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A STUDY OF GLUTATHIONE S-ARYLTRANSFERASE FROM *Costelytra zealandica*

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TO MARGARET

and MY PARENTS

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ABSTRACT

An investigation has been made of the stability, purification and properties of Glutathione *S*-aryltransferase (EC 2.5.1.13) from the grass-grub, *Costelytra zealandica*.

The enzyme was found to be extremely unstable in crude homogenates of grass-grubs that had been stored frozen at -20°C, but was considerably more stable in homogenates of live grass-grubs. The instability increased with increase of pH. Glutathione gave some protection against inactivation.

Selective fractionation of crude homogenates with $(NH_4)_2SO_4$ provided some evidence for the presence of an endogenous inhibitor of the enzyme.

DEAE-cellulose chromatography and isoelectric focusing studies showed the presence of two major GSH *S*-aryltransferases with isoelectric points of 4.6 and 8.7. Both enzymes were present in the homogenate from a single, live, grass-grub. The molecular weight and optimum pH of each enzyme was identical within experimental error.

A brief comparative study of GSH *S*-transferases showed the presence of GSH *S*-alkyl- and GSH *S*-alkene-transferase, but in only very small amounts compared with GSH *S*-aryltransferase. Differences in stability were demonstrated and some cross-specificity was indicated.

Several inhibitor-substituted Sepharoses were prepared in an attempt to purify GSH *S*-aryltransferase by affinity chromatography. Although columns of the inhibitors removed the enzyme from solution an active enzyme could not be recovered.

The effects of pH and temperature on the enzyme-catalysed reaction of GSH and 1,2-dichloro-4-nitrobenzene (DCNB) were investigated in detail. Analysis of the variation of pK_{GSH} with pH showed the presence of active site groups with p*K* approximately 9 involved in GSH binding. Calculation

of the heat of ionization of these groups in the pI 8.7 enzyme, from the effect of temperature on their pK, suggested that the groups may be Lysine ε-NH_2. Values for the enthalpy, free energy and entropy of GSH-binding to the pI 8.7 enzyme and of DCNB-binding to the enzyme-GSH complex were also obtained.

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INTRODUCTION

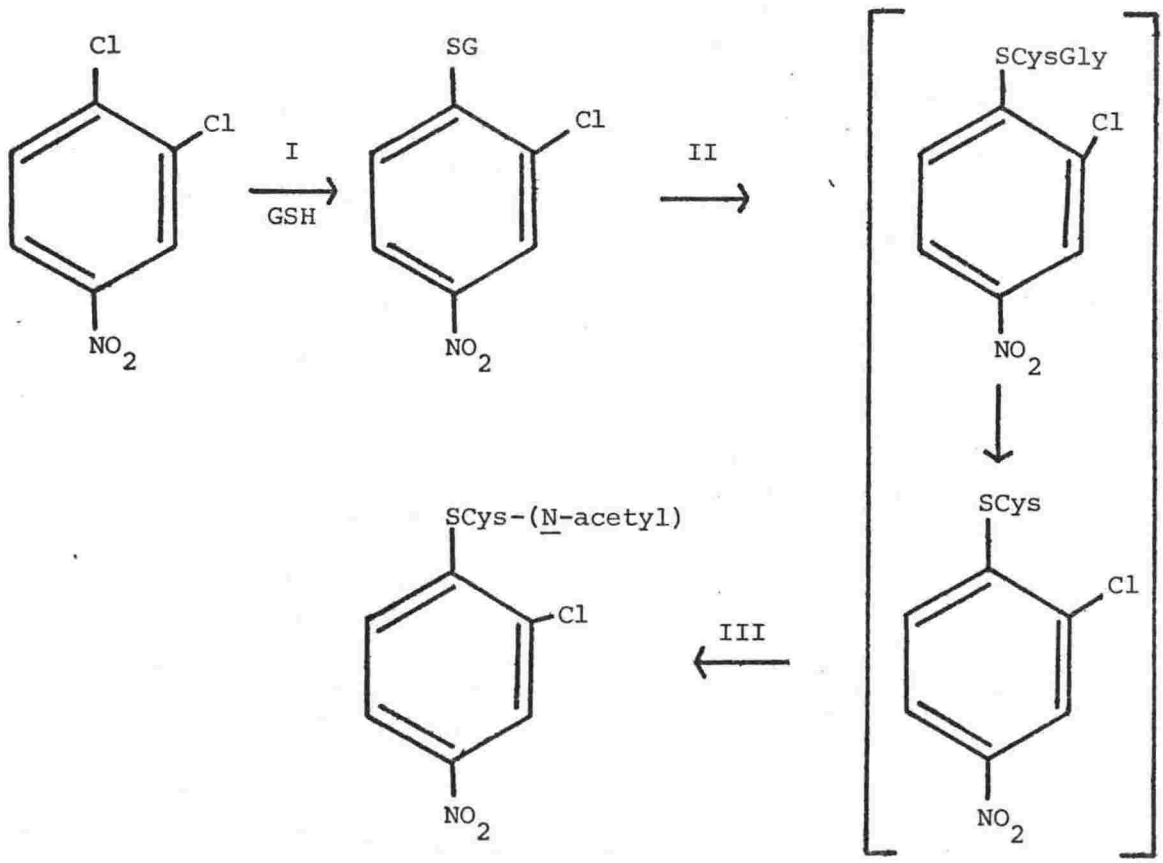
Glutathione S-aryltransferase catalyses the reaction of the endogenous tripeptide glutathione (GSH) with aromatic compounds containing labile halogen atoms or nitro groups (Boyland & Chasseaud, 1969a). The enzyme is one of a group of widely distributed GSH S-transferases involved in the detoxication of non-polar xenobiotic compounds. The conjugation is the first step in the excretion of the xenobiotic compound as a mercapturic acid, i.e. N-acetyl-S-substituted cysteine.

Formation of Mercapturic Acids

The immediate source of the cysteine required for conjugation with the mercapturic acid precursor was not established until many years after the first detection of mercapturic acid formation in mammals. Conjugation with tissue protein (Stekol, 1939; Smith et al, 1950), cysteine (Gutmann & Wood, 1950) or cystine (Marsden & Young, 1958) were favoured initially but were shown to be the immediate source of only a small fraction of the cysteine used. Barnes & James (1957) showed that administration of cysteine, cystine or methionine did not augment mercapturic acid synthesis in the rabbit. Indirect evidence for the participation of GSH in the conjugation was provided by Stekol (1940) who showed that rats excreted S-benzyl glutathione as benzyl mercapturic acid, and that p-bromobenzyl mercapturic acid was formed from p-bromobenzyl bromide, S-p-bromobenzyl glutathione and S-p-bromobenzyl cysteine (Stekol, 1941). A fall of the liver GSH level occurred when rabbits and rats were dosed with mercapturic acid precursors

(Nakashima, 1934; Barnes & James, 1957; Barnes et al, 1959), the fall being roughly equivalent to the amount of mercapturic acid formed (Barnes et al, 1959). The fall was not accompanied by formation of GSSG and was assumed to be the result of conjugation of GSH or of a secondary effect following conjugation of cysteine with the precursor (Barnes et al, 1959).

The complete synthesis of a mercapturic acid was demonstrated by Booth et al (1961) using 1,2-dichloro-4-nitrobenzene (DCNB), which was known to be excreted by rabbits as the mercapturic acid (Bray et al, 1957). The reaction was shown to occur in three stages:-



The first reaction, displacement of the activated para-Cl atom by GSH, was catalysed by the soluble fraction of rat liver. The second stage, conversion of S-(2-chloro-4-nitrophenyl)glutathione to S-(2-chloro-4-nitrophenyl)cysteine, catalysed by kidney homogenate, was probably due to the two enzymes which catalyse hydrolysis of GSH to its constituent amino acids (Olson & Binkley, 1950). The acetylation was demonstrated using liver slices.

The first two stages have been demonstrated in the locust (Schistocerca gregaria) with several mercapturic acid precursors (Cohen & Smith, 1964).

