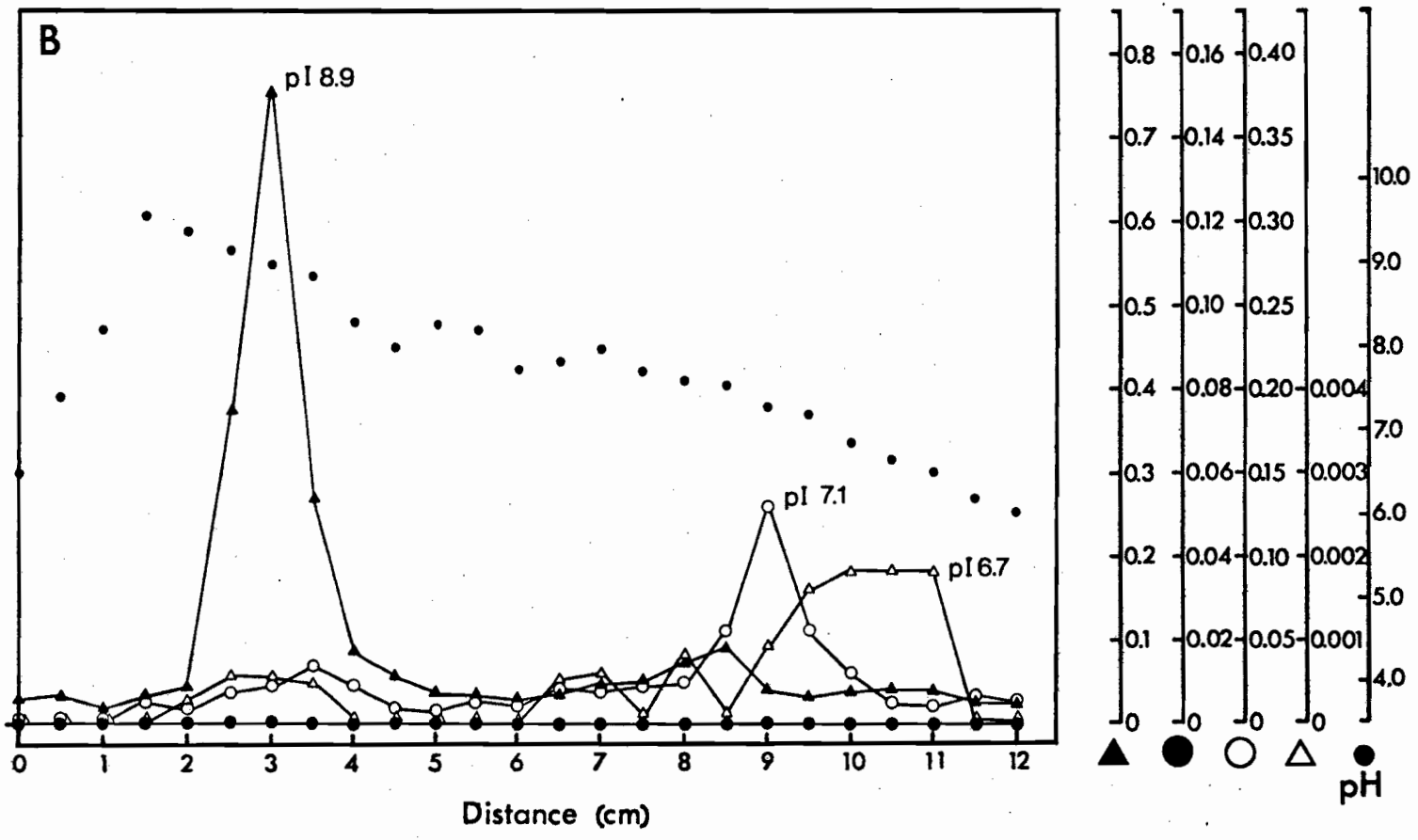

B
pI 8.9
pI 7.1
pI 6.7
0 1 2 3 4 5 6 7 8 9 10 11 12
Distance (cm)
0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0
0.16 0.14 0.12 0.10 0.08 0.06 0.04 0.02 0
0.40 0.35 0.30 0.25 0.20 0.15 0.10 0.05 0
0.004 0.003 0.002 0.001 0
10.0 9.0 8.0 7.0 6.0 5.0 4.0
pH

Figure 17. ELECTROFOCUSING PROFILE OF GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOL FROM MALE RATS

Male rat kidney cytosol (6 ml) was applied to a column of Sephadex G-75 Superfine and electrofocused as described in the text. Sections (0.5 cm) of the extruded Sephadex bed were eluted into 3 ml of 20% (v/v) glycerol and assayed for glutathione S-transferase activity toward (▲) CDNB, (●) DCNB, (o) NBC and (Δ) TPBO. (•) indicates pH. Activity is expressed as μmoles min^{-1} per ml of eluate.

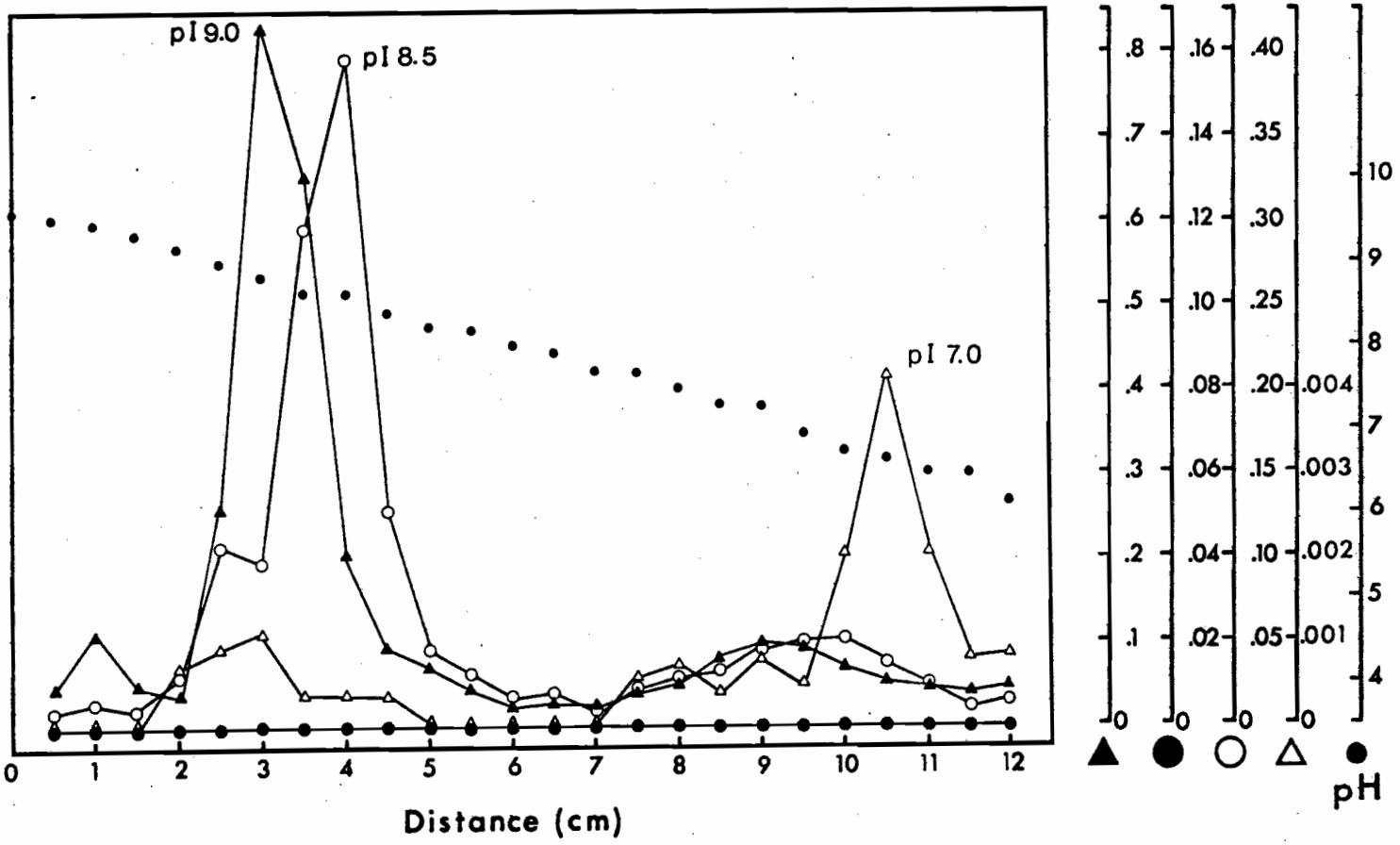

pI 9.0
pI 8.5
pI 7.0
0 1 2 3 4 5 6 7 8 9 10 11 12
Distance (cm)
.8 .7 .6 .5 .4 .3 .2 .1 0
.16 .14 .12 .10 .08 .06 .04 .02 0
.40 .35 .30 .25 .20 .15 .10 .05 0
.004 .003 .002 .001 0
10 9 8 7 6 5 4
pH

Table 8. ISOELECTRIC POINTS AND SUBSTRATE SPECIFICITIES OF GLUTATHIONE S-TRANSFERASES FROM MALE AND FEMALE RAT KIDNEY

Peak	pI		CDNB/NBC		CDNB/TPBO	
	Male	Female	Male	Female	Male	Female
K1	9.0 ± 0.1	8.9 ± 0.1	52 ± 41	27 ± 3	>1000	>1000
K2m	8.5 ± 0.1	trace	1.1 ± 0.2	-	>1000	-
K2f	trace	7.1 ± 0.1	-	1.2 ± 0.3	-	45 ± 3
K3	7.0 ± 0.2	6.7 ± 0.3	1.6 ± 0.3	2.2 ± 0.6	52 ± 16	22 ± 3

Enzyme activities toward 1-chloro-2,4-dinitrobenzene (CDNB), p-nitrobenzylchloride (NBC) and trans-4-phenylbut-3-ene-2-one (TPBO) were assayed as described in the text and are expressed for comparative purposes as ratios of activity toward CDNB to the other two substrates. Values are means ± S.E.M. for three animals.

toward CDNB. This peak did not react with an antibody directed against purified hepatic transferase B (Figure 24, well 5). The two forms of transferase with activity toward NBC, K2m and K2f, were both present to some extent in each sex.

It is possible that peaks K2m and K2f represent different forms of the same transferase. In Table 8 it can be seen that the ratios of activities toward CDNB and NBC are the same for peaks K2m and K2f. The large difference in the ratios of activities toward CDNB and TPBO, >1000 for K2m versus 45 for K2f is probably an artifact of the proximity of peaks K2f and K3, a peak with high activity toward TPBO. Fractions from the peak of K2f coincide with those from the down slope of K3 and therefore contain significant activity toward TPBO. It is this difference between having measurable activity toward TPBO in K2f and having activity toward TPBO which approaches zero in K2m that makes a difference in the denominator of the CDNB to TPBO ratio that drastically affects the final ratio. The isoelectric points of the two renal peaks with activity toward NBC did differ significantly between males and females. This form had a pI of 8.5±0.1 in male kidney and 7.1±0.1 in female kidney. However these results are representative for only four out of the five males tested and for four out of the five females tested. In the remaining two animals, NBC activity in the male focused predominantly at pIs 7.0 and 5.6 while that in the female focused predominantly at 8.5. The distribution of the pIs of the major peaks with activity toward NBC in male and female rat kidneys is

shown in Figure 18. Subsequent experiments confirmed the anomalous behaviour of the activity towards this substrate.

In order to assess the possibility that some forms of transferase activity especially those towards NBC might interconvert, focused peaks were pooled and refocused on acrylamide gels after dialysis against 20% v/v glycerol. In males and females, the peaks with activity toward CDNB, K1, and those with activity toward TPBO, K3, refocused with the same isoelectric points and substrate specificities as the source peaks (Figures 19 and 20). However when peak K2m was refocused the majority of the activity toward NBC was not found at the pI of the source peak. Rather, activity toward NBC was spread along the gel with most activity being found at pH 7.1. Similarly, when peak K2f was refocused the activity was not recovered in a single peak but in five smaller peaks. Recovery of activity toward NBC after re-electrofocusing was low for both sexes. This result was not a function of the electrofocusing technique since simple dialysis or storage of the sample overnight in the cold resulted in the loss of up to 80% of activity towards NBC. The form with a 'pI' of 8.5 seems to be the more unstable of the two main forms with activity toward NBC.

At this stage the question of whether to use Sephadex columns or polyacrylamide gels for subsequent electrofocusing work arose. The use of polyacrylamide gels rather than Sephadex columns allowed many samples to be run simultaneously and so permitted more direct comparison of samples. Also the pH gradients obtained

Figure 18. DISTRIBUTION OF THE pI's OF THE MAJOR PEAKS WITH ACTIVITY TOWARD NBC

The male rats are numbered 1-5, the females 6-10. The pI's of the major peaks with activity toward NBC from male rat kidneys are indicated by •, those from females by o. With the exception of rat #4, in all cases only 1 major peak with activity toward NBC was found.