Many organic compounds showing great diversity of structure (Table I) are detoxified by one or more of the steps outlined for mercapturic acid formation with DCNB (for recent reviews see Boyland & Chasseaud, 1969a; Wood, 1970). However, with many of the compounds this is not the primary method of detoxication, e.g. in rabbits dosed with halobenzenes the ratio oxidation/mercapturic acid formation was 3/1, 20% of the dose being converted to p-halophenylmercapturic acid (Spencer & Williams, 1950). The main metabolite of chlorobenzene was 4-chlorocatechol, excreted as 4-chloro-2-hydroxyphenyl glucuronide; 3,4-dihydro-3,4-dihydroxychlorobenzene was also formed (Smith et al, 1950).

The only mercapturic acid precursors produced in normal vertebrate metabolism are the oestrogen derivatives. Kuss (1967) demonstrated the conjugation of GSH with 2-hydroxyoestradiol in rat liver homogenates. The product, (2-hydroxyoestradiol-1-yl)glutathione, was converted to the cysteine derivative by rat kidney microsomes then acetylated by liver or kidney homogenates fortified with acetyl-CoA (Elce, 1970).

Table 1[a] MERCAPTURIC ACID PRECURSORS

Precursor	Example	Group reacting with thiol
Aromatic hydrocarbon	Naphthalene	Epoxide intermediate
Arylamine	Aniline	Hydroxylamine intermediate
Arylhalide	Halobenzenes	Epoxide intermediate
Halogenonitrobenzene	1,2-Dichloro-4-nitrobenzene	1-Cl
	Pentachloronitro-benzene	NO_2
Aralkyl halide	Benzyl chloride	Cl
Aralkyl ester	1-Menaphthyl acetate	$O.CO.CH_3$
Alkyl phenol	3,5-Di-tert-butyl-4-hydroxy-toluene	H of ring methyl
Alkylhalide	Iodomethane	I
Nitroalkane	1-Nitropropane	NO_2
Cycloalkene	Cyclopentene	Epoxide intermediate
Halogenocycloalkane	Bromocyclohexane	Br
Ester	Ethyl methane-sulphonate	$O_3S.CH_3$
	Urethane	$O.CO.NH_2$
Sulphonamide	Benzothiazole-2-sulphonamide	$SO_2.NH_2$
αβ-Unsaturated compound	Crotonaldehyde[b]	αβ-Double bond
	Ethacrynic acid	αβ-Double bond
	Arecoline[c]	αβ-Double bond
	Maleic (or fumaric) acid	αβ-Double bond
s-Triazine	2-Chloro-4-ethyl amino-6-isopropyl amino-s-triazine[d]	Cl
Oestrogen	2-Hydroxyoestradiol[e]	H of A-ring

a: Adaptation of Table IV from Boyland & Chasseaud, (1969a).
b: Gray & Barnsley, (1971).
c: reaction probably non-enzymic.
d: Lamoureux et al, (1970).
e: Kuss, (1967); Elce, (1970).

Jellinck *et al* (1967) showed *in vitro* formation by rat liver fractions of an oestradiol-glutathione conjugate, and Nambara & Numazawa (1971) characterized a similar conjugate in rat urine after large dosage of 3-deoxyoestrone. Elce (1971) showed that horse-radish peroxidase catalysed conjugation of GSH and oestradiol.

Quinones are important naturally occurring αβ-unsaturated compounds, and although some quinones react with GSH (Mason, 1955) and there is evidence that reaction of GSH with menadione is catalysed by rat liver preparations (Chasseaud, 1967), Bray & Garrett (1961) found no mercapturic acid excreted after administration of menadione and related quinones to animals.

Grover (1965) and Johnson (1966) have shown that thyroxine and related compounds are not substrates for GSH *S*-transferases.

It is possible that several naturally occurring insect juvenile hormones may be substrates for GSH *S*-transferases. Two such hormones which have been isolated from the adult cecropia moth are sesquiterpenoid αβ-unsaturated methyl esters with an epoxy group at the penultimate position of the chain (for a review on juvenile hormones see Bowers, 1971).

The best substrates for the GSH *S*-transferases also have the fastest non-enzymic reactions with GSH. The non-enzymic reactions are usually favoured by high pH, which suggests nucleophilic attack by the $GS^{\ominus}$ ion on the second substrate. In neutral solution the thiol group is almost completely unionized (Waley, 1966) and the enzymes may act in part by lowering the p*K* of this group. However, Webb (1966) has suggested that reaction with double bonds may occur more readily when the thiol group is unionized and this is supported by Boyland & Nery (1969) who

showed the non-enzymic reaction of arecoline and GSH to proceed optimally at neutral pH.

Glutathione-dependent Enzymes

Several enzymes are known for which GSH is a cofactor, e.g. the glyoxalase complex, formaldehyde dehydrogenase, maleylacetoacetate isomerase, DDT dehydrochlorinase. With the exception of DDT dehydrochlorinase, which resembles the GSH S-transferases in its inhibition by various organic dyes (Balabaskaran & Smith, 1970), these enzymes will not be considered further.

Several GSH S-transferases have been identified and appear to be closely related in their binding of GSH, but differ in specificity towards the second substrate.

Glutathione S-aryltransferase (EC 2.5.1.13)

The substrates for this enzyme have been defined (Boyland & Chasseaud, 1969a) as aromatic compounds containing labile-halogen atoms, e.g. 1,2-dichloro-4-nitrobenzene (Booth et al, 1961), or -nitro groups, e.g. 1,2,3,4,5-pentachloro-6-nitrobenzene (Betts et al, 1955). The reactivity of a particular centre on the aromatic ring is governed by electronic and steric effects exerted by neighbouring groups. It seems that the nitro group is lost when flanked by halogen atoms and there is another halogen atom in the ring; in other cases the halogen atom ortho to the nitro group is replaced, e.g. 1,3-dichloro-4-nitrobenzene, but para-replacement occurs when an ortho group is absent, e.g. 1,2-dichloro-4-nitrobenzene (Boyland & Chasseaud, 1969a).

Booth et al (1961) partially purified an enzyme from rat liver soluble fraction which catalysed reaction between GSH and DCNB. The

preparation was specific for GSH; GSSG, cysteine or N-acetylcysteine were not substrates. Several mercapturic acid precursors could replace the dichloronitrobenzene and it has become apparent since that the preparation contained other GSH S-transferases. Small amounts of activity were found in the nuclear, mitochondrial and microsomal fractions of liver, and in the soluble fraction of other tissues. The liver/kidney activity ratio was about 20/1. Grover & Sims (1964) showed wide variation in specific activity of the enzyme from a large number of vertebrate species. In general, livers of the rodents and carnivores examined had the highest activities whereas those of birds, fish and amphibia were low. The distribution of the enzyme in different species was generally similar to that of other enzymes concerned with the metabolism and renal excretion of foreign compounds.

GSH S-aryltransferase activity has been demonstrated in a wide range of insect species (Cohen & Smith, 1964; Cohen et al, 1964; Balabaskaran, 1972), a non-insect arthropod (Cohen et al, 1964), and the snail (Helix aspersa), peripatus (Peripatoides novaezealandiae), earthworm (Lumbricus rubellus)and planarian (Dugesia genus) (Balabaskaran, 1972). In the locust (Schistocerca gregaria) the highest activity was found in the fat body, malpighian tubes and gut, with small amounts in other tissues.

Bromosulphonphthalein (BSP) forms a conjugate with GSH by displacement of one $Br^{\ominus}$ ion and is probably a substrate for vertebrate liver GSH S-aryltransferase (Coombes & Stakelum, 1961). An enzyme catalysing this reaction has been purified 100-fold from rat liver by a combination of ammonium sulphate fractionation, gel filtration and DEAE-cellulose ion exchange chromatography (Troxler et al, 1973). The

purified preparation also accepted ethyl methanesulphonate as a substrate; measurements were not made with the usual aryltransferase substrates.

Al-Kassab et al (1963) demonstrated the reaction of 4-nitropyridine-N-oxide and 4-nitroquinoline-N-oxide with GSH in rat liver soluble fraction; one $NO_2^{\ominus}$ ion was released per GSH molecule conjugated. Comparison of the rates of heat inactivation and activity ratios of the soluble fraction and the ammonium sulphate-purified fraction suggested that the same enzyme catalysed reaction of GSH with DCNB and 4-nitropyridine-N-oxide. The affinity of the enzyme for 4-nitropyridine-N-oxide and 1,2,3,5-tetrachloro-6-nitrobenzene was low and the authors suggested that the aromatic substrate did not bind to the enzyme-GSH complex (Theorell-Chance mechanism; Theorell & Chance, 1951).