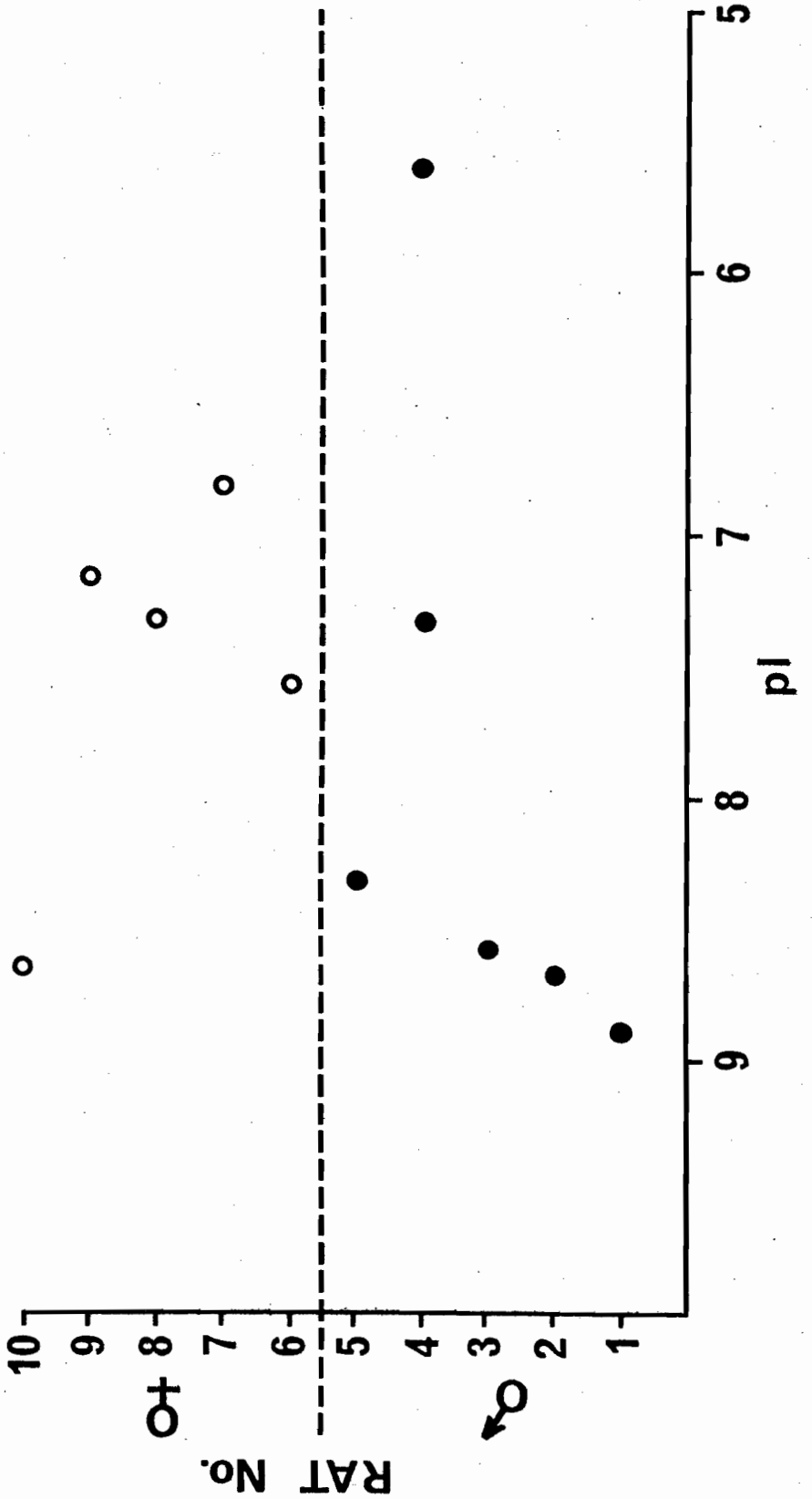

RAT No.
♀
♂
pl
1
2
3
4
5
6
7
8
9
10
5
6
7
8
9

Figure 19. REFOCUSING OF THREE ELECTROFOCUSED PEAKS OF GLUTATHIONE S-TRANSFERASE ACTIVITY FROM RENAL CYTOSOL OF MALE RATS

The peak fractions with glutathione S-transferase activity from an isoelectric focusing column of male rat kidney cytosol were pooled. Each peak was then dialyzed for 24 hours against 3x2 litres of 20% (v/v) glycerol. Ampholine mix was added to a final concentration of 2% (v/v) and the sample refocused overnight on acrylamide gels. Enzyme activity was eluted and assayed as in the Methods section. Glutathione S-transferase activities toward (●) CDNB, (o) NBC and (Δ) TPBO are expressed as μmoles min^{-1} per ml of eluate.

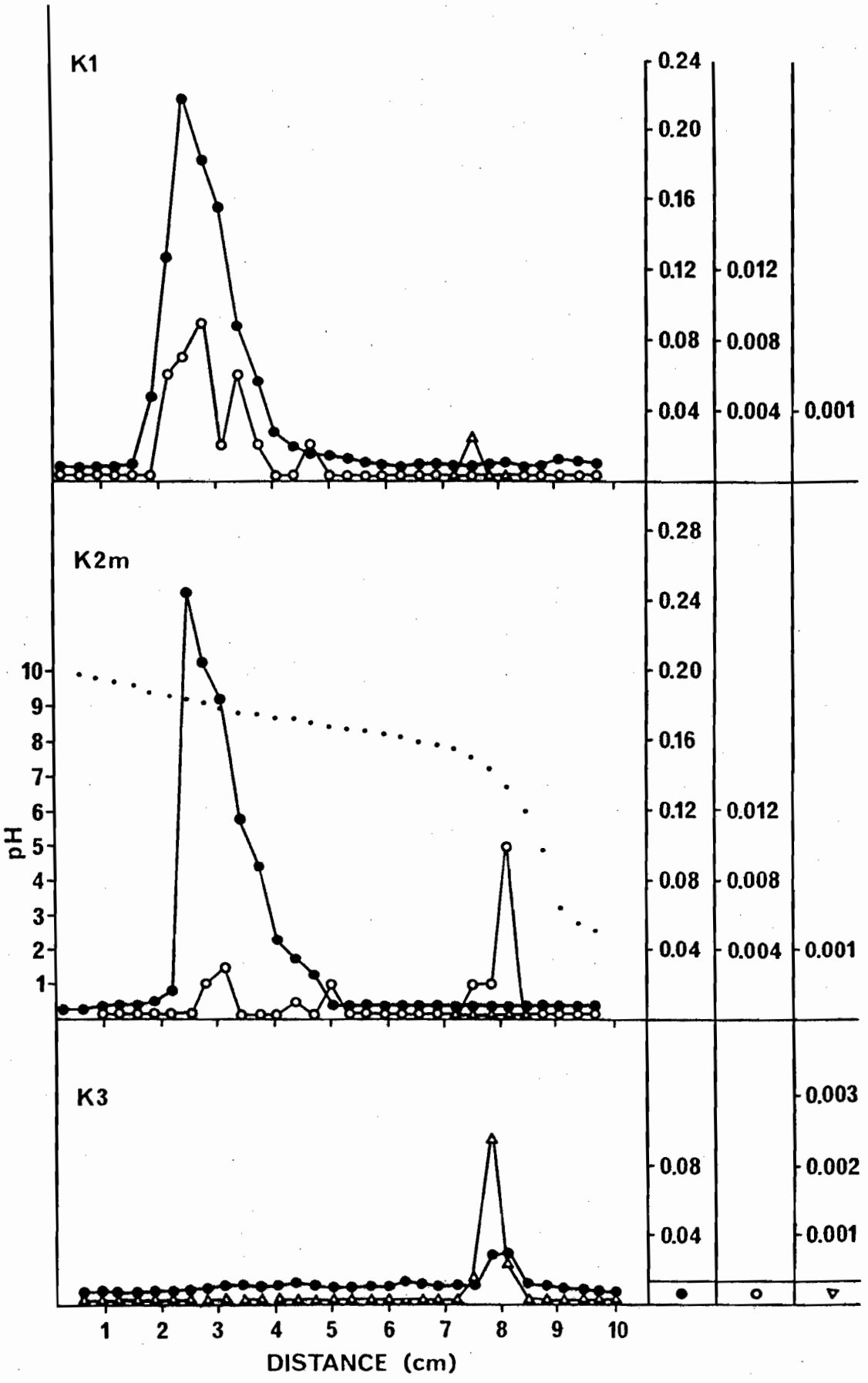
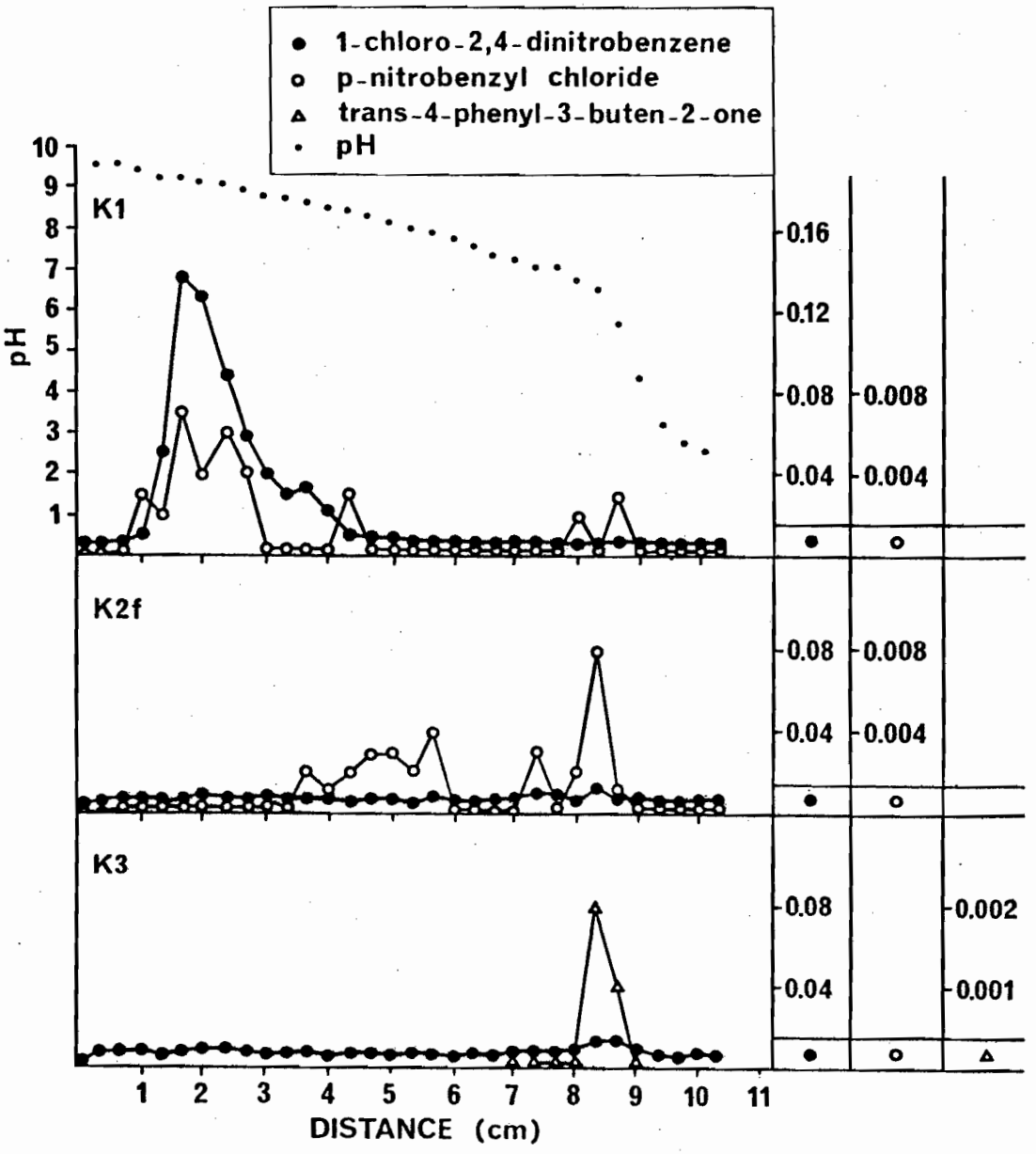

K1
K2m
K3
pH
10
9
8
7
6
5
4
3
2
1
0.24
0.20
0.16
0.12
0.08
0.04
0.012
0.008
0.004
0.001
0.28
0.003
0.002
1
2
3
4
5
6
7
8
9
10
DISTANCE (cm)

Figure 20. REFOCUSING OF THREE ELECTROFOCUSED MAJOR PEAKS OF GLUTATHIONE S-TRANSFERASE ACTIVITY FROM RENAL CYTOSOL OF FEMALE RATS

The peak fractions with glutathione S-transferase activity from an isoelectric focusing column of female kidney cytosol were pooled. Each peak was then dialyzed for 24 hours against 3x2 litres of 20% (v/v) glycerol. Ampholine mix was added to a final concentration of 2% (v/v) and the sample refocused overnight on acrylamide gels. Enzyme activity was eluted and assayed as in the Methods section. Glutathione S-transferase activity is expressed as μmoles min^{-1} per ml of eluate.


1-chloro-2,4-dinitrobenzene
p-nitrobenzyl chloride
trans-4-phenyl-3-buten-2-one
pH
K1
K2f
K3
pH
DISTANCE (cm)
0.16
0.12
0.08
0.04
0.008
0.004
0.002
0.001

with the gels were more consistent and more linear than those obtained with the columns. When aliquots of renal cytosol from one animal were electrofocused concurrently on Sephadex columns and on acrylamide gels very little difference was observed in the pattern of activity toward CDNB and TPBO. The recovery of activity toward these two substrates was as high after electrofocusing on gels as after electrofocusing on columns. However, recovery of activity toward NBC was lower after electrofocusing on gels and the activity recovered was always found at pI 7.0 whether the cytosol was from a male or a female. This latter observation probably reflects the fact stated earlier that the peak with pI of 8.5 is the more unstable of the two predominant forms with activity towards NBC. The reason for the increased instability of the forms of transferase with activity toward NBC when focused on gels as opposed to columns is unknown. Acrylamide gels were used for all subsequent experiments dealing with induction and development since the advantages of direct comparison and linear pH gradient were seen to outweigh the disadvantage of lower recovery of activity toward NBC.