Boyland & Speyer (1970) demonstrated enzyme-catalysed reaction between GSH and several 2-substituted 5-nitrofuran derivatives using rat- and human-liver soluble fraction; the nitro group was released as NO_2^-. A ten-fold purification in specific activity of GSH S-aryltransferase gave a similar increase in specific activity with the nitrofuran derivatives.

Frear & Swanson (1970) have partially purified a GSH S-transferase from corn leaves which catalyses reaction of substituted 2-chloro-s-triazines with GSH; one mole $Cl^{\ominus}$ is released per mole GSH consumed. Specificity for GSH was demonstrated. The enzyme was present in leaf tissues of other s-triazine-tolerant plants but not in leaves from susceptible species. Very little activity was found in the root tissues. Enzyme activity was inhibited by 2-amino-4-nitrophenol, DCNB and BSP.

Hollingworth et al (1973) have identified and assayed an enzyme in vertebrate liver that converts parathion (O,O-diethyl-O-p-nitrophenyl-phosphorothionate) and related insecticidal organophosphate triesters to

S-p-nitrophenylglutathione and the corresponding dialkyl phosphorothioic or phosphoric acids; the enzyme has been named "phosphoric acid triester: glutathione S-aryltransferase" by analogy with the related (Hutson et al, 1972) alkyltransferase. With rat liver soluble fraction, ethyl paraoxon (O,O-diethyl-O-p-nitrophenylphosphate) gave predominately aryl transfer to GSH whereas methyl paraoxon gave predominately alkyl transfer. This effect was due to competing enzymes since a partially purified aryltransferase, considerably reduced in alkyltransferase activity, gave a high rate of production of S-p-nitrophenylglutathione with methyl paraoxon. Separate enzymes were shown to effect the two types of transfer.

There is some evidence for induced formation of GSH S-aryltransferase. Sims & Grover (1965) found more enzyme activity in a dieldrin-resistant strain of housefly than in a dieldrin-susceptible strain, and Grover & Sims (1964) showed that activity in rats increased initially after phenobarbitone treatment but then declined in a few days to a value 40% less than that of untreated animals. Darby & Grundy (1972) found a small but significant increase in specific activity (with 1-chloro-2, 4-dinitrobenzene, CDNB) in phenobarbitone-treated male rats, but not in females, and there was no change in apparent K_m for CDNB or DCNB. In control and phenobarbitone-treated animals the specific activity with DCNB and BSP was three times higher in males than females, but with CDNB it was only marginally higher. K_m (DCNB) was similar for males and females, but K_m (CDNB) was significantly higher for females.

Ion-exchange chromatography on CM-cellulose of a housefly (Musca domestica) extract revealed the presence of two enzymes, when assayed with CDNB and several other substrates (Ishida, 1968). Electrophoresis

showed the enzymes were negatively charged at pH 7.4. The activity in rat liver homogenate moved towards the cathode in two peaks during electrophoresis at pH 7.4. Goodchild & Smith (1970) showed that housefly GSH S-aryltransferase migrated as a single peak during electrophoresis at several pH values, although a symmetrical activity peak was not obtained. Extrapolation of the 'mobility against pH' plot gave a value below pH 5 for the isoelectric point. The enzyme was not detected after isoelectric focusing.

The molecular weight of GSH S-aryltransferase from vertebrates and invertebrates is approximately 36 000 - 40 000 as measured by gel chromatography (e.g. Ishida, 1968; Goodchild & Smith, 1970; Balabaskaran, 1972).

Glutathione S-alkyltransferase (EC 2.5.1.12)

Enzyme-catalysed reaction between GSH and alkyl halides was first demonstrated by Booth et al (1961). Johnson (1963) showed the presence of a separate GSH S-alkyltransferase in rat liver- and kidney-homogenates which catalysed this reaction and which could be separated from GSH S-aryltransferase activity. The liver/kidney activity ratio was 1.4/1 using iodomethane, and in each tissue greater than 80% of the activity was in the soluble fraction (Johnson, 1966). Several alkyl halides and related compounds could replace the iodomethane, but with varying efficiency, suggesting that the enzyme has considerable specificity for the second substrate. Both liver- and kidney-alkyltransferases were inactivated by dialysis against distilled water, and were inhibited competitively by GSSG and S-methylglutathione.

The dealkylation of organophosphorus triesters may be catalysed by GSH S-alkyltransferase. Fukami & Shishido (1966) examined an enzyme from rat liver and insect mid-gut that catalysed reaction of methyl parathion (O,O-dimethyl-O-p-nitrophenylphosphorothionate) with GSH to give desmethyl parathion (O-methyl-O-p-nitrophenylphosphorothionate) and S-methylglutathione. Hutson et al (1968) demonstrated demethylation of 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethylphosphate (cis-isomer) by a GSH-dependent enzyme in mammalian liver soluble fraction, and have recently (Hutson et al, 1972) purified the enzyme 45-fold, as assayed with dimethyl 1-naphthylphosphate. O-ethyl groups were removed at $\frac{1}{50}$-$\frac{1}{100}$ of the rate of methyl groups; S-methyl groups were not readily removed. The authors suggested the name "phosphoric acid triester-GSH-S-alkyltransferase" for the enzyme although it was not separated from GSH S-alkyltransferase (iodomethane) by their procedure. Hollingworth (1969) showed that iodomethane inhibits P-O-alkyl cleavage by GSH S-alkyltransferase.

Ohkawa & Casida (1971) showed the presence in mouse liver- and housefly-homogenates of GSH-dependent enzymes which catalyse liberation of hydrogen cyanide from a variety of organic thiocyanates. The mechanism may involve formation of an S-alkylglutathione or glutathione thiocyanate as an intermediate; GSH was converted ultimately to GSSG. Activity chromatographed on DEAE-cellulose similarly to the enzyme catalysing GSH-dependent demethylation of Sumithion [O-O-dimethyl-O-(4-nitro-m-tolyl)phosphorothionate].

Glutathione S-epoxidetransferase

Booth et al (1961) found that rat liver soluble fraction formed GSH conjugates with compounds such as iodobenzene, naphthalene, 1-chloronaphthalene and phenanthrene only if microsomes and NADPH also were added. Boyland & Williams (1965) showed that preparations of rat-liver and -kidney and ferret liver catalysed reaction of GSH with a variety of epoxides to give an S-(dihydrohydroxy -aryl or -alkyl)glutathione and proposed the name GSH S-epoxidetransferase for the enzyme. A similar enzyme has been found in the liver of several species of wild bird (Wit & Snel, 1968). Experimental evidence suggests that microsomal epoxide-formation is a prerequisite in the formation of mercapturic acids from aromatic hydrocarbons and halogenated aromatic and aliphatic hydrocarbons, although epoxide formation from such compounds has not been detected in vivo. Several epoxides have been isolated from mammalian systems but they do not yield mercapturic acids (Wood, 1970).

Glutathione S-epoxidetransferase is specific for GSH, but several aromatic and aliphatic epoxides are effective second substrates. Proximity of an aromatic ring to the epoxy group increases the rate of reaction (Boyland & Williams, 1965). Diepoxides appear to give only one conjugate, even in the presence of excess GSH. Conjugation of 1,2,3,4-tetrachloro-5,6-epoxycyclohexane (Sims & Grover, 1965) and cycloalkene epoxides (James et al, 1971) with GSH has been shown.