IDENTIFICATION OF PEAK K3

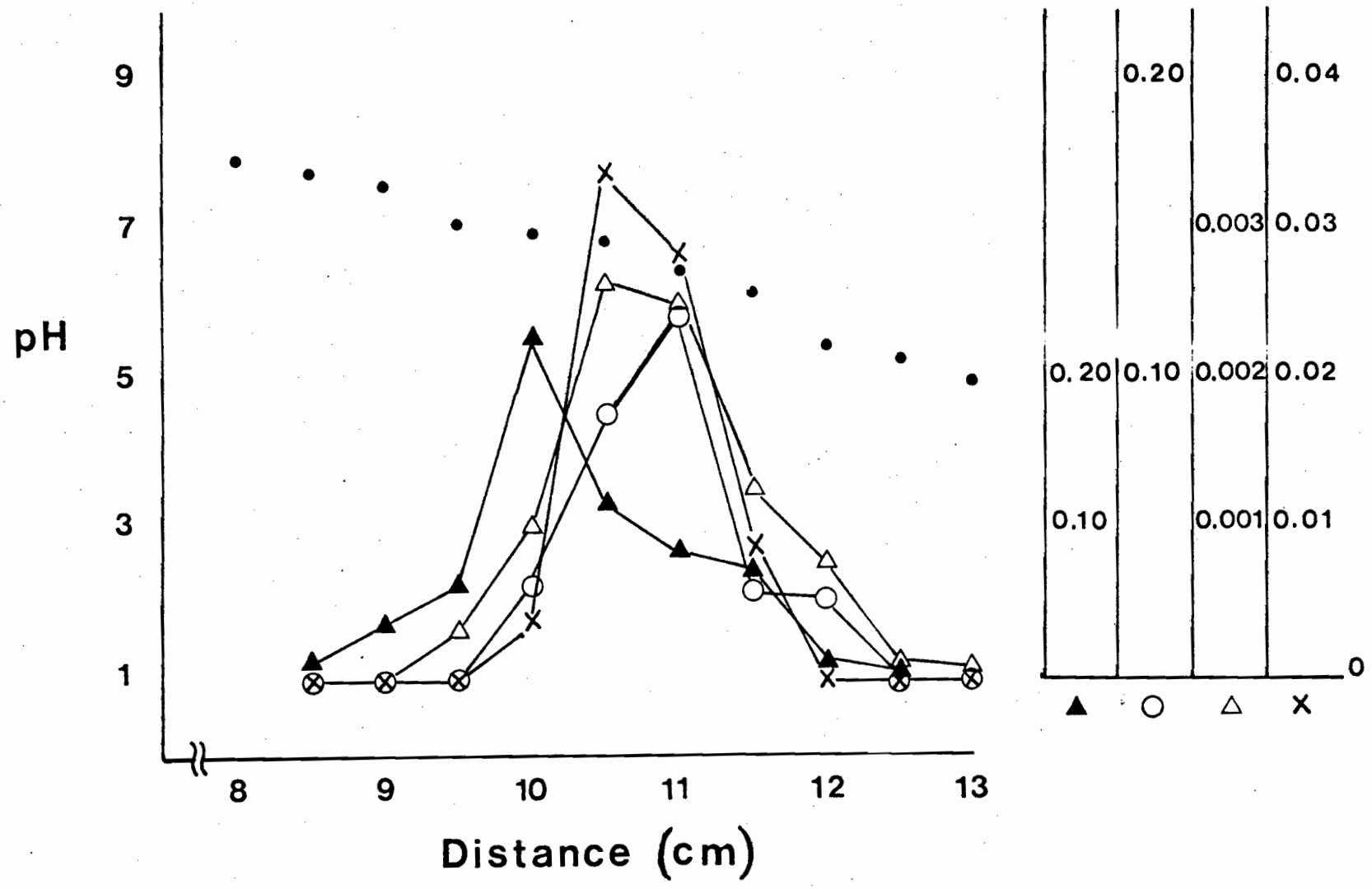
In 1973 Gillham isolated a glutathione S-transferase, pI 6.9, from rat liver which was active towards 1-menaphthyl sulfate. This was named glutathione S-transferase M. Since the pI for peak K3 was 6.9 we used 1-menaphthyl sulfate in an attempt to identify this renal transferase. Renal cytosol possessed activity toward 1-menaphthyl

sulfate. The average value obtained was 0.331 $\pm$ .028 μmoles min^{-1}/gm wet weight of tissue (n=5). When renal cytosol was passed through a Sephadex G-100 column activity toward 1-menaphthyl sulfate was recovered in the same fractions as that towards CDNB, NBC, and TPBO.

Renal cytosols from two rats were pooled and 8 ml of the mixture were electrofocused on a Sephadex G-75 column as described in the Methods. Only one peak of activity toward 1-menaphthyl sulfate was found. This peak had a pI of 6.9 and corresponded to the peak with activity toward TPBO, peak K3 (Figure 21). The peaks with activity toward CDNB and NBC had different pIs, 6.5 and 7.05 respectively, and different activity profiles than the peak with activity toward 1-menaphthyl sulfate. Therefore it seems that in addition to having a transferase equivalent to hepatic transferase B, rat kidney also possesses one equivalent in pI and substrate specificity to transferase M.

Figure 21. ELECTROFOCUSING PROFILE OF PEAK K3 FROM MALE RATS

Renal cytosol (6 ml) from male rats was applied to a column of Sephadex G-75 Superfine and electrofocused as described in the text. Sections (0.5 cm) of the extruded Sephadex bed were eluted into 3 ml of 20% (v/v) glycerol and assayed for glutathione S-transferase activity toward (▲) CDNB, (o) NBC, (Δ) TPBO and (X) MNS. Activity is expressed as μmoles min^{-1} per ml of eluate. Only the activities of the fractions from the acidic end of the column are graphed.


pH
9
7
5
3
1
8
9
10
11
12
13
Distance (cm)
0.20
0.04
0.003
0.03
0.20
0.10
0.002
0.02
0.10
0.001
0.01
0
▲
○
△
×

RESULTS

SECTION III

XENOBIOTIC INDUCTION OF RENAL GLUTATHIONE S-TRANSFERASES FROM MALE RATS

COMPARISON OF OVERALL GLUTATHIONE S-TRANSFERASE ACTIVITY OF RENAL CYTOSOLS FROM INDUCED AND CONTROL RATS

The glutathione S-transferase activities toward CDNB, NBC, and TPBO in renal cytosols from male rats pretreated with phenobarbital, β-naphthoflavone, Aroclor 1254 or appropriate vehicle are presented in Table 9. The time intervals between injecting and killing the animals were based on those reported for studies of the effect of inducing agents on hepatic glutathione S-transferases and cytochromes P-450 (Kaplowitz et al., 1975; Ecobichon, personal communication) and on results of preliminary experiments conducted in this laboratory. Phenobarbital pretreatment did not significantly increase the transferase activity toward any of the substrates tested whereas both β-naphthoflavone and Aroclor 1254 increased glutathione S-transferase activity toward CDNB by at least 40% relative to controls. Of those transferase activities tested only that towards CDNB was increased by any of the potential inducers. These conclusions do not depend on whether the data are expressed per mg protein rather than per gm wet weight of tissue since there was no significant difference in protein concentration of cytosols from induced and control animals.

One of the highest set of control values obtained was with commercial peanut oil and it was deemed necessary to determine

Table 9. EFFECT OF PRETREATMENT WITH PHENOBARBITAL, β-NAPHTHOFLAVONE AND AROCLOR 1254 ON GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOL FROM MALE RATS

TREATMENT	GLUTATHIONE S-TRANSFERASE ACTIVITY TOWARDS CDNB	NBC	TPBO
Saline	12.50 ± 1.32	4.50 ± 0.45	.0412 ± .0042
Phenobarbital	12.25 ± 0.50	4.65 ± 0.75	.0443 ± .0051
Corn Oil	11.71 ± 1.96	9.01 ± 1.03	.0434 ± .0031
β-naphthoflavone	21.30 ± 2.26	7.43 ± 0.63	.0434 ± .0025
Peanut oil	19.15 ± 1.20	10.16 ± 1.30	.0238 ± .0010
Aroclor [1]	27.56 ± 4.56	7.66 ± 1.33	.0291 ± .0041
Aroclor [2]	33.03 ± 2.05	12.80 ± 0.70	.0252 ± .0013

Enzyme activities toward CDNB, NBC and TPBO assayed as described in the text are expressed in μmoles/min per gm wet weight of tissue. Values are means ± S.E.M. for cytosol preparations from three to six animals.

[1] Animals were injected with Aroclor 1254, 50 mg/Kg/day for three days

[2] Animals were given a single injection of Aroclor 1254, 500 mg/Kg, in corn oil.

whether this oil alone could be having an inductive effect on transferase activity. To test the intra-experimental variability of controls and determine whether type of vehicle oil alone might have exerted an effect on activity toward CDNB, groups of rats were concurrently injected with corn oil or peanut oil or received a sham injection. When the renal cytosols of these animals were tested for glutathione S-transferase activity no significant difference was found between the three groups (Table 10). In general, although from time to time throughout the year control values varied quite considerably, within any given set of experiments there was little variation in control values obtained.

ELECTROFOCUSING PROFILES OF GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM XENOBIOTIC-TREATED AND CONTROL MALE RATS

Aroclor 1254 and β-naphthoflavone were found to increase overall cytosolic glutathione S-transferase activity toward CDNB. If the technique of electrofocusing is a valid one for examination of the individual glutathione S-transferases, changes observed in the electrofocused peaks of transferase activity should explain the observed changes in cytosolic activity. Cytosols from phenobarbital, β-naphthoflavone, Aroclor 1254 pretreated and control animals were electrofocused on polyacrylamide gels (Figures 22 and 23) to determine (i) whether the observed increase in activity toward CDNB seen in β-napthoflavone and Aroclor treated

Table 10. EFFECT OF INJECTION OF VEHICLE ALONE ON GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOL FROM MALE RATS

TREATMENT	GLUTATHIONE S-TRANSFERASE ACTIVITY TOWARDS		
	CDNB	NBC	TPBO
Sham injection	13.00 ± 1.66	7.15 ± .590	.0299 ± .00234
Corn oil injection	13.15 ± .900	6.95 ± .184	.0315 ± .00150
Peanut oil injection	14.45 ± 2.01	9.65 ± 1.49	.0262 ± .00337

Enzyme activities towards C DNB, NBC and TPBO assayed as described in the text, are expressed as μmol/min/gm wet weight of tissue. Values are means ± S.E.M. for cytosol preparations from three animals.

Figure 22. ELECTROFOCUSING PROFILES OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM RATS TREATED WITH PHENOBARBITAL AND RATS TREATED WITH SALINE

Male rat kidney cytosols (300 µl) from either saline or phenobarbital injected animals were applied to the top of acrylamide gels and electrofocused as described in text. Gel sections were eluted and assayed as in the Methods section. Glutathione S-transferase activities toward (●) CDNB, (o) NBC and (Δ) TPBO are expressed as µmoles min^{-1} per ml of eluate. (•) indicates pH of each section.

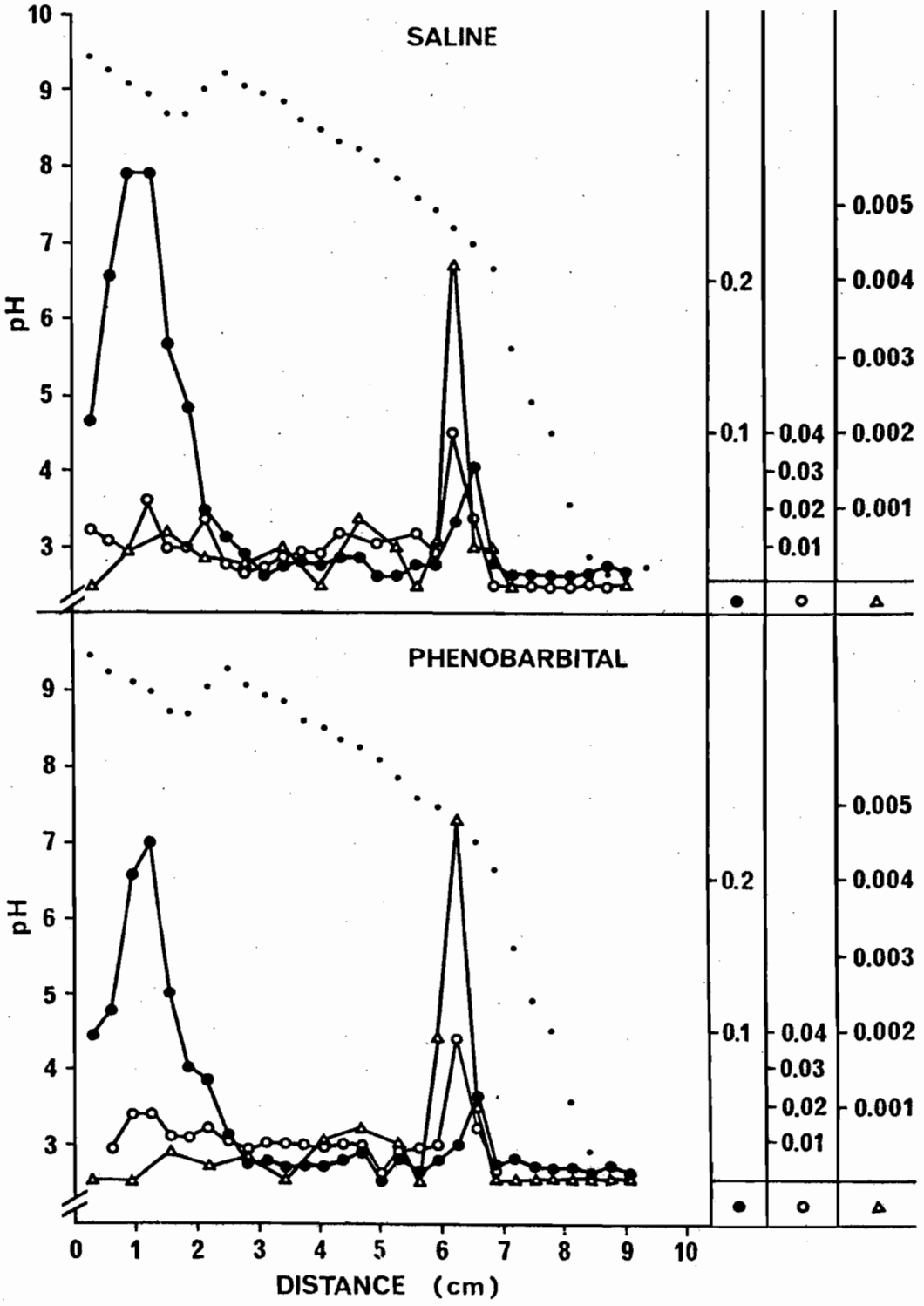
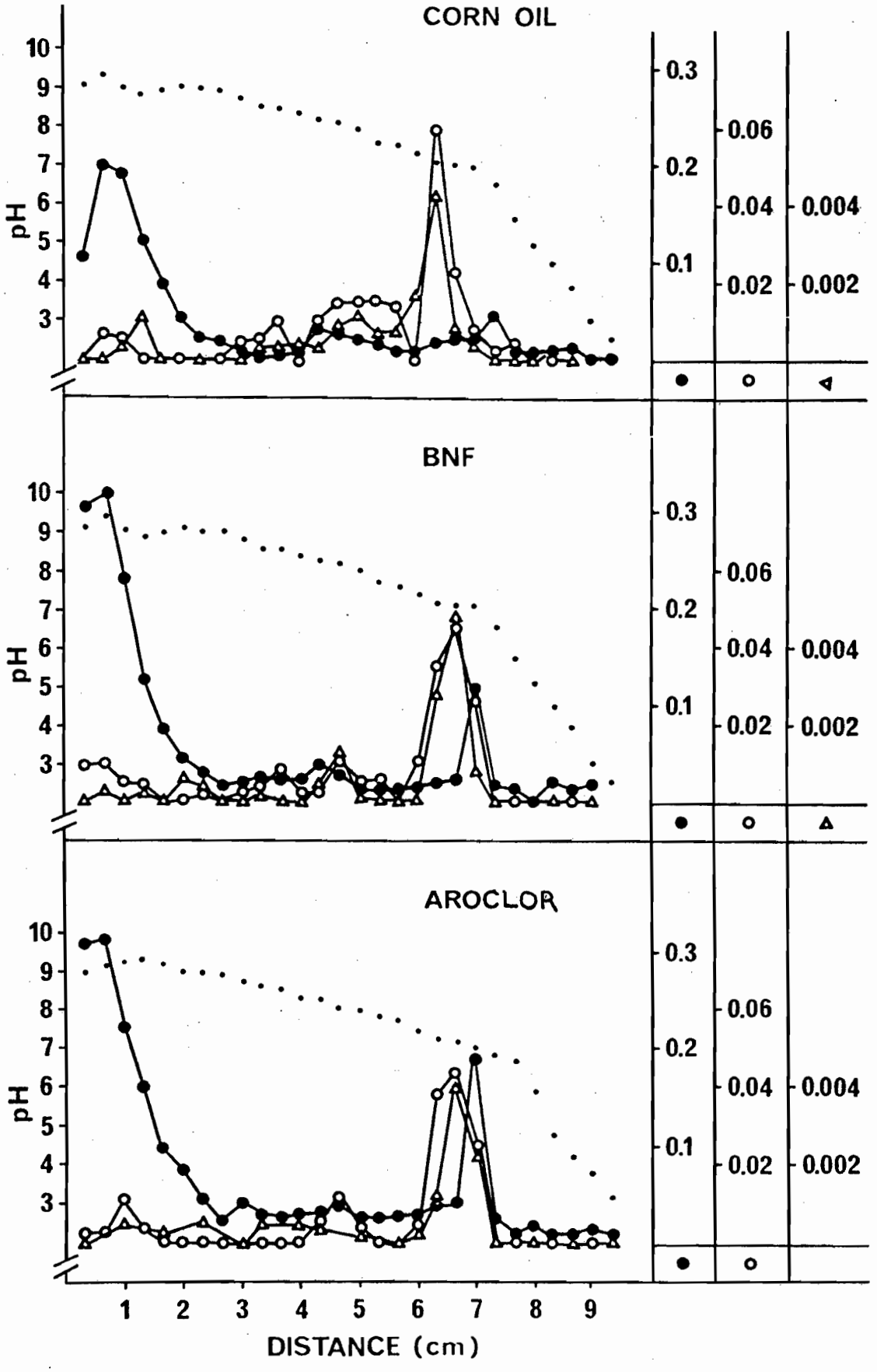

SALINE
PHENOBARBITAL
pH
10
9
8
7
6
5
4
3
0.2
0.1
0.04
0.03
0.02
0.01
0.005
0.004
0.003
0.002
0.001
●
○
△
0 1 2 3 4 5 6 7 8 9 10
DISTANCE (cm)

Figure 23. ELECTROFOCUSING PROFILES OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM RATS TREATED WITH CORN OIL, RATS TREATED WITH β-NAPHTHOFLAVONE AND RATS TREATED WITH AROCLOR 1254

Cytosol (300 µl) from rats treated with βnaphthoflavone, Aroclor 1254 or corn oil alone was applied to acrylamide gels and electrofocused as described. Gel sections (3 mm) were eluted into 20% (v/v) glycerol and the eluate assayed for glutathione S-transferase toward (●) CDNB, (o) NBC and (Δ) TPBO. Activities are expressed in µmoles min^{-1} per ml of eluate. (•) indicates pH of each section.


CORN OIL
BNF
AROCLOR
pH
10
9
8
7
6
5
4
3
0.3
0.2
0.1
0.06
0.04
0.02
0.004
0.002
1
2
3
4
5
6
7
8
9
DISTANCE (cm)

rats was due solely to an increase in peak K1 or to the induction of another distinct form of transferase with activity toward CDNB and (ii) whether phenobarbital had caused a redistribution of activity between the renal transferase peaks without affecting overall cytosolic activity. The results showed that a) only the areas of the two transferase peaks with activity toward CDNB already present in control animals were increased by treatment with Aroclor or β-naphthoflavone and b) there was no significant difference in areas of any of the transferase peaks from phenobarbital pretreated rats when compared with the saline.

The area of peak K1 from both β-naphthoflavone and Aroclor 1254 treated rats was significantly greater than that in corresponding controls. In the Aroclor 1254 treated rats only, electrofocusing revealed that the minor peak with activity toward CDNB, pI 7.0, was also induced. The area of this peak increased approximately 50% over control animals. Ouchterlony immunodiffusion tests (Figure 24) showed that Aroclor 1254 was causing the induction of a form of transferase which was antigenically different from peak K1. Peak tubes #2 and #21 (corresponding to distances of 0.6 and 7 cm respectively) from the Aroclor treated rat whose electrofocusing profile has been shown and peak tubes #2 and #22 from the corresponding control were tested against an antibody to purified hepatic transferase B. In concentrations containing equivalent amounts of activity toward CDNB, Aroclor peak K1 reacted with the

Figure 24. OUCHTERLONY IMMUNODIFFUSION OF THE TWO PEAKS FROM AROCLOR TREATED AND CONTROL RATS WHICH SHOWED ACTIVITY TOWARD CDNB

The ability of the peak tubes from K1 and peak pI7.0 with activity toward CDNB from Aroclor treated and control rats to cross react with an antibody to purified rat liver transferase B was tested. The wells were filled as follows:

C - 40 µl antiserum

1 - 25 µl of K1 (diluted 1:4) from control rats

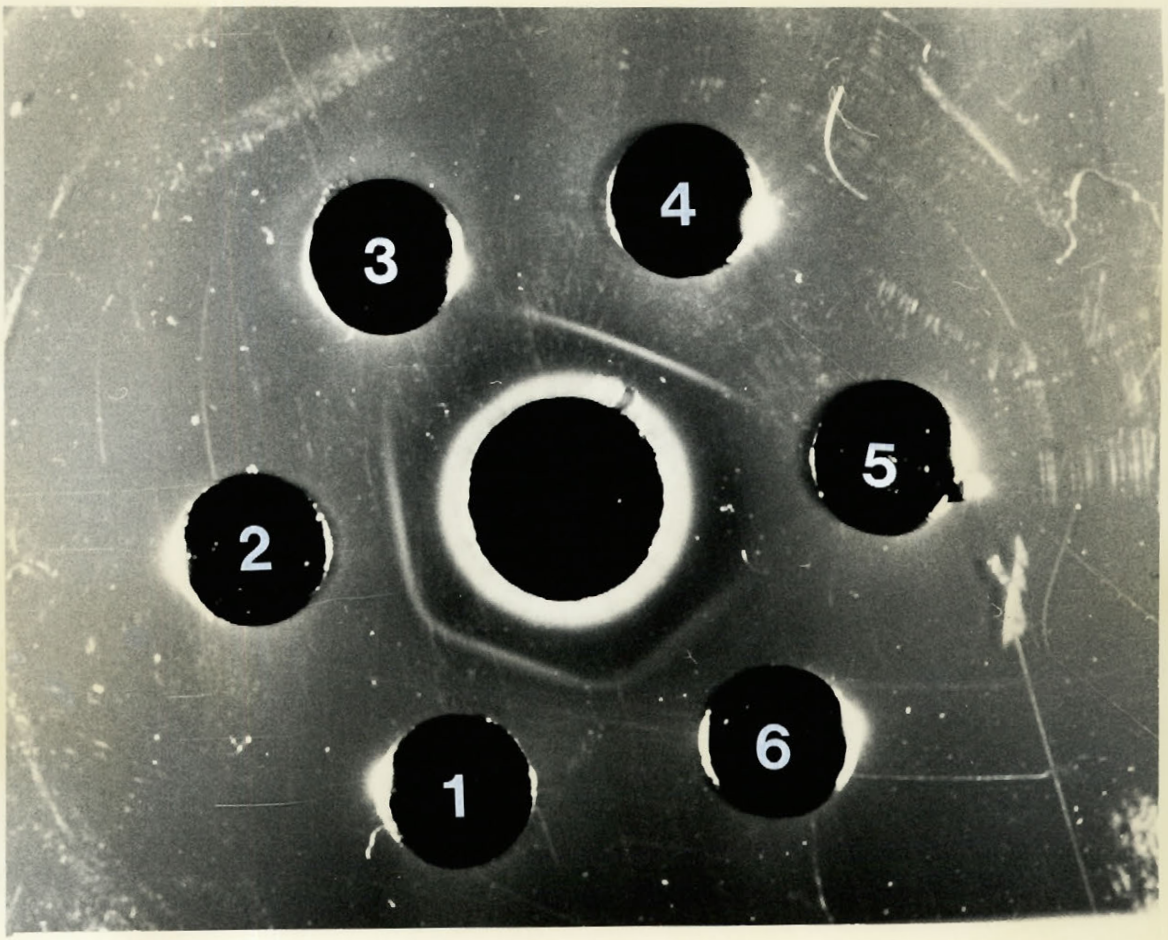
2 - 25 µl of K1(diluted 1:2) from Aroclor treated rats

3 - 25 µl of peak pI7.0 from Aroclor treated rats

4 - 25 µl of K1 (diluted 1:2) from control rats

5 - 25 µl of peak pI7.0 from control rats

6 - 25 µl of K1 (diluted 1:4) from Aroclor treated rats


3
4
5
2
6
1

antibody and gave a line of identity with peak K1 from control animals while tubes #21 (Aroclor treated) and #22 (control animal) did not react with the antibody. Therefore, Aroclor 1254 seems to induce two forms of transferase, of distinct pI and antigenicity, with activity toward CDNB while βnaphthoflavone significantly induces only one of these forms, peak K1.

COMPARISON OF OVERALL GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOLS FROM ALCOHOL TREATED AND CONTROL FEMALE RATS

This study was done in collaboration with the laboratory of Dr. Gilles Joly, Hôpital St-Luc, Montreal, who raised the alcohol treated rats and their corresponding controls. The glutathione S-transferase activities of the kidneys of female rats who had been fed a liquid diet containing alcohol were compared to those of controls who received a similar diet without the alcohol. The following values were obtained:

	CDNB	NBC	TPBO
Alcohol pretreated	12.0 ± 0.65	9.0 ± 1.24	0.075 ± 0.005
Control liquid diet	13.9 ± 0.32	9.5 ± 1.00	0.065 ± 0.010

The values represent means ± S.E.M. for four animals and are expressed as μmoles/min per gm weight of tissue. There was no significant difference in glutathione S-transferase activities toward any of the tested substrates between the two groups.

RESULTS

SECTION IV

DEVELOPMENTAL PATTERNS OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES

OVERALL TRANSFERASE ACTIVITIES OF RENAL CYTOSOL FROM IMMATURE RATS

All the glutathione S-transferase activities in this section are expressed per gm wet weight of tissue or per ml of 100,000g supernatant. Prior to birth, the transferase activities towards all substrates tested were low. On fetal day 20 of a 21-day gestation, the percent of adult activity toward each substrate in renal cytosol was as follows: CDNB 10%, NBC 16%, DCNB 22% and TPBO 24%. Overall glutathione S-transferase activities of renal cytosol were measured in at least three groups of 4 rats on each of days 1, 5, 10, 16, 22, 30 and 42 after birth. The results are shown in Table 11 and Figure 25. Our animals were still ingesting substantial amounts of milk on day 16 and were removed from their mothers on day 21. Although activity towards all substrates had approximated the adult level by day 30, the course of development to that level was very different for the different transferase activities. That towards CDNB showed a gradual and continuous increase from day 1 postnatal until adult values were reached. Activity toward TPBO and NBC remained relatively constant from day 1 to day 16 and then increased sharply until about day 30. Since the activity toward DCNB in immature rats was at the limit of detection the values obtained may be imprecise. Perhaps as a result of this, the developmental pattern of activity toward DCNB appeared more erratic. In

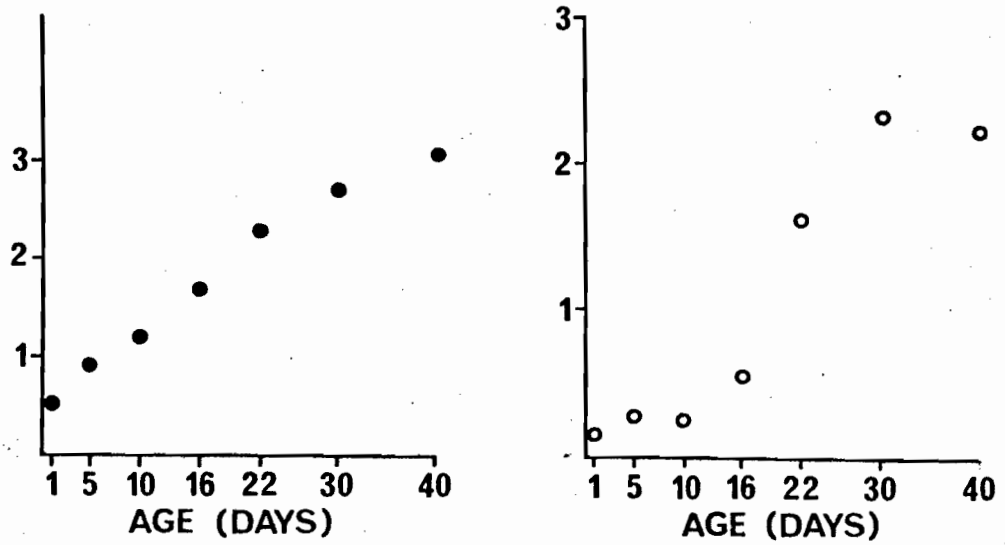
Table 11. DEVELOPMENTAL PATTERN OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES

AGE (Days)	GLUTATHIONE S-TRANSFERASE ACTIVITY TOWARD CDNB	NBC	TPBO	DCNB
1	2.540 ± .157	.831 ± .0995	.0265 ± .00231	.036 ± .003
5	4.570 ± .474	1.442 ± .103	.0252 ± .00211	.055 ± .004
10	5.950 ± .338	1.270 ± .0830	.0280 ± .00201	.080 ± .013
16	8.500 ± .745	2.940 ± .720	.0275 ± .00480	.065 ± .012
22	11.500 ±1.755	7.805 ± .560	.0360 ± .00670	.070 ± .005
30	13.555 ±1.560	11.600 ± 1.075	.0600 ± .00421	.115 ± .012
42	15.350 ± .900	11.200 ± 1.120	.0578 ± .00679	.069 ± .004

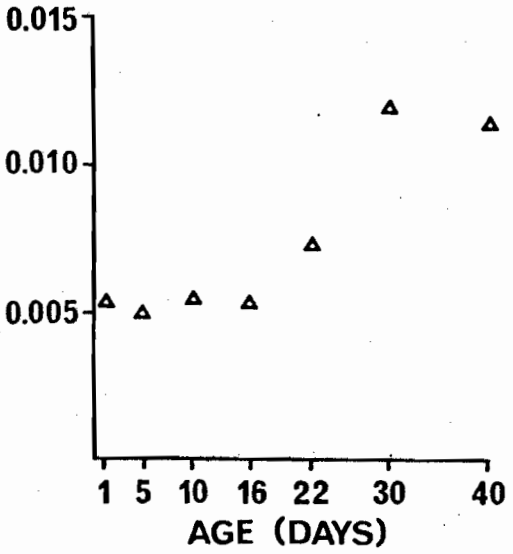
On the days indicated kidneys were excised and homogenized and the cytosols assayed for glutathione S-transferase activity towards CDNB, NBC, TPBO and DCNB. Activities are expressed as μmoles/min per gm wet weight of tissue. Values are means ± S.E.M. for 3 groups of 4 animals each.

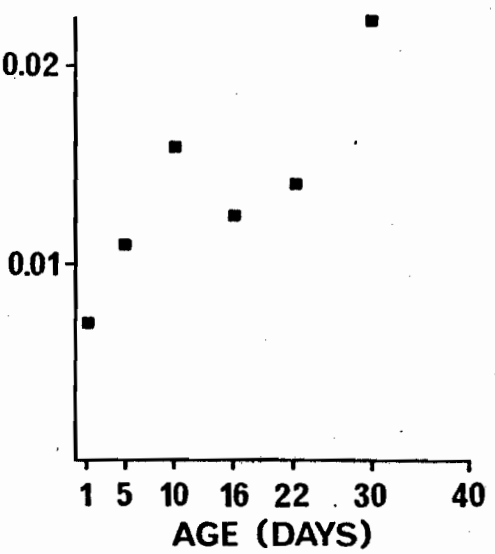
Figure 25. PATTERNS OF DEVELOPMENT OF GLUTATHIONE S-TRANSFERASE ACTIVITIES IN RENAL CYTOSOL FROM MALE RATS

Cytosols from male rats of the indicated ages were assayed for glutathione S-transferase activity toward (●) CDNB, (o) NBC, (▲) TPBO and (■) DCNB and the results graphed as a function of age. Activities are expressed as μmoles min^{-1} per ml of 100,000g supernatant. Values are means for 3 groups of 4 animals.


3
2
1
1 5 10 16 22 30 40
AGE (DAYS)

3
2
1
1 5 10 16 22 30 40
AGE (DAYS)


0.015
0.010
0.005
1 5 10 16 22 30 40
AGE (DAYS)


0.02
0.01
1 5 10 16 22 30 40
AGE (DAYS)

overall shape it more closely resembled that of CDNB than that of either TPBO or NBC.

ELECTROFOCUSING PATTERNS OF GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOLS FROM IMMATURE RATS

Renal cytosols from rats ages 5, 16 and 30 days were selected for electrofocusing on polyacrylamide gels. As early as day 5 all of the transferases later present in the adult were seen (Figure 26) albeit in decreased amounts. No forms of transferase not present in the adult were found. The comparative electrofocusing patterns from days 5, 16 and 30 (Figures 26, 27 and 28) showed a gradual increase in the height of peak K1 with activity toward CDNB, but a sudden increase in the height of the peaks with activity towards NBC and TPBO. Thus, the developmental patterns seen in overall cytosolic glutathione S-transferase activities towards these three substrates seems to reflect the developmental pattern of the individual transferase peaks as determined by electrofocusing. Although activity toward DCNB is measurable in whole cytosol, in gel slices it is beyond the limit of detection and no peaks with activity toward this substrate could be recovered.

OUCHTERLONY IMMUNODIFFUSION STUDIES OF RENAL CYTOSOLS FROM IMMATURE RATS

In order to determine whether cytosol from young rat kidneys

Figure 26. ELECTROFOCUSING PROFILE OF GLUTATHIONE S-TRANSFERASE ACTIVITIES OF RENAL CYTOSOLS FROM MALE RATS AGED 5 DAYS

Kidneys from three 5 day old male rats were pooled and homogenized. Cytosol (300 µl) prepared from this homogenate was electrofocused on acrylamide gels as described in the Methods section. Gel sections (3mm) were eluted and assayed for glutathione S-transferase activity towards (●) CDNB, (o) NBC and (Δ) TPBO. Activities are expressed in µmoles min^{-1} per ml of eluate.

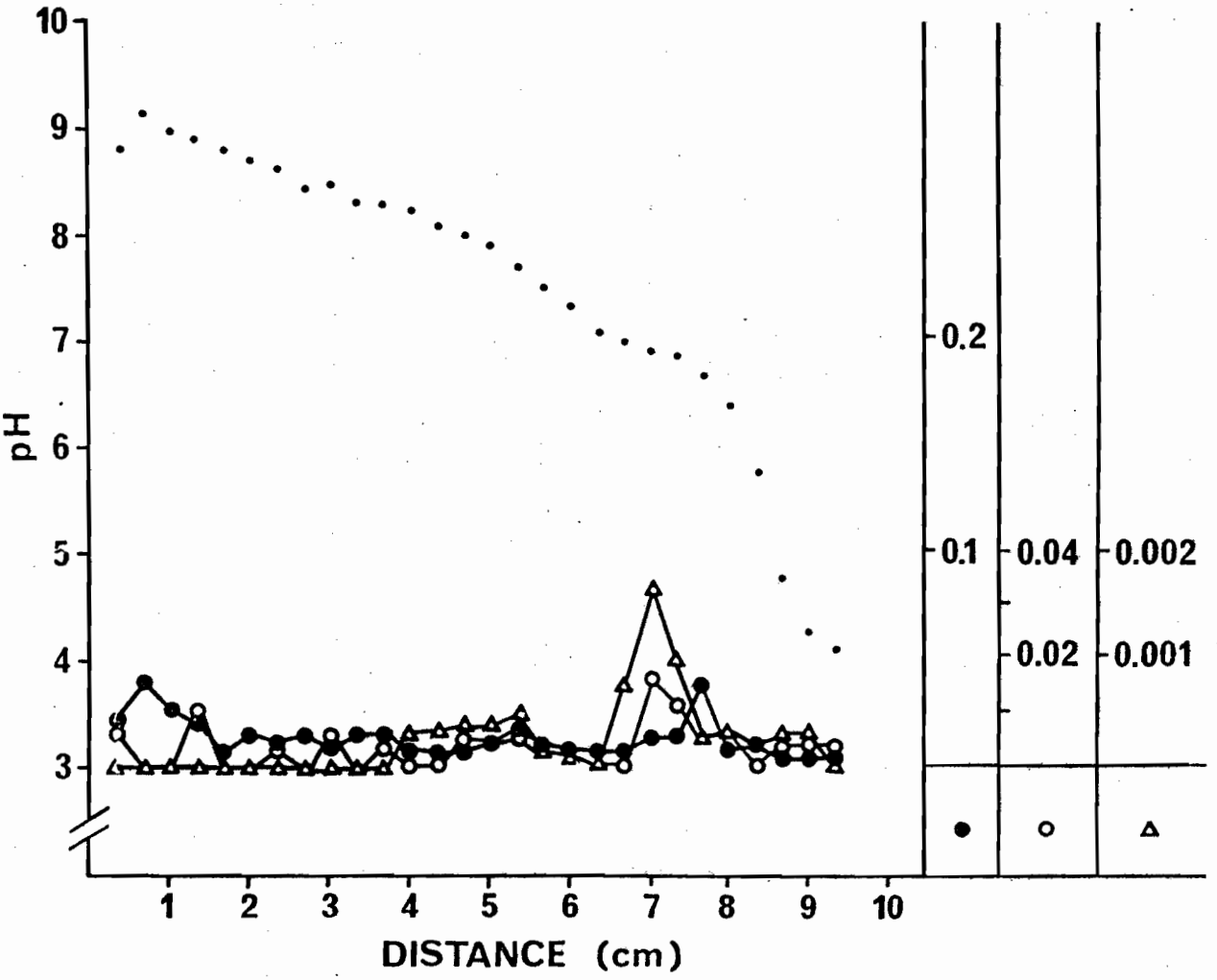

10
9
8
7
6
5
4
3
pH
1
2
3
4
5
6
7
8
9
10
DISTANCE (cm)
0.2
0.1
0.04
0.002
0.02
0.001
●
○
△

Figure 27. ELECTROFOCUSING PROFILE OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM MALE RATS AGED 16 DAYS

Kidneys from three 16 day old male rats were pooled and homogenized. Cytosol (300 µl) prepared from this homogenate was electrofocused on acrylamide gels as described in the Methods section. Gel sections (3mm) were eluted and assayed for glutathione S-transferase activity towards (•) CDNB, (o) NBC and (▲) TPBO. Activities are expressed in µmoles min^{-1} per ml of eluate.

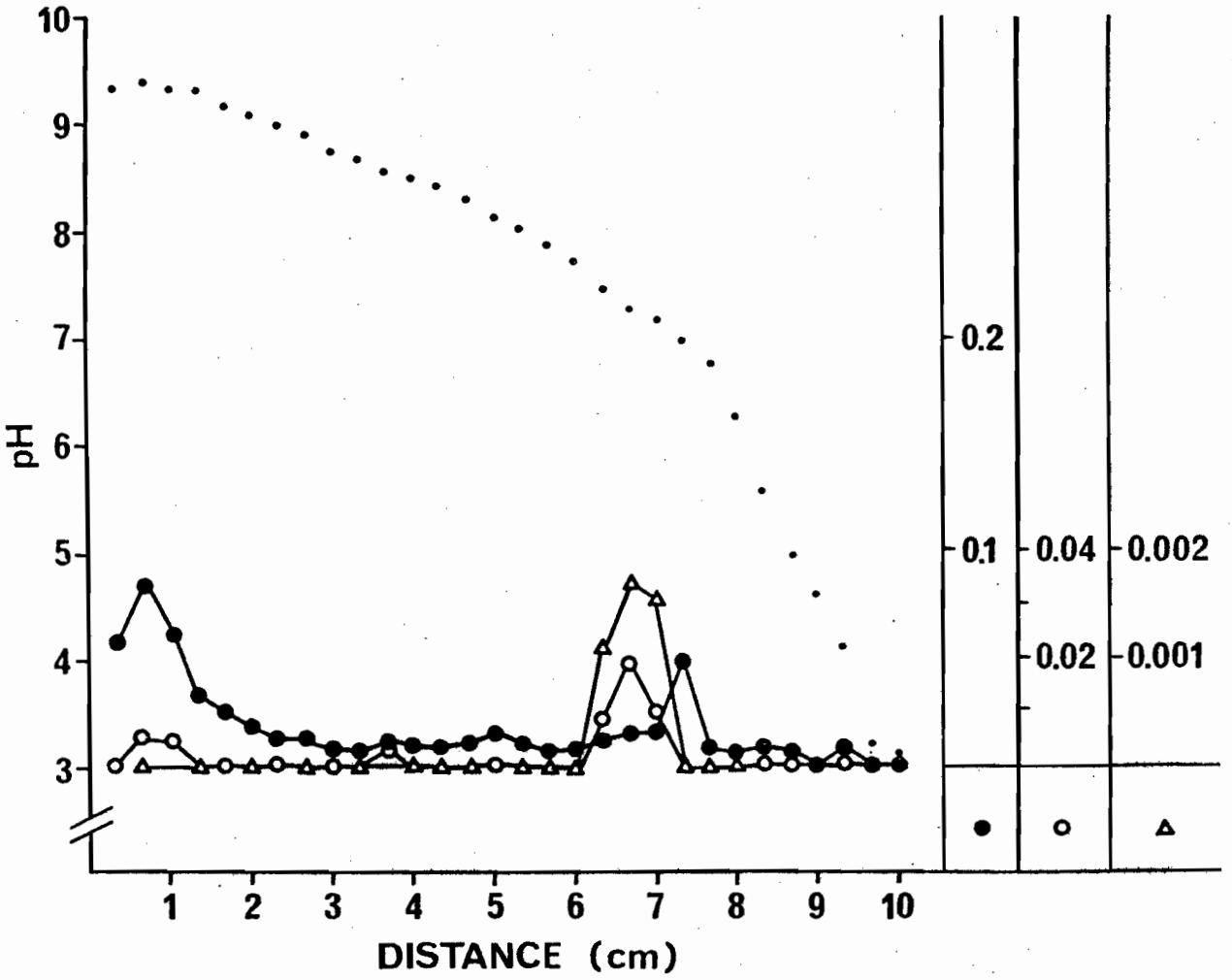
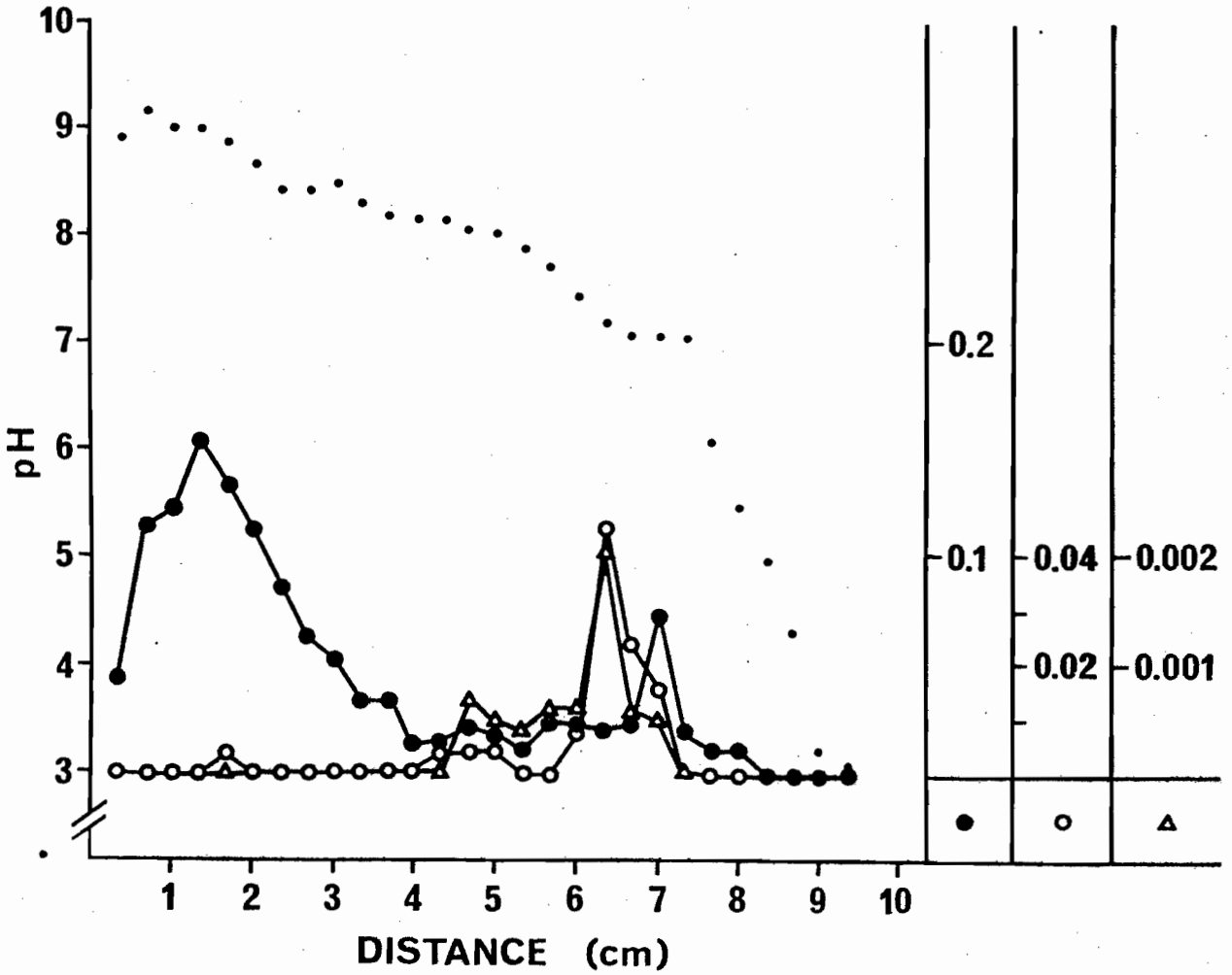