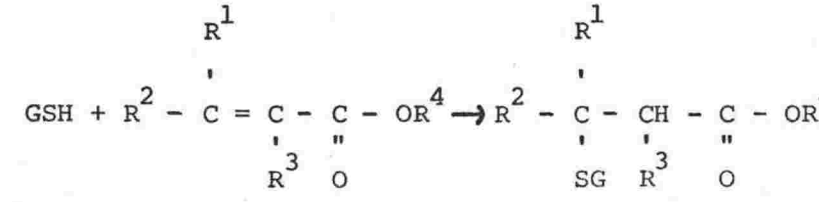
Several enzymes that catalyse reaction of GSH with epoxides occur in the liver of male Sprague-Dawley rats (Fjellstedt et al, 1973). One of these has been isolated in homogeneous form (EC 4.4.1.7., S-(hydroxylalkyl)-glutathione alkyl-epoxidelyase) and had a molecular

weight of 40 000 from sedimentation equilibrium centrifugation data and 39 500 from Sephadex G-100 filtration. Treatment with 6M guanidine HCl - 0.1M mercaptoethanol split the enzyme into two subunits of molecular weight about 25 000. Activity increased with pH similarly to the non-enzymic reaction up to pH9. Storage of the purified enzyme frozen in the presence of 30% glycerol-5mM-GSH resulted in loss of activity and the appearance of additional protein bands upon gel isoelectric focusing.

Stability studies and activity ratios after partial purification of GSH S-transferase activity from rat liver (Boyland & Williams, 1965) suggested that activity with 2,3-epoxypropyl phenyl ether was distinct from GSH S-aryl- and S-alkyl- transferase. However, no significant separation of activity towards epoxides and DCNB was obtained by fractional precipitation with ammonium sulphate, acetone or ethanol. 2,3-epoxypropyl phenyl ether and hexane-1,2-5,6-diepoxide did not inhibit aryltransferase activity with DCNB (Boyland & Williams, 1965) although Booth et al (1961) showed that 1,2-epoxy-1,2,3,4-tetrahydronaphthalene was an inhibitor.

Glutathione S-alkenetransferase

Boyland & Chasseaud (1968) proposed this name for the enzyme catalysing reaction of GSH with a range of α,β-unsaturated compounds which includes aldehydes, esters, ketones, lactones, nitriles and nitrocompounds.



Reaction of GSH with αβ-unsaturated compounds involves addition of the nucleophile $GS^{\ominus}$ to the β-carbon atom polarized by conjugation with strongly electron-withdrawing groups. The reaction probably is governed by electronic and steric effects (Boyland & Chasseaud, 1967).

Gray & Barnsley (1971) showed *in vivo* formation by rats of a GSH conjugate of crotonaldehyde.

The enzyme is present in liver preparations from a number of vertebrate species (Boyland & Chasseaud, 1967) and in some insects (Speir, 1972). Heat inactivation experiments, ammonium sulphate fractionation, inhibition studies with *S*-(αβ-diethoxycarbonylethyl) glutathione, and the distribution of activities in rat liver, rat kidney and in the livers of other animals suggests there are at least six GSH *S*-alkene-transferases which are different from *S*-alkyl-*S*-aryl- and *S*-epoxide-transferase, and that the *S*-alkenetransferases may be specific for one conformation (Boyland & Chasseaud, 1968; Chasseaud, 1973).

Speir & Barnsley (1971) have partially purified an enzyme from rat liver supernatant which catalysed conjugation of several αβ-unsaturated acyl thiol esters with GSH. Speir (1972) has shown that the partially purified preparation may be resolved further by isoelectric focusing into several activity peaks and demonstrated cross-specificity of aryl-, alkyl- and alkene-transferases. With crotonyl-*N*-acetylthioethanolamine the product was *S*-(2-carboxy-1-methylethyl)glutathione (Speir & Barnsley, 1971). The reaction was catalysed to a significant extent only by the soluble fraction of rat liver and kidney, which were equally active on the basis of wet weight of tissue. The non-enzymic reaction of GSH with *S*-crotonyl-*N*-acetylthioethanolamine was complicated; neither the enzymic nor the non-enzymic reaction, measured with equimolar amounts of substrates

or with excess GSH, went to completion. S-(2-carboxy-1-methylethyl)-glutathione and GSSG inhibited the reaction competitively with respect to GSH. It was suggested that reaction between GSH and naturally occurring 2,3-unsaturated acyl-CoA thiol esters, or their partial hydrolysis products, may have significance for the increased excretion of carboxyalkylcysteines in some pathological conditions.

Glutathione S-aralkyltransferase (EC 2.5.1.14)

The enzymic reaction of GSH with benzyl chloride in rats (Booth et al, 1961; Suga et al, 1967; Boyland & Chasseaud, 1969b), and with p-nitrobenzyl chloride in locusts and other insects (Cohen et al, 1964) has been shown. Boyland & Chasseaud (1969b) suggested the reaction may be of SN_1 type:

$$Ph.CH_2Cl \longrightarrow [Ph.CH_2^+] \xrightarrow{GSH} Ph.CH_2.SG + H^+ + Cl^-$$

Gillham (1971) suggested that such a mechanism would display Ping-Pong kinetics (Cleland, 1963). The concerted SN_2 reaction was considered less likely because GSH is a relatively large molecule (Boyland & Chasseaud, 1969b). 3,5-Di-tert-butyl-4-hydroxybenzyl acetate and some other aralkyl esters were substrates but reacted only slowly with GSH (Boyland & Chasseaud, 1969b).

Using the same techniques as in their study of GSH S-alkene-transferase (Boyland & Chasseaud, 1967), Boyland & Chasseaud (1969b) concluded that reaction of benzyl chloride with GSH was catalysed by an enzyme different from the aryl- alkyl-, epoxide- and alkene-transferases. Suga et al (1967) found the activity ratio with benzyl chloride in rat liver/kidney to be 100/73 compared with 100/8 for aryltransferase, and Booth et al (1961) showed non-competitive

inhibition by benzyl chloride in the reaction of DCNB with GSH. The alkyl-, aralkyl- and alkene-transferases were inhibited by GSSG and the nature of the inhibition varied in each case, but partial purification of rat liver supernatant abolished this inhibition (Boyland & Chasseaud, 1969b).

Gillham (1971) has partially purified from rat liver a GSH S-aralkyltransferase for which some aralkyl sulphate esters are substrates. The substrates include benzyl sulphate, 1-menaphthyl sulphate and phenanthr-9-ylmethyl sulphate; 1-menaphthyl alcohol was neither a substrate nor an inhibitor (Gillham et al, 1970). The enzyme may be different from GSH S-aralkyl(Benzyl chloride) transferase.

Enzyme catalysing reaction of halogenocycloaliphatic compounds with GSH

It is possible that the non-planar halogenocycloaliphatic compounds are conjugated with GSH by a specific enzyme, rather than by GSH S-aryltransferase which is concerned with planar aromatic molecules. Clark et al (1969) showed inhibition of the metabolism of γ-hexachlorocyclohexane (γ-HCH) and γ- and δ-pentachlorocyclohex-1-ene (-PCCH) in flies and grass grubs by tetrabromophenolphthalein ethyl ester or bromophenol blue, and provided evidence for an S-(pentachlorocyclohexyl)glutathione being the initial metabolite of γ-HCH. James et al (1971) showed that rabbits and rats formed mercapturic acids from bromocycloalkenes, the amount formed decreasing with increasing ring size. The 3-hydroxy isomer was the main mercapturic acid excreted. Sims & Grover (1965) concluded that chlorocyclohexane and the α-, β-, γ- and δ-isomers of HCH were not substrates for rat liver GSH S-aryltransferase, but suggested that α-3,4,5,6-tetrachlorocyclohex-1-ene and γ-2,3,4,5,6-pentachlorocyclohex-1-ene were substrates.

However, inhibition of GSH S-aryltransferase by γ-PCCH, using DCNB as substrate, could not be shown.

DDT-dehydrochlorinase (EC 4.5.1.1.)

This enzyme, which catalyses dehydrochlorination of 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane (DDT) to 1,1-dichloro-2,2-bis-(p-chlorophenyl)ethylene (DDE), was purified from DDT-resistant houseflies by Lipke & Kearns (1959a); considerably less of the enzyme was present in DDT-susceptible flies. Material prepared from flies stored for long periods had a yellow-brown appearance, even at the purest stage, which was probably due to oxidation of tyrosine residues by tyrosinase in the frozen flies. Of a wide range of thiols, only GSH and cysteinylglycine initiated the reaction. The dipeptide activated the dehydrochlorination in the presence of GSH. γ-Glutamylcysteine showed some inhibitory action (Lipke & Kearns, 1959b).