10
9
8
7
6
5
4
3
pH
0.2
0.1
0.04
0.002
0.02
0.001
1
2
3
4
5
6
7
8
9
10
DISTANCE (cm)

Figure 28. ELECTROFOCUSING PROFILE OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES FROM MALE RATS AGED 30 DAYS

Kidneys from three 30 day old male rats were pooled and homogenized. Cytosol (300 µl) prepared from this homogenate was electrofocused on acrylamide gels as described in the Methods sections. Gel sections (3mm) were eluted and assayed for glutathione S-transferase activity towards (•) CDNB, (o) NBC and (Δ) TPBO. Activities are expressed in µmoles min^{-1} per ml of eluate.


10
9
8
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3
pH
1
2
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4
5
6
7
8
9
10
DISTANCE (cm)
0.2
0.1
0.04
0.002
0.02
0.001

contained antigenically reactive but catalytically inactive forms of transferase, Ouchterlony immunodiffusion studies were carried out. As noted above, cytosol from 5-day-old rats exhibited approximately 10% of the adult activity towards CDNB. Adult renal cytosol diluted 1:10 reacted with the antibody prepared against purified hepatic transferase B (Figure 29) while cytosol prepared from the kidneys of 5-day-old rats gave an immunoprecipitin line against this antibody only when tested in undiluted form. When this cytosol was diluted 1:5 or 1:10 no precipitin line was visible. Therefore it is very unlikely that there are present significant amounts of antigenically reactive but catalytically inactive forms of the renal transferase K1. To test for this in the development of the other transferases requires specific antibodies directed against these transferases.

INDUCIBILITY OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES WITH DEVELOPMENT

Renal glutathione S-transferase activity toward CDNB was inducible by β-naphthoflavone in immature rats as early as the fifth day of life (Figure 30). As in the adult, only the transferase activity toward CDNB was increased. The transferase activities toward DCNB, TPBO and NBC were no different from those in control animals. The developmental patterns of those animals given corn oil injections were no different from those who had received no

Figure 29. OUCHTERLONY IMMUNODIFFUSION OF PEAK K1 FROM RATS OF VARIOUS AGES

Peaks K1 from rats aged 1, 5 and 42 days were tested by the method of Ouchterlony for the presence of antigenically reactive forms of transferase. The wells were filled as follows:

Centre well - 40 µl antiserum

1 - 25 µl K1 (diluted 1:10) from 1 day old rats

2 - 25 µl K1 (diluted 1:10) from 5 day old rats

3 - 25 µl K1 (diluted 1:10) from 42 day old rats

4 - 25 µl K1 (undiluted) from 1 day old rats

5 - 25 µl K1 (undiluted) from 5 day old rats

6 - 25 µl K1 (diluted 1:10) from 42 day old rats

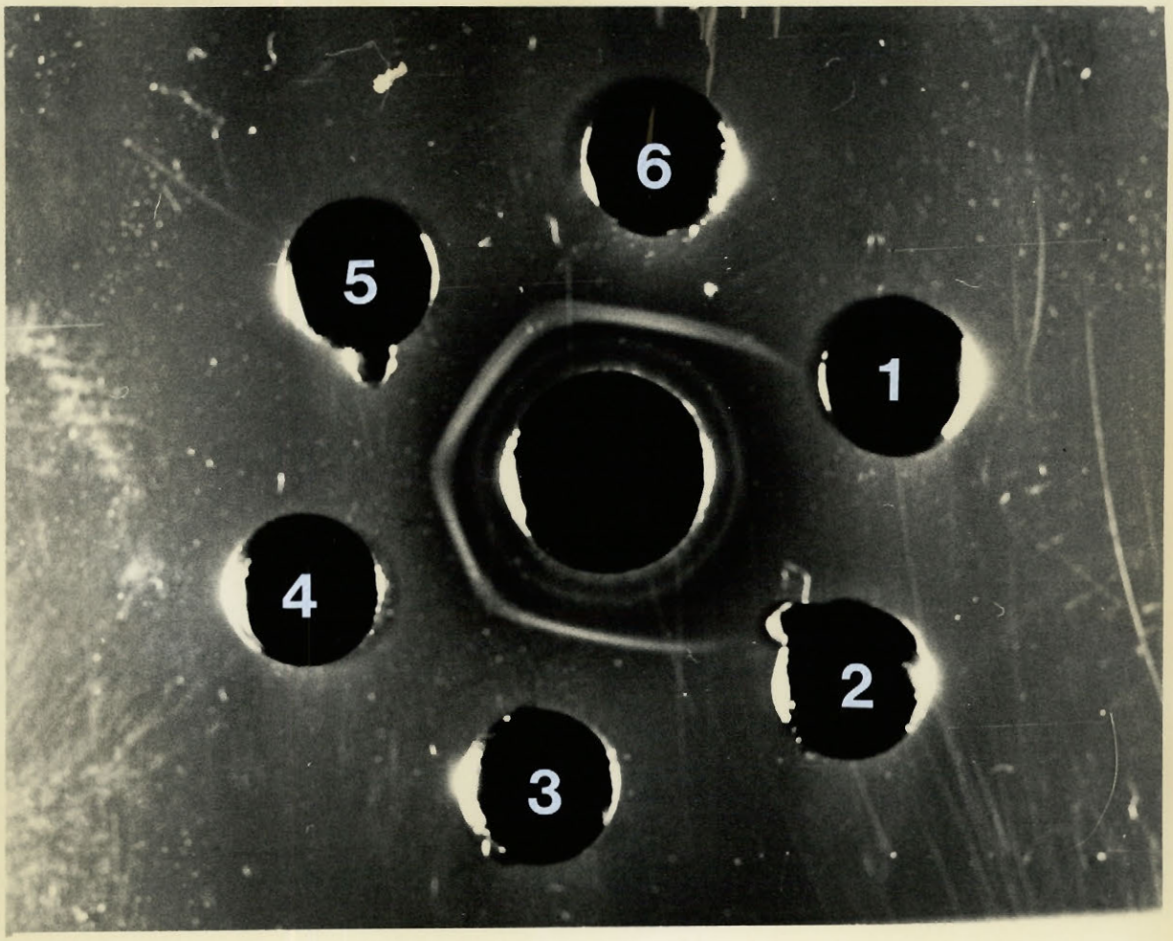
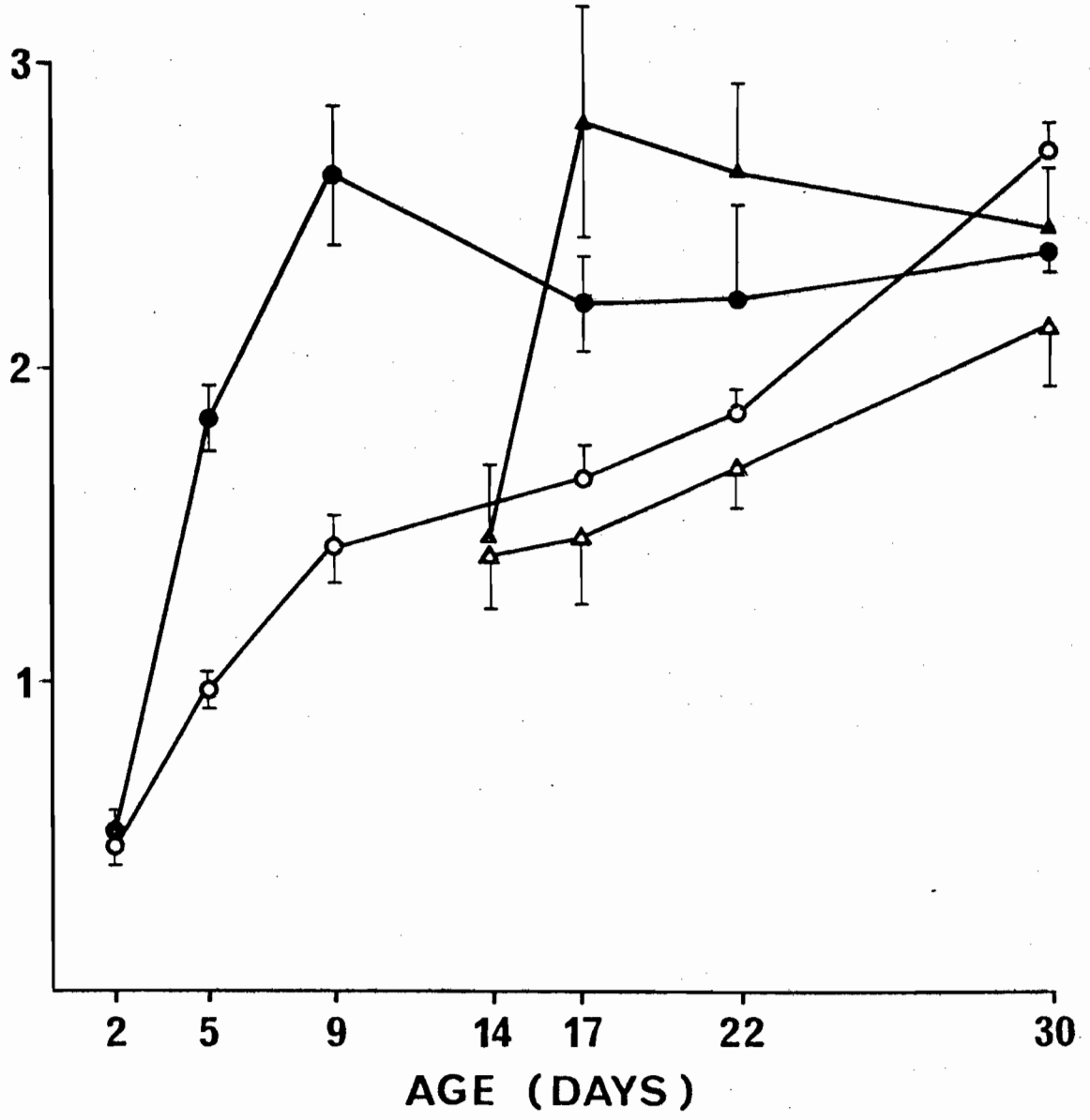

6
5
1
4
2
3

Figure 30. INDUCIBILITY OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITIES WITH DEVELOPMENT

Group I rats were injected with β-naphthoflavone in corn oil (●) or corn oil alone (o) on days 2, 3, 4. Group II rats were injected with β-naphthoflavone in corn oil (▲) or corn oil alone (△) on days 14, 15, 16. Rats were killed on the days indicated and their renal cytosols assayed for activity towards CDNB as described in the Methods section. Numbers on the vertical axis indicate activity towards CDNB in terms of μmoles min^{-1} per ml of cytosol.

Values are means ± S.E.M. for 4 groups of 2 animals.


3
2
1
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5
9
14
17
22
30
AGE (DAYS)

injection. In those animals (Group I) who had received β-naphthoflavone injections on days 2, 3 and 4 activity toward CDNB continued to increase relative to untreated animals at least until five days after the last injection. In adults and in those animals (Group II) injected on days 14, 15 and 16 activity towards CDNB had already started to return to control values by the fifth day after the last injection. The levels of activity towards CDNB had returned to control levels by age 30 days in both experimental groups of animals.

Table 12 shows the percent induction over corresponding corn oil controls for adult rats, immature pups injected in the first week of life (Group I) and immature animals injected in the third week of life (Group II). Transferase activity toward CDNB was increased by approximately the same amount (80 - 90%) in all three groups. As evident from Figure 30 activity towards CDNB remained elevated in Group I longer than in Group II. The time course of return to control levels after induction by β-naphthoflavone in Group II was essentially the same as in the adult. In these latter two groups the difference between control and experimental values had lost statistical significance by thirteen days after the last injection.

Table 12. TIME COURSE OF THE INDUCTION OF ACTIVITY TOWARDS CDNB IN IMMATURE AND ADULT RATS

	DAYS AFTER LAST INJECTION			
	1	5,6	13,14	18
Group I				
Day 2,3,4	89.6%	82.4%(5)	34.8%(13)	12%
Group II				
Day 14,15,16	90.9%	58.5%(6)	16.3%(14)	
Group III				
Adults	81.9%	48.9%(5)	18.3%(13)	

Activities are expressed as percent increase over corresponding corn oil injected controls and are the averages of four groups of 2 animals. Groups I and II received β-naphthoflavone 25 mg/Kg/day at the ages indicated. Group III received 40 mg/Kg/day for three consecutive days.

CHAPTER IV

DISCUSSION

GENERAL CONSIDERATIONS

In this thesis, the glutathione S-transferases of rat kidney were examined with respect to the forms present, their individual responses to inducing agents and their developmental patterns. Comparison to the more extensively studied and complex hepatic transferases was included where appropriate.

For some studies such as determination of the overall ability of a tissue to handle xenobiotics and/or detoxify active metabolites, measurement of total glutathione S-transferase catalytic activity of cytosol toward a given substrate is sufficient. Using such an approach, Pinkus et al. (1977) found that glutathione S-transferase activity toward CDNB is greatest in the duodenum and jejunum and lowest in the colon and stomach. The authors suggest that this finding may be related to the colon's sensitivity to neoplastic growth but many other factors must also be considered.

For studies of the mechanisms involved in the developmental, genetic and hormonal control of the transferases, it is advantageous to measure actual gene products and/or post-translational modifications thereof rather than the overall activity toward one or another substrate. It follows that quantitation of discrete proteins is desirable for such purposes. From the viewpoint of toxicology one may speculate that such an approach may even be more economical in the long run. For example, when it is known which glutathione S-transferases a tissue possesses, and how they are regulated, one need only determine for which transferases a novel chemical is a substrate in order to know which tissues do or do not have the ability to conjugate gluta-

thione to that chemical. There are of course a number of limitations to any simple approach attempting to predict in vivo events from in vitro data. These include the fact that even though a tissue may possess the transferases necessary for the conjugation of glutathione to a certain compound, such conjugation need not occur in vivo. This will depend in part on competitive pathways, compartmentalization, substrate (eg. GSH) availability, etc. Also, the number of transferases observed in vitro is to a certain extent a function of the number and type of substrates used experimentally. For example, in the kidney, if only CDNB had been used as a substrate only one major and one minor peak of transferase activity would have been noted. There definitely remains the possibility that there exist more transferases in the kidney than are being revealed by the substrates we and others have used.

A means of assessing and comparing the various forms of glutathione S-transferase that contribute to catalytic activity in rat liver cytosol has been developed in this laboratory using preparative electrofocusing on Sephadex G-75 (Hales et al., 1978). This technique served to distinguish five different transferase forms in hepatic cytosol of male rats. On the basis of pI and substrate specificity four of these correspond to glutathione S-transferases AA, B, A, and C described by Jakoby et al. (1976a) (see Table 13). The fifth hepatic peak may correspond to transferase M but its activity toward 1-menaphthyl sulfate has not been determined. The electrofocusing technique described herein is rapid and allows some quantitation of the relevant proteins. Electrofocusing had been used previously to

study the glutathione S-transferases (Clark et al., 1973) but data pertaining to only one substrate, CDNB, can be compared with our work. Their electrofocusing profile of hepatic transferase activity toward CDNB is in good agreement with the one presented above.

The hepatic transferases exhibit overlapping substrate specificity with respect to electrophilic substrates. Observed peaks of catalytic activity toward some substrates are so close that activity does not return to baseline between peaks. These factors make studies of the development and induction of individual transferases in liver difficult at best. Because of this complexity, we did not choose liver as the first tissue for use in studies of the regulation of the multiple forms of the glutathione S-transferases.

A simpler pattern of glutathione S-transferase activity peaks was obtained when renal cytosol was electrofocused. As in hepatic cytosol, five distinct forms of transferase were found upon electrofocusing on Sephadex G-75 but there was much less overlap of catalytic activity between adjacent peaks. For example, peaks K1 and K2m had similar pI's 9.0 $\pm$ 0.01 and 8.5 $\pm$ 0.01, but K1 was active towards CDNB with scarcely any activity toward NBC, while for K2m the opposite was true. Drug-metabolizing enzymes were once described as enzymes with incredibly broad substrate specificities. Work with the glutathione S-transferases and with other enzymes shows that even though whole cytosol may be active toward a large number of substrates, the substrate specificity of each enzyme (or form) may be relatively narrow. Kidney seems to be a good tissue in which to study the forms of glutathione S-transferase present and their response to various perturbations in order to contribute to the understanding of the

regulation of the various forms of such drug metabolizing systems.

GLUTATHIONE S-TRANSFERASES OF UNTREATED MALE RATS

When comparing the renal transferases with those found in liver a number of interesting points emerge. Rat renal transferases, here designated K1 and K3, correspond to hepatic transferases B and M, respectively, on the basis of pI and substrate specificity. The major transferases we observed in rat liver and kidney using electrofocusing are listed in Table 13 and, where possible, identified as previously described transferases. It should be noted that the terms 'major' and 'minor' refer only to the relative amount of activity toward a given substrate in each peak and do not say anything about the actual amount of transferase protein in the peaks or the biological importance of each protein. The notation, 'K1' or 'L1,' is not suggested as an alternate nomenclature to that proposed by Jakoby et al. but is used to distinguish the peaks found in this laboratory using electrofocusing from those described by Pabst et al., 1974. Transferase B, as ligandin, has previously been identified in and purified from rat kidney (Fleischner et al., 1976; Arias et al., 1976). Its concentration was estimated at between 22 and 31 µg/mg soluble protein as opposed to 45 µg/mg in the liver and 17 µg/mg in small intestine (Jakoby, 1978; Kirsch et al., 1975). Assuming a specific activity of 11 µmoles/min/mg protein for transferase B with CDNB as substrate (Jakoby et al., 1976) we estimate its concentration in kidney to be approximately 15 µg/mg soluble protein. We found no renal transferase activity corresponding to that of transferase E but the results are not considered definitive

Table 13. IDENTIFICATION OF RENAL AND HEPATIC GLUTATHIONE S-TRANSFERASES

	LIVER	KIDNEY
AA	L1	
B	L2	K1
A	L3	K2(?)
C	L4	
	L5(?)	
M		K3

due to the background noise of the ENPP assay in our hands. Bend et al. (1976) do state that renal cytosol is active toward benzo(a)-pyrene-4,5-oxide, having about the same specific activity as liver, and they emphasize that the kidney has the ability to detoxify epoxides. This does not specifically indicate the presence of transferase E since transferase E has relatively low activity toward benzo(a)pyrene-4,5-oxide (Nemoto et al., 1975). It is interesting that the two hepatic transferases (A and C) that have not been distinguishable from each other immunologically and which have very similar amino acid compositions (difference index 2.2) were both not detected in rat kidney. This perhaps may be taken as another indication of the close relationship between these two. The absence of transferases A and C does account for the low renal activity toward DCNB. We found a kidney to liver ratio of 1:27 for activity toward DCNB (versus 1:4 for CDNB). This result is intermediate between the ratio of 1:20 found by Booth et al. (1961) and that of 1:47.6 reported by Boyland and Chasseaud (1969b).

The multiplicity of the renal transferases and the fact that more than one protein may contribute to cytosolic transferase activity towards any one substrate may partially explain the conflicting results concerning the role of the renal glutathione S-transferases in organic anion transport. If, for example, only CDNB is used as an indicator of cytosolic transferase activity and peak K3 happened to be responsible for the transport of a particular organic anion, then a relationship between glutathione S-transferase activity and organic anion transport would be missed since K3 is active towards TPBO and

not CDNB.

The nature of the differences between the renal transferases is not clear. It is known that proteins may differ in many ways, including amino acid sequence, secondary or tertiary structure, subunit interactions, prosthetic groups, post-translational covalent modifications and partial proteolysis.

The transferases could differ in all or part of their primary sequence. The amino acid compositions of a number of the hepatic transferases have been determined and the difference indices (Metzger, 1968) ranged from 12 for transferases AA and C to 2.2 for transferases A and C (Habig et al., 1974b). The amino acid sequences of the glutathione S-transferases have not been reported. It is possible that even those peaks with the largest difference indices may share homologous sequences.

Proteins may differ in important respects besides amino acid sequence. For example, the microenvironment of the proteins inside the cell could affect the state of oxidation of the enzymes' sulfydryl groups and so alter tertiary structure. It is possible that, like the γ-glutamyl cyclotransferase of rat kidney, the heterogeneity of the renal glutathione S-transferase forms relates to the state of oxidation of sulfhydryl groups. We suspect that this may be the case for the transferase(s) with activity toward NBC. It is unlikely that the other renal transferases differ only with respect to the state of sulfhydryl group oxidation as they are quite stable, do not cross react immunologically and do not interconvert.

Selective addition of prosthetic groups or other secondary

covalent modifications may also cause heterogeneity of a family of enzymes. Reports concerning the glutathione S-transferases have not mentioned the presence of carbohydrate, lipid or other prosthetic groups (Jakoby, 1978). Phosphorylation or adenylation of amino acid side chains is important in regulation of activity of some proteins while deamidation and phosphorylation may influence secretion, uptake and degradation as well as subcellular localization. Immunological studies of the glutathione S-transferases suggest but do not prove that the differences between these proteins are due to more than presence or absence of a phosphoryl or amide residue.

One interesting possibility is that the transferases differ from each other because of proteolysis of a precursor peptide. An example of a mammalian enzyme partly regulated by proteolysis is fructose biphosphatase (Horecker, 1975). Available evidence neither supports nor denies that the multiplicity of the glutathione S-transferases is due to proteolysis, although there are indications with hepatic transferase B that the nonidentity of its subunits could be due to cleavage of a 3,000 mol. wt. fragment from one of the two (Bhargava et al., 1978). There is no evidence for the presence of a large precursor. If partial and selective proteolysis were contributing to heterogeneity here the fragments cleaved must be small since all of the renal transferases elute together from Sephadex G-100 and therefore have very similar molecular weights. The observed multiplicity is not likely to be an artifact of the action of proteolytic enzymes during the separation procedures, since addition of the common protease inhibitor, phenylmethanesulfonylfluoride, did not alter

results (Habig et al., 1974b).

SEX DIFFERENCES IN GLUTATHIONE S-TRANSFERASES OF KIDNEY

With the exception of glutathione S-transferases with activity toward NBC, sex differences in the renal glutathione S-transferases were not striking. The same five forms are found in kidney of both sexes. Male to female ratios of activities toward CDNB, DCNB, and TPBO were 1.37:1.00, 1.02:1.00, and 0.77:1.00, respectively. There was no sex difference in the isoelectric points of the transferase peaks with activity toward these three substrates. Clifton et al. (1975) likewise did not observe prominent sex differences in activities toward three substrates; the male to female activity ratios for ENPP, methyl iodide and DCNB were 1.52:1.00, 1.33:1.00, and 0.74:1.00, respectively. An exception was NBC, where the male to female activity ratio was 2.37:1.00. We observed a ratio of 0.95:1.00. The sex difference we observed in isoelectric points of the forms with activity toward NBC disappeared when cytosol was electrofocused on polyacrylamide gels rather than on Sephadex G-75. In addition, M. K. Johnson (1966) found that the activity of renal cytosol toward NBC was extremely variable from experiment to experiment and diminished rapidly at 2°C. We therefore refrain from drawing definite conclusions concerning a sex difference in activity toward this substrate.

Sex steroids may act as inducers or repressors of enzyme activity. Glutathione S-transferases are present in ovary and testis and are involved in the transport and metabolism of steroids in the liver (Smith et al., 1977; Ohl and Litwack, 1977). Sex

differences are known for the hepatic transferases: activity toward DCNB is lower in female rats (Darby and Grundy, 1972) while the proportion of transferase B is greater in males (Hales and Neims, 1976a). The sex differences in the renal transferases described herein are at most small but need not be unimportant. Differences in microsomal oxidative enzyme activities relative to sex in the rat are well known (Kuntzman et al., 1966). Sex differences in the ratio of oxidative to conjugative enzymes may account for the observed difference in susceptibility to certain toxic agents between males and females. This applies especially to an agent with a threshold for observable toxicity: a small change in steady state levels of active metabolites from just below threshold to just above threshold could cause a large change in observable toxicity.

DEVELOPMENT OF THE RENAL GLUTATHIONE S-TRANSFERASES

There exist both inter-species and inter-organ variations in the rate at which the glutathione S-transferases and the other enzymes involved in drug metabolism mature. Even the rates at which different forms of the "same" enzyme system mature in one tissue may vary. Since many metabolic pathways are parallel and competitive, there may be differences in the maturational pattern of the metabolism of a particular compound within any one organ. For example, the specific activities of glutathione S-epoxide transferase in extrahepatic organs of neonatal guinea pig and rabbit are relatively higher than those of epoxide hydrase. In such organs detoxification by glutathione S-epoxide transferase may be more important than by epoxide hydrase

in the perinatal period despite the fact that the reverse is probably applicable in the adult (James et al., 1977). Therefore, it is clear that the significance of a given metabolic pathway in toxification/detoxification may vary as a function of age.