Dinamarca et al (1969; 1971) have shown that the enzyme from a strain of DDT-resistant houseflies may be isolated either as a monomer of molecular weight about 30 000 or as a tetramer of molecular weight about 120 000, depending on whether or not it is prepared in the presence of a reducing agent - addition of dithiothreitol, 2-mercaptoethanol or GSH to the preparative solutions results in isolation of the monomer. The monomers appear to be chemically and physically identical. DDT converts monomers into tetramers which are stabilized by GSH but disaggregated by dithiothreitol or 2-mercaptoethanol. Monomers slowly aggregate into tetramers when in relatively high concentration at low ionic strength. Conversely, at high ionic strength and low protein concentration, in the absence of GSH, the tetramers tend to dissociate. The enzyme contains 32

cysteinyl residues per tetramer; none appears to be associated with the active site, none is detectable with p-chloromercuribenzoate after DDT-induced aggregation and there are no disulphide bonds. The enzyme appears to be associated with phospholipid which is essential for activity. The GSH requirement for activity remains unexplained, but appears to be more complex than maintenance of protein thiol groups in the reduced state.

Goodchild & Smith (1970) demonstrated the presence of several DDT dehydrochlorinases by electrophoresis and isoelectric focusing of crude preparations from houseflies selected, without insecticide pressure, to have a normal low resistance to DDT. Some of the enzyme activity and activity peaks disappeared upon extending the time of electrofocusing from three to nine days. In electrophoretic experiments different preparations of the enzyme did not always have the same proportions of activity in the two DDT dehydrochlorinase peaks, and it was suggested that one of these may be an artifact.

Comparative Studies of Glutathione S-transferases

From studies using rat liver preparations previous workers (Boyland & Williams, 1965; Johnson, 1966; Boyland & Chasseaud, 1967, 1968, 1969a, 1969b) have concluded that aryl-, alkyl-, epoxide-, alkene- and aralkyl-transferases are all different. The following table (Table 2) is typical of the evidence upon which these conclusions are based:

Table 2. Relative stability of Glutathione S-transferases. (from Boyland & Chasseaud, 1969b).

Enzyme Preparation and Treatment	% activity remaining towards				
	alkyl	aralkyl	aryl	alkene	epoxide
Undialysed rat liver supernatant (control)	100	100	100	100	100
Dialysed rat liver supernatant	67	68	90	80	67
Supernatant after lowering to pH5.0 and raising to pH6.5	44	69	65	77	39
Supernatant after lowering to pH5.0 and acetone drying	17	35	100	71	22
Ratio of activity of undialysed rat liver supernatant to that of ammonium sulphate-fractionated acetic acid-precipitated rat liver homogenate	11.2	2.3	0.99	2.7	4.7
Ratio of activity of dialysed rat liver supernatant to that of dialysed rat kidney supernatant	8.3	5.1	47.6	2.6	4.1

Boyland & Chasseaud (1968) also suggested that there were at least six different GSH S-alkenetransferases based on differences in substrate specificities.

A detailed study has been made (Speir, 1972; Clark et al, 1973) of GSH S-transferases from several vertebrates and insects using ammonium sulphate fractionation, ion exchange chromatography and isoelectric focusing techniques. Sheep liver 60-100% ammonium sulphate precipitate contained major enzyme peaks with isoelectric points (pI) of 7.1 and 9.5. Rat liver 60-100% precipitate had four

major peaks with pI values of 7.6, 8.0, 8.6 and 9.4, and the similar fraction from mouse liver had two peaks with pI of 8.2 and 9.8 respectively. The enzymes with pI greater than 9 had predominately alkyltransferase activity with smaller amounts of aryl- and alkene-transferase; enzymes with pI values less than 9 had aryl- and alkene-transferase activity with some alkyltransferase activity. In the insects examined the pI values of the major enzymes were generally lower than for the vertebrate enzymes. Three major zones of activity were obtained from housefly 50-70% ammonium sulphate precipitate (pI values 5.1, 5.9 and 6.8) and from cockroach (Periplaneta americana) 40-70% ammonium sulphate precipitate (pI values 4.5, 6.6 and 8.5); in both insects alkyl- and alkene-transferase activity were associated with aryltransferase. In grass grubs (Costelytra zealandica; 50-63% ammonium sulphate precipitate) two major peaks (pI values 4.5 and 8.8) were obtained with very high aryltransferase activity relative to the other enzymes; several smaller peaks containing relatively minor amounts of activity with each substrate were evident in the region between the major peaks when this was refocused over a narrow pH range. The results are thus consistent with the presence in all species of several GSH S-transferases each showing some degree of cross specificity towards the three substrates.

More conclusive evidence of cross specificity is provided by Pabst et al (1973) in studies of three GSH S-transferases that have been purified to homogeneity from rat liver. Each enzyme was active with p-nitrobenzylchloride; one was also active with epoxides and p-nitrophenethylbromide, the second with iodomethane and the third with 3,4-dichloronitrobenzene and 4-nitropyridine N-oxide. The

authors concluded that differences in specificity are not directly related to the nucleophilic leaving group and that it is not accurate to classify the enzymes by their preference for substrates with a particular type of hydrocarbon skeleton.

Kinetic Studies of Glutathione S-transferases

Studies by Clark (1967) of grass grub GSH S-aryltransferase suggested that rapid equilibrium kinetics apply and that the substrates combine in random order. In contrast, recent work (Gillham, 1973) with a partially purified enzyme from rat liver that catalyses reaction of 1-menaphthyl sulphate with GSH demonstrated that an Ordered Bi Bi mechanism applied, in which GSH added before 1-menaphthyl sulphate and $SO_4^{2\ominus}$ was released before the conjugate.

Oxidised glutathione inhibits all known GSH S-transferases present in rat liver supernatant preparations (E. Boyland, L.F. Chasseaud & K. Williams, unpublished work; cited in Boyland & Chasseaud, 1969b). 'Dixon plots' using GSSG as inhibitor were non-linear for S-alkene(cis-ester)-, S-alkyl- and S-aralkyl-transferase but the inhibition was abolished by partial purification (Boyland & Chasseaud, 1969b). Using S-(αβ-diethoxycarbonylethyl)-glutathione as inhibitor, Boyland & Chasseaud (1968) obtained a linear 'Dixon plot' with diethylmaleate as substrate, but non-linear plots with diethylfumarate and trans-benzylidene acetone.

Inhibition of GSH S-transferases by phthaleins and similar compounds has been studied by several workers. Cohen et al (1964) showed that insect aryl- and aralkyl-transferases were clearly distinguished from the vertebrate liver enzymes by their inhibition by phthaleins, particularly BSP. The enzyme from the cattle tick (Boophilus), a non-insect arthropod, was similar to the vertebrate

enzyme and was little affected by BSP. Inhibition of locust enzyme was usually complex and may have indicated the presence of more than one enzyme with different inhibitor- and substrate-affinities. BSP appears to be a substrate for vertebrate GSH S-aryltransferase (Coombes & Stakelum, 1961) and apparently competes with DCNB for the binding site (Grover & Sims, 1964). Inhibition experiments with GSH S-aryltransferase from houseflies and grass grubs using phthalein and fluorescein derivatives gave simple patterns when plotted according to the method of Lineweaver & Burk (1934) or Dixon (1953), which suggested that the inhibitors were competing with GSH (Clark et al, 1967; Balabaskaran & Smith, 1970), but not with CDNB or DCNB. Sheep liver enzyme was not inhibited by dicarboxylic acids or phthalein derivatives at concentrations up to 0.8 mM except for BSP which gave weak inhibition at 0.2 mM.