The renal glutathione S-transferase activities towards all four substrates used in this study were measurable before birth. On fetal day 20 of a 21-day gestation, the percent adult glutathione S-transferase activity toward each substrate in renal cytosol was as follows: CDNB 10%, NBC 16%, DCNB 22% and TPBO 24%. No large increase in enzyme activity was observed immediately following birth. These data are in reasonable agreement with previous studies in which it was observed that at birth renal ligandin comprised less than 2 µg/mg soluble protein (Kirsch et al., 1975) and that renal glutathione "S-epoxide" activity in neonatal rats was less than 10% of adult values (James and Pheasant, 1978). The possibility always exists that an enzyme may be present in forms unique to the fetus or newborn. An obvious example is fetal hemoglobin in which one of the two types of chains present, designated γ, is structurally distinct from those usually found in the adult (Schroeder et al., 1963).

In agreement with James et al. (1977), we found that all glutathione S-transferase activities measured in renal cytosol approximated adult levels by day 30. In addition, we found that the time course of development to adult level differed for the activity toward each substrate. The activities toward TPBO and NBC (see Figure 26) follow a pattern similar to that described by Greengard (1971) for enzymes belonging to the late suckling cluster. Table 14 depicts

Table 14. DEVELOPMENT OF RENAL GLUTATHIONE S-TRANSFERASE ACTIVITY TOWARD CDNB WITH RESPECT TO THAT TOWARDS NBC, TPBO and DCNB

	AGE (DAYS)					
RATIO	1	5	10	16	22	30
CDNB/NBC	3.06	3.17	4.69	2.89	1.47	1.17
CDNB/TPBO	95.85	183.80	212.50	309.00	319.00	225.83
CDNB/DCNB	69.58	83.090	74.37	130.77	164.29	117.83

the ratios of activity toward CDNB to activities toward each of NBC, TPBO and DCNB on days 1, 5, 10, 16, 22 and 30 postnatal in order to give indication of how the transferases are changing in relation to each other. For example, activity toward CDNB matures faster than that towards NBC until around day 10 at which point the latter starts to catch up. From day 22 onwards, the ratio of these two activities remains fairly constant. These results reveal that the transferases are individually regulated at least in part but do not establish total independence or the absence of feedback control of one transferase activity on another.

It is becoming quite clear that the maturational patterns of the different enzyme systems involved in xenobiotic metabolism are different. This implies that from day to day during maturation the ratio of one set of enzymes, products and intermediates to another may change. The uneven maturation of the individual renal transferases may have evolved in response to developmental variations in the type of compounds presented to the kidney for excretion, although speculation ascribing a cause-effect relationship is largely without support.

INDUCTION OF RENAL GLUTATHIONE S-TRANSFERASES IN IMMATURE RATS

Organisms are exposed to xenobiotics from the time of conception. The enzyme systems of neonates do not necessarily respond to potential inducers in the same way as do those of the adult. It is interesting in the context of the issues of this thesis to know whether the renal glutathione S-transferases are inducible in immature rats and, if so, does one see the same pattern

of response as in the adult. Renal glutathione S-transferase activity toward CDNB was induced by β-napthoflavone as early as day 5 postnatal. As in mature rats, only glutathione S-transferase activity toward CDNB was induced. The percent but not the absolute induction relative to vehicle-treated controls was approximately the same for adult rats, pups injected in the first week of life (Group I) and pups injected in the third week of life (Group II). In a similar experiment Hales and Neims (1977) found that phenobarbital increased hepatic transferase activity toward CDNB and DCNB in pups and adults to the same percent of basal levels. The fact that the transferases are induced by a certain percentage of basal levels and not by a fixed increment presents an interpretative challenge relative to the possible mechanisms of induction.

Once induced, glutathione S-transferase activities of Group I and Group II rats did not return to the levels existing at the time of initiation of treatment with β-napthoflavone. Rather, the glutathione S-transferase levels in kidneys of both groups of rats came to coincide with the levels in vehicle treated controls by the age of 30 days postnatal. The increase and decrease in glutathione S-transferase levels in Group II animals were similar to the pattern seen in adults in that transferase levels peaked within three days of the initiation of treatment and thereafter decreased to come to coincide with the vehicle treated controls. In Group I rats, on the other hand, renal transferase activity seemed to follow a distinctive developmental curve until day 30 when it also coincided with that of the vehicle treated control. Why transferase activity

in Group I was slower to peak and slower to return to the normal developmental curve is not known. It may be because β-napthoflavone is eliminated very slowly in such immature rats or it may be that the transferases of immature rats have longer half-lives than those of adults. Another possibility is that the level of activity recognized by the cell as "correct" for a particular age has been reset, directly or indirectly, by the action of the inducer. Few reports are available in which both the increase and decrease of drug metabolizing enzymes have been followed after treating a developing animal with a potential inducer; the pattern exhibited by hepatic nitroreductase upon treatment of immature rats with phenobarbital resembled closely that described herein for glutathione S-transferase in the Group II rats (Müller et al., 1971).

INDUCTION OF RENAL GLUTATHIONE S-TRANSFERASES IN ADULT RATS

Phenobarbital increased hepatic glutathione S-transferase B in parallel with other hepatic glutathione S-transferases with activity toward CDNB (Hales and Neims, 1977; Kaplowitz et al., 1975; Klaasen and Plaa, 1968) but had no effect on extrahepatic glutathione S-epoxide transferase (Bresnick et al., 1976). Kluwe et al., (1978) found that neither phenobarbital, penicillin nor 3-methyl-cholanthrene increased renal glutathione S-transferase activity toward CDNB in adult rabbits.

Pretreatment with phenobarbital had no effect on renal glutathione S-transferase activities towards any of the four substrates we tested. Our results are in contrast to those of Clifton et al. (1975) who found that parenterally administered phenobarbital caused

a selective 50% increase in renal transferase activity toward NBC. These results are difficult to interpret considering the instability of activity toward this substrate since the value for induced activity reported by these authors is less than our value for control renal cytosol. Recent reports from the same laboratory state that orally administered phenobarbital exerted no inductive effect on the renal transferases (Clifton and Kaplowitz, 1978).

Earlier investigations into the effects of ethanol on the hepatic glutathione S-transferases indicated that classical 'aryl,' 'aralkyl' and 'epoxide' transferase activities were induced but that glutathione S-transferase B was not induced (Joly et al., 1977). We found that renal glutathione S-transferases of female rats were not induced by ethanol. This raises the possibility that some metabolite of ethanol produced in liver but not in kidney causes the induction. Another possibility is that some receptor necessary for transferase induction by ethanol and/or phenobarbital is absent from the kidney.

β-Napthoflavone and Aroclor 1254, a commercially available mixture of polychlorinated biphenyls, caused a marked induction of renal glutathione S-transferase activity toward CDNB in our study. Treatment of rats with β-napthoflavone caused a 91% induction of overall cytosol activity toward CDNB while treatment with Aroclor 1254 resulted in a 74% increase in such activity. Electrofocusing of renal cytosol from rats treated with β-napthoflavone showed that the increased cytosolic activity towards CDNB seemed to be due to an induction of only peak K1. In rats treated with Aroclor 1254, not only peak K1 but also the second ("minor") transferase peak with activity

toward CDNB (pI, 6.7) was found to be induced. Measurement of overall cytosolic activity alone had not served to distinguish the inductive effects of these two agents.

The inductive effect of a polychlorinated biphenyl mixture on liver "glutathione S-epoxide transferase" activity had been described earlier (Parkki et al., 1977). Aroclor 1254 contains both high and low chlorinated analogs as well as a number of contaminants. Some of these contaminants may be partly responsible for Aroclor's inductive effect. In one study, commercially available biphenyl nearly trebled hepatic bromosulfophthalein conjugating activity while purified biphenyl less than doubled it. Stimulation of bromosulfophthalein-glutathione conjugating enzyme activity seems to be more closely linked to the biphenyl nucleus than to the position and number of chlorine substituents (Ecobichon and Comeau, 1975). The purpose of our study was not to examine structural features of the inducing agents per se but rather to use these agents as tools to gain information about the regulation of the renal glutathione S-transferases. We have not pursued investigations into the causative inducing agent in the Aroclor 1254 mixture.

The potential inducing agents used in this study also act to induce other drug-metabolizing enzymes. β-Napthoflavone and polychlorinated biphenyls are known inducers of the microsomal mixed function oxidase system both in liver and kidney (Gillette, 1971; Boobis et al., 1977; Alvares et al., 1973). Epoxide hydrase in mice is also induced by these xenobiotics (Oesch et al., 1972). Jakobsson et al., (1970) report that phenobarbital has no measurable effects either on the amount

of CO-binding hemoprotein and drug hydroxylases in kidney or on the cytochrome P-454 level and ω-oxidation activity of the kidney cortex microsomes. The differential response of the sets of drug-metabolizing enzymes in individual organs is one of many factors determining selective liability of organs to toxicity.

The mechanism of induction of the glutathione S-transferases is not known. In phenobarbital treated rats, induction of hepatic transferases appears to be due to an increase in the rate of mRNA translation rather than to an increase in the amount of mRNA present (Daniel et al., 1977). Studies of this issue have not been reported for other tissues or transferases. Kaplowitz and Clifton (1976b) state that induced activities are characterized by increased Vmax with no change in Km but since these experiments were performed on cytosol and therefore on mixtures of at least five enzymes, such statements are difficult to interpret.

CONCLUSIONS

The questions of 'How well does the whole i.e. cytosol give us information concerning its constituent parts?' was posed in the Introduction. In kidney where transferases with relatively narrow substrate specificities have been successfully separated, the answer seems to be, 'Not very well.'

The work presented in this thesis emphasizes the advantages surrounding the study of the individual members of a family of drug-metabolizing enzymes as well as overall activity toward a given substrate. This derives from the fact that the glutathione S-transferases have overlapping substrate specificities. For example

there appear to be at least two unstable forms of renal transferase with activity toward NBC and two forms with activity toward CDNB. Measurements of the activities in cytosol cannot reveal the percent contribution of the individual proteins to overall activity. In addition, it was shown that the renal transferases are individually regulated with respect to both their developmental patterns and responses to inducing agents. Again, measurements of the activities of cytosol toward any few substrates cannot adequately describe the individual responses of the proteins or predict activity toward another potential substrate.

In order to further pursue the problem of the nature of the multiplicity of drug-metabolizing enzymes, specifically of the renal glutathione S-transferases, it seems requisite to purify the major transferases of kidney K1, K3 and K2m/K2f. The problem of the instability and interconversion of K2m and K2f must be solved before purification is attempted. Preliminary experiments indicate that this is possible using ion exchange chromatography on DE-52. The same number and pattern of transferase peaks are obtained using this technique as with electrofocusing and the recovery of activity towards NBC is much greater.

Although this thesis deals specifically with multiple forms of the glutathione S-transferases, the principle of the necessity to examine individual proteins applies to the cytochromes P-450 and other enzymes of drug metabolism. We do not yet understand the details of how these different enzyme systems relate to each other or whether they are in any way coupled, whether with respect to cell location, function

or regulation. Such coupling is not unreasonable considering that enzyme systems such as the cytochromes P-450 and glutathione S-transferases are known to function in sequence in the metabolism of some xenobiotics. Part of the difficulty lies in the fact that the test substrates used are artificial and would not necessarily reveal an existing relationship between different sets of enzymes. With purified proteins it should be possible to determine not only the degree of homology between the individual glutathione S-transferases but also to begin exploring the relationship between the individual members of the glutathione S-transferase family and those of other drug-metabolizing enzyme systems such as the cytochromes P-450.

CHAPTER V

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Isoelectrofocusing on Sephadex G-75 and on polyacrylamide gels can be used as a tool to study the multiple forms of the glutathione S-transferases. The technique is rapid and allows reasonable quantitation with most substrates used. The substrate specificities and the pI's of the hepatic transferase peaks recovered correspond very well to those found by Jakoby et al. using CM-cellulose chromatography. In addition, the technique revealed the presence of previously undescribed glutathione S-transferases.
2. Both renal and hepatic cytosols from rats contain multiple forms of the glutathione S-transferases. Five forms of hepatic and five forms of renal glutathione S-transferase were revealed with the electrofocusing technique. Glutathione S-transferases B and M are present in the kidney but glutathione S-transferases A and C appear to be absent. With the exception of the two forms of transferase with activity toward NBC, the renal transferases are stable and do not interconvert.
3. In general, the renal glutathione S-transferases exhibit less overlapping substrate specificity than do the hepatic transferases. They are therefore more suitable for many studies of the glutathione S-transferases including those of induction, development and genetic and hormonal control.
4. Male and female rats possess the same spectrum of renal glutathione S-transferases. However, the contribution of some transferases to overall catalytic activity is different for males and females. In males, most of the glutathione S-trans-

ferase activity toward NBC is found in a peak which on Sephadex G-75 focuses with a pI 8.5 while in females the peak with most such activity focuses with pI 7.1.

5. Of the four agents tested, only β-naphthoflavone and Aroclor 1254 increase renal glutathione S-transferase activity and that only toward CDNB. β-naphthoflavone induced only glutathione S-transferase B while Aroclor 1254 stimulated transferase B plus a second more acidic transferase also with activity toward CDNB. Generally then, the renal glutathione S-transferases respond selectively to different inducing agents. This shows that they are individually but not necessarily independently controlled.

6. At day 1 post-natal all renal glutathione S-transferase activities are less than 25% of adult levels and all such activities have approximated adult level by day 30. However, the course of development to that level is very different for the different glutathione S-transferase activities: activity towards CDNB increases gradually and continually while that towards TPBO and NBC remains relatively constant for about two weeks after birth and then increases sharply. The developmental studies confirm that the renal glutathione S-transferases are under individual control.

7. Renal glutathione S-transferase activity is inducible by β-naphthoflavone at least as early as the first week of life. Percent induction over corresponding corn oil control is approximately the same for adults as for immatures. Renal glutathione S-transferase activity toward CDNB, of animals treated with

β-naphthoflavone in the first week of life, does not immediately return to control values. Rather, the glutathione S-transferase activity follows its own 'induced developmental pattern' until adult values are reached.

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