Studies with a number of compounds structurally related to phenolphthalein have clarified some of the requirements for an inhibitor of GSH S-aryltransferase (Clark et al, 1967; Balabaskaran & Smith, 1970). Substitution of halogen atoms ortho to the oxygen atoms decreased the K_i values of phthalein derivatives about 20-fold with grass grub and housefly enzyme. Reduction of the quinonoid form, ionization of the carboxyl group in the phthaleins or the sulphonic acid group in the sulphonphthaleins, esterification of the carboxyl group, or loss of the phthalein structure in the dihydroxydiphenylmethane derivatives had little effect on the K_i. It was suggested (Clark et al, 1967) that the phthaleins, sulphonphthaleins and fluoresœins can be regarded as isosteric with GSH and in models it was possible to superimpose their phenolic

oxygen atoms on those of the carboxyl groups of GSH. Inhibition by simple dicarboxylic acids was negligible until the distance between the carboxyl groups approached that between the carboxyl groups in GSH. However, inhibition was weak even with the most effective of the dicarboxylic acids, traumatic acid (trans-dodec-2-en-1,12-dioic acid) and 3,3'-dicarboxydiphenylmethane. Behaviour of the phthalein and dicarboxylic acid inhibitors towards GSH S-aryltransferase was a useful qualitative guide towards their action on DDT dehydrochlorinase (Balabaskaran & Smith, 1970).

Boyland & Grover (1967) studied several inhibitors of human and rat liver GSH S-aryltransferase. The coronary vasodilator Benziodarone [2-ethyl-3-(4-hydroxy-3,5-diiodobenzoyl)benzofuran] was the best inhibitor of the rat liver enzyme, being non-competitive with the aromatic substrate with a K_i of 1.25×10^{-7}M. At 1 mM, Benziodarone inhibited the enzymes catalysing conjugation of GSH with halogenonitrobenzenes (100%), epoxides (81%), αβ-unsaturated esters (52%) and alkyl halides (31%) (L.F. Chasseaud, unpublished data; cited in Boyland & Grover, 1967). At the same concentration 3,5-diiodotyrosine inhibited aryltransferase 30.6%.

The effect of pH on the kinetic constants v_o, V_m, K_m and K_i suggested that grass grub GSH S-aryltransferase may have two ionizing groups, and sheep liver enzyme one such group, with pK about 9.2 involved in binding of GSH to the enzyme (Clark et al, 1967). Basic groups such as ε-NH_2 of lysine, free α-NH_2, phenolic -OH of tyrosine and -SH of cysteine normally ionize in this region and may be involved.

Frear & Swanson (1970) observed non-enzymic catalysis of S-(4-ethylamino-6-isopropylamine-2-s-triazino)glutathione formation with tris buffer systems. The rate of this reaction increased with

increase of pH from 7.0 - 9.0. Catalysis was not observed with N-tris-(hydroxymethyl)glycine (Tricine) buffer systems under the same conditions. This suggested that the free primary amino group of tris may be functioning as a model nucleophilic catalyst.

Boyland & Chasseaud (1968) obtained a break at 32-35°C in the Arrhenius plot for the enzyme-catalysed reaction of diethylmaleate and GSH. The activation energy was 43.1 kJ/mole below 32°C and 15.5kJ/mole above 35°C.

Purification of Glutathione S-transferases

Purification procedures have used conventional methods based on the physical and chemical properties of proteins in general (Table 3). Several of the enzymes have been isolated from rat liver as homogeneous species (Fjellstedt et al, 1973; Pabst et al, 1973). In most instances cross specificity of the purified preparations has not been examined.

Table 3. Purification of GSH S-transferases

GSH S-transferase	Reference
Aryl	Booth, Boyland & Sims, (1961); Speir, (1972); Pabst et al,(1973).
Alkyl (alkyl halide) (phosphoric acid triester)	Johnson, (1963, 1966); Pabst et al, (1973); Hutson et al, (1972); Speir, (1972).
Epoxide	Boyland & Williams, (1965); Fjellstedt et al, (1973); Pabst et al, (1973)
Alkene	Boyland & Chasseaud, (1967, 1968); Speir & Barnsley, (1971); Speir, (1972).
Aralkyl (aralkyl halide) (aralkyl sulphate ester)	Boyland & Chasseaud, (1969b); Pabst et al, (1973); Gillham, (1971, 1973).
'Substituted nitrofuran'	Boyland & Speyer, (1970).
'Substituted triazine'	Frear & Swanson, (1970).

Affinity Chromatography

Purification of enzymes and other biological macromolecules by affinity chromatography exploits the unique property of these molecules to bind ligands specifically and reversibly. Recent advances have made it possible to approach the purification of any given enzyme, or other macromolecule, systematically and with reasonable chance of success. The rapid increase in publications in this field testifies to the acceptance of the method.

Baker (1967) has explained the necessity for determining regions of the ligand not in contact with the enzyme (bulk-tolerance regions) so that attachment to the solid support may be made at this point. General considerations concerning the nature of the insoluble support, covalent-linking techniques for ligand attachment, and conditions for protein adsorption and elution have been reviewed by Cuatrecasas & Anfinsen (1971) and Cuatrecasas (1971).

EXPERIMENTAL

Chemicals not specified were generally of laboratory reagent grade. Glass-distilled water was used for the preparation of all aqueous solutions. pH measurements were made with a Radiometer pH meter 22, or when greater accuracy was required, with the pH meter 26 (Radiometer, Copenhagen, Denmark) using the expanded scale.

Source of Enzyme

New Zealand grass grubs [third-instar larvae of Costelytra zealandica (White)] were collected from pastures in Canterbury in August 1969 and placed individually, in moist soil, in the compartments of small ice-cube trays for transport to the laboratory. After separation from soil the grubs were frozen at -20°C in 50g amounts in polythene bags. Grubs which had pupated or were dead upon sorting from the soil were discarded. The live grubs used in some experiments were from farms in the Nelson district and were stored in the trays at 4°C until required.

Determination of Enzyme Activity

Measured reaction rates were corrected for the non-enzymic contribution, (see Table 17).

1. Glutathione S-aryltransferase

The spectrophotometric method of Cohen et al (1964) was used when CDNB (1-chloro-2,4-dinitrobenzene,'OAS', BDH Chemicals Ltd, Poole, England), 1-bromo-2,4-dinitrobenzene or 1-iodo-2,4-dinitrobenzene was the second substrate. The bromo- and iodo- compounds were synthesized by Dr. A.G. Clark using standard methods and recrystallized before use. BDNB (1-bromo-2,4-dinitrobenzene) m.p.73-75°C (lit.74°C); IDNB (1-iodo-2,4-dinitrobenzene) m.p.87°C (lit.87.5-88.5°C).

The spectrophotometric method of Booth et al (1961), adapted for continuous assay, was used with DCNB (1,2-dichloro-4-nitrobenzene, BDH Chemicals Ltd; recryst. once from ethanol) as second substrate.

Buffers were prepared from 0.1M-sodium acetate adjusted with acetic acid (acetate buffer, pH 5-6), NaH_2PO_4 and Na_2HPO_4 (phosphate buffer, I=0.1, pH 6-8) or 0.1M-$Na_4P_2O_7$ adjusted with conc. HCl (pyrophosphate buffer, pH 8-10). The stock GSH (Sigma Chemical Co.) solution was prepared in buffer immediately before use and adjusted to pH 8 with 2M-NaOH.

The reaction mixture normally contained 0.05ml GSH (final concn.2mM), 0.05ml second substrate (stock solutions in ethanol; final concn.0.1mM for CDNB, 0.75mM for DCNB), enzyme, and buffer (pyrophosphate pH 8.3, unless otherwise specified) to give a total volume of 3.00ml. Small volumes (0.05ml or less) were pipetted with "Auto-zero high precision micropipettes" (E-mil Works, Glamorgan, Wales). Reactions were initiated by final addition of the second substrate (CDNB, unless otherwise specified). Enzyme was omitted for the non-enzymic reactions. Extinction changes at 344nm were measured at 37°C in the Beckman KINTRAC VII™ spectrophotometer, using the single-cell mode with CDNB and the continuous cycling mode with DCNB. Recorder chart speed and extinction span across the chart were selected, when possible, to give full scale deflection for each cell over the period of measurement. The maximum sensitivity obtainable was 0.10 E per 10in chart width, i.e. 0.0010 E per chart division. Reaction rates were calculated from the linear recorder traces.

Kinetic Studies

The activation energy of the GSH S-aryltransferases isoelectric at pH 4.6 (pI 4.6 enzyme) and pH 8.7 (pI 8.7 enzyme) was measured at pH 8.30 using CDNB and DCNB as second substrate.

An investigation was made of the effect of pH and temperature on the 'substrate-kinetics' of the pI 8.7 enzyme using DCNB as second substrate. The pH was varied from 8-10.4 and the temperature from 15-37°C. The effect of variation of pH, at 37°C, on the 'substrate-kinetics' of the pI 4.6 enzyme was also studied.

Buffers of constant ionic strength were prepared from $Na_4P_2O_7$ and conc. HCl for pH values from 8.0 - 9.9. A graph showing the variation with pH of the concentration of $Na_4P_2O_7$ required to give an ionic strength of 0.755M at 25°C (the value given by 0.100M-$Na_4P_2O_7$/HCl at pH 8.3) may be found in Appendix I, (Fig. 57). Buffers at pH 10.4 were prepared from 0.200M-Glycine and NaOH; the desired ionic strength was obtained by addition of NaCl before pH adjustment. All pH adjustments were made at room temperature, and for the Glycine buffers allowance was made for the temperature dependence of pH:-

viz. $\frac{dpH}{dT} = -0.026$ pH units/°C (Dawson et al, 1969; p.482)

The continuous cycling mode of the KINTRAC was used for all measurements with DCNB, and for each run a range of six GSH concentrations was used at constant DCNB concentration. Pipetting errors were minimized by preparing a series of stock solutions of each substrate such that an equal volume of each was taken. Reaction temperature was controlled (±0.1°C) by water circulation through the cell basket and basket housing using a Churchill 'Laboratory Thermocirculator' (Churchill Instrument Co. Ltd, Middlesex, England). At reaction temperatures of 30°C or less a Grant thermocirculator and refrigerating unit (Grant Instruments, Cambridge, England) were used to circulate cold water around the heating coils of the Churchill circulator. Reactions were followed for up to 20min, depending on the rate, and in this time about 16 readings of extinction in each cell were obtained.

2. Glutathione S-methyltransferase

Production of desmethyl parathion from methyl parathion was measured by the discontinuous spectrophotometric assay of Speir (1972). The reaction mixture contained

0.2M-tris/HCl buffer, pH 8.0 (20°C)

1.5mM GSH

enzyme solution

methyl parathion

in a total volume of 4.0ml. The reaction was started by addition of 0.05ml 24mM-methyl parathion (solution in ethanol). The solution was incubated at 20°C for 90min then stopped by addition of 2ml freshly prepared 10% (v/v) titanous chloride solution (prepared from stock 12% (w/v) $TiCl_3$/15% (w/v) HCl by 10-fold dilution with water). After at least 1h, 2ml chloroform and 1ml 0.75M-NaOH were added and the mixture was shaken vigorously until the intense blue colour of titanous hydroxide disappeared leaving a white suspension of titanic hydroxide. After centrifugation to remove protein and titanic hydroxide 2ml of the aqueous supernatant was added to 1ml 0.5% (w/v) p-dimethylaminobenzaldehyde (in 50% (v/v) acetic acid). Extinction at 440nm was measured after 15min. Each enzymic assay was corrected for the non-enzymic reaction and for controls in which GSH and enzyme plus GSH were omitted.

3. Glutathione S-crotonyl-N-acetylthioethanolamine transferase

Assays for this activity were done by Mr T.W. Speir by continuous spectrophotometric assay (Speir, 1972). Loss of S-crotonyl-N-acetyl-thioethanolamine (CrSE) at 37°C was measured in 1cm cuvettes in a Perkin Elmer 137 UV spectrophotometer. The cuvettes contained 0.05M phosphate buffer pH 7.4, 0.15mM CrSE, enzyme solution and the reaction was started by addition of GSH (final concentration 0.5mM). Total reaction volume

was 3.0ml. Extinction at 263nm was measured for 1min, or longer if the reaction rate was low. Reaction rates were corrected for the non-enzymic rate of 0.003µmole per min.

4. Acid Phosphatase

(i) Preparation of Enzyme Solutions. Frozen grass grubs were homogenized in an MSE top-drive blender with stainless steel blades (Measuring and Scientific Equipment Ltd., London, England) in water, 0.25M- and 0.44M-sucrose (25g/50ml) and the homogenate was centrifuged at 10 000g for 15min. Aliquots were kept for measurements and the rest was centrifuged at 23 000g for 30min. Supernatant and precipitate from the second centrifugation were assayed.

(ii) Acid Phosphatase activity. The method was based on that of Hsu et al (1966). Enzyme activity was measured at pH 5.0 in 0.2M-acetate buffer, both in the presence and absence of Triton-X-100, using p-nitrophenyl-o-phosphate (BDH Chemicals Ltd) as substrate. Reaction solutions contained 1.0ml p-nitrophenyl-o-phosphate (stock solution 5.5mM in buffer), 0.2ml enzyme and some contained 0.1ml of a 1.5% (v/v) solution of Triton-X-100 in water. Substrate, with or without Triton-X-100, was equilibrated at 37°C before addition of enzyme. Control solutions lacked enzyme. After incubation at 37°C for 30min, 10.0ml 0.02M-NaOH was added to each solution then enzyme was added to the controls. Extinction at 405nm was measured with the Beckman KINTRAC spectrophotometer. The amount of p-nitrophenol formed was calculated from a curve prepared with standard solutions of p-nitrophenol.

Protein Measurements

Extinction at 280 and 260nm was measured in 1cm or 0.5cm cuvettes and the protein concentration was calculated from the formula (Layne, 1957):

$$\text{Protein (mg/ml)} = 1.55\underline{E}_{280} - 0.76\underline{E}_{260}$$

Molecular Weight Measurements

Molecular weights were determined by gel chromatography at 4°C on BioGel P150, particle size 50 - 100 mesh (wet), (Calbiochem), in a glass column (dimensions 1.5cm x 135cm) identical to that used by Balabaskaran (1972). The gel was equilibrated with 0.02M-pyrophosphate buffer, pH 7.5, and packed into the column as described by Balabaskaran (1972).

The column was calibrated by chromatography of 1ml solutions in the column buffer (10 or 20 mg/ml) of cytochrome c (Horse heart, Type II; Sigma), ovalbumin (5X cryst; Mann Research Laboratories, Inc, New York, N.Y., U.S.A.) and bovine serum albumin (cryst., lyophilized; Sigma). Buffer was pumped upwards through the column by an LKB peristaltic pump (LKB Produkter AB, Stockholm, Sweden). Collection of approximately 2ml fractions (30 drops) was commenced as soon as the protein solution was loaded. The elution position of cytochrome c was measured by its extinction at 414nm, and that of the other proteins by their extinction at 280nm.

Solutions of GSH S-aryltransferase from isoelectric focusing experiments were dialysed against column buffer before loading into the column. The molecular weights of the GSH S-aryltransferases were calculated by interpolation of the standard curve prepared using the values of molecular weight of the standard proteins given by Mahler & Cordes (1966; p.46, 75, 594).

Ion-Exchange Chromatography

The following ion-exchange celluloses were used:

Mannex DEAE-cellulose (exchange capacity 0.93 mequiv./g; Mann Research Laboratories);

Whatman CM-cellulose (Advanced Fibrous CM23, fines reduced, exchange capacity 0.6 mequiv./g dry powder; W. & R. Balston Limited, Maidstone,

Kent, England);

Cellex SE (sulphoethyl)-cellulose (exchange capacity 0.2 ± 0.06 mequiv./g; BIO-RAD Laboratories, Richmond, California, U.S.A.);

Cellex T (TEAE, triethylaminoethyl)-cellulose (exchange capacity 0.5 ±0.1 mequiv/g; BIO-RAD).

Buffers of the desired pH and ionic strength were prepared from ethylenediamine and conc. HCl, sodium acetate and acetic acid, or NaH_2PO_4 and Na_2HPO_4. Details are given in the Results section.

All ion-exchange celluloses were cycled each time before use as described by Thompson (1970). The procedure was identical to that supplied with the advanced grades of Whatman DEAE- and CM- cellulose. The celluloses were equilibrated at the desired pH with a buffer ten-times more concentrated than the starting buffer, and washed thoroughly in a Buchner funnel with starting buffer. The column (Pharmacia K15/30, Pharmacia Fine Chemicals, Uppsala, Sweden) was packed at room temperature by the aliquot method, consolidated under pressure (Thompson, 1970), then equilibrated at 4°C by passage of several column-volumes of starting buffer using a Watson-Marlow H.R. Flow Inducer (Watson-Marlow Ltd, Bucks, England) to pump the buffer onto the column. Samples were dialysed against starting buffer then pumped onto the column at the same flow rate as used for elution, about 30ml/h. In most experiments a linear gradient of ionic strength was produced using two identical cylindrical vessels (Bock & Ling, 1954), the mixing vessel initially containing starting buffer and the reservoir starting buffer plus NaCl. In some experiments starting buffer development was used before commencing the gradient. The linearity of the gradient was established initially by measurement of the refractive index of the fractions using an Abbe refractometer, and thereafter its emergence was predicted using the void volume of the column. Fractions were collected

dropwise using an LKB UltroRac 7000 fraction collector.

Elution of GSH S-aryltransferase from DEAE-cellulose was attempted by inclusion of GSH, in addition to NaCl, in the reservoir vessel. NaCl concentration and pH were corrected to give the same ionic strength and pH as in the absence of GSH.

Isoelectric Focusing

Isoelectric focusing was performed in a column made to the specifications of the LKB 8100 (110ml) isoelectric focusing column. Ampholine® carrier ampholytes were obtained from LKB Produkter AB and were used at a concentration of 1%. A stepwise sucrose density gradient was prepared as described by Haglund (1971) with the anode at the bottom of the column. When the method was used on an analytical scale enzyme solution replaced some of the light solution for the gradient, but when the method was used on a preparative scale enzyme solution was used instead of water in preparing both solutions for the gradient. The sample was dialysed against distilled water to reduce the salt concentration to an acceptable value*. Protein precipitated during dialysis was removed by centrifugation and the supernatant was loaded onto the column. Focusing was carried out at 5-6°C at constant voltage until the current decreased to a constant value and then for a further 24h. Stepwise increases of the voltage were made periodically, the power being kept below 0.7W. At the completion of focusing the voltage was turned off, the central electrode compartment closed and the anode solution siphoned out of it. Fractions (2 or 3ml) were collected manually from the bottom of the column at a flow rate of about 2ml per min and the pH of each fraction was measured immediately at 5°C. *(no greater than 0.5 mmole in total per run).

Enzyme Preparations

Unless otherwise specified, frozen grass grubs were used. All

steps were performed at 0-5°C. Frozen grass grubs were not thawed before use. As conditions were varied in the initial investigations, in an attempt to define the optimum parameters for purification, the composition and pH of the buffers used is described in the Results section.

Homogenates

Grass grubs were homogenized in water or buffer (1g/2ml) in an MSE top-drive blender for 30 seconds at maximum speed and the homogenate was centrifuged in an MSE 18 centrifuge (8 x 50ml head) at 13 000g for 15 min. The supernatant was filtered through cotton wool to remove fat.

Acetone Powder

The 13 000g supernatant was adjusted to pH 5.0 with 2M-acetic acid and centrifuged at 13 000g for 15 min. The residue was discarded, the supernatant adjusted to pH 7 with 2M-NaOH and added slowly, with rapid stirring, to 10 volumes of acetone at -15°C. The precipitate was removed by filtration under reduced pressure and the residual acetone was removed under vacuum in a desiccator. The dry powder was stored under vacuum at room temperature. Solutions of the powder in buffer were centrifuged at 2 000g for 5 min before use.

Ammonium Sulphate Fractionation

Ammonium sulphate concentrations were calculated after noting the volume change due to addition of the salt. Solid ammonium sulphate was added gradually to the 13 000g supernatant, the suspension was stirred for 20 min after addition was completed and the precipitate was removed by centrifugation at 10 000g for 10 min. Precipitates were redissolved in water.

Purification of Glutathione S-aryltransferase

A total of 400g grass grubs was homogenized in lots of 50g/100ml 0.4M-acetate buffer, pH 5.5, the combined homogenate was centrifuged in

the MSE 18 (6 x 250ml head) at 13 000g for 15 min and the supernatant was filtered through cotton wool. Solid ammonium sulphate was added to the supernatant and the fraction precipitating between 2.2 - 3.3M was collected and stored as a suspension in 3.5M-ammonium sulphate. The pH did not change appreciably during this fractionation. The active precipitates from five 400g fractionations were combined (total volume 500ml) and centrifuged at 10 000g for 15 min. The precipitate was dissolved in the minimum quantity of 0.025M-acetate buffer, pH 5.5, dialysed against 5 litre of the same buffer for 6h and then against 5 litre distilled water for 16h. Protein precipitated during dialysis was removed by centrifugation and discarded. The supernatant was adjusted to pH 8 with NH_3 (sp.gr. 0.91) and kept at this pH value during a second fractionation with ammonium sulphate. The fraction precipitating between 2.3 - 3.4M was dissolved in 0.01M-acetate buffer, pH 5.5, and dialysed against two 5 litre portions of the buffer for a total of 24h. Precipitated protein was removed by centrifugation and discarded. The supernatant from dialysis was halved and each half further purified by isoelectric focusing using pH 3-10 "Ampholines". Two GSH S-aryltransferases were obtained from the column, one with a pI of 8.7 and the other with a pI of 4.6. For each of these enzymes, fractions containing 10% or more of the activity of the peak fraction were pooled, and each enzyme was dialysed against 0.025 M acetate buffer, pH 5.5. The pI 8.7 enzyme was stored in 2ml aliquots at -20°C until required for kinetic studies. The pI 4.6 enzyme was refocused using pH 4-6 "Ampholines", the peak fractions were pooled, dialysed against 10 litre 0.025M-acetate buffer, pH 5.5, for 24h and stored in 7ml aliquots at -20°C.

Affinity Chromatography

(1) Synthesis of 5'-carboxyfluorescein and 5'-carboxyeosin

5'-carboxyfluorescein (6-carboxy-3', 6'-dihydroxyfluoran) was prepared from 1,2,4-benzenetricarboxylic acid anhydride (Eastman Organic Chemicals, Rochester, New York, U.S.A.) and resorcinol by the method of Drechsler & Smagin (1965) and brominated as described, but without further purification, to yield 5'-carboxyeosin (6-carboxy-2',4',5',7'-tetrabromo-3',6'-dihydroxyfluoran).

(2) Preparation of Inhibitor - substituted Sepharose

(a) Activation of Sepharose

Reactions involving cyanogen bromide (CNBr) were carried out in a fume cupboard. Sepharose-6B (Pharmacia Fine Chemicals Ltd) was activated with CNBr (Koch-Light Laboratories Ltd, Bucks, England) essentially as described by Cuatrecasas (1970). 5ml Sepharose-6B was washed with water (4°C), suspended in 5ml water, and 1g finely powdered CNBr was added. The solution was adjusted to pH 11, and kept at this pH during the course of the activation (about 25min), by titration with 2M-NaOH. The activated Sepharose was filtered rapidly on a sintered glass funnel, washed with 75ml water (4°C), then 10 mmoles 1,6-diaminohexane in 5ml water (previously adjusted to pH 10 with conc. HCl) was added to the funnel. The suspension was mixed, transferred to a small stoppered bottle, and stirred at 4°C for 20h. The suspension was then filtered and washed thoroughly with 1 litre water, 500ml 0.1M-Na_2CO_3 and a further 500ml water.

(b) Reaction of Aminohexylamino-Sepharose with 5'-carboxyeosin

5'-Carboxyeosin (0.1 mmoles, 70mg) was dissolved in 4ml dimethylformamide (DMF) and added to the aminohexylamino-Sepharose (AHA-Sepharose) from (a). The suspension was stirred for 5min

to allow the DMF to penetrate the Sepharose then 0.5 mmoles (90mg) 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (Ott Chemical Co., Muskegon, Michigan, U.S.A.) was added and the pH adjusted to 5 with 2M-HCl. A further 2ml DMF was added and the suspension was stirred at 4°C for 20h. The suspension was then filtered on a sintered glass funnel and unreacted inhibitor removed by thorough washing with 1 litre DMF-0.2M-NaOH (1:1,v/v), 500ml 0.1M-NaOH and then water until the filtrate was neutral. The Eosin-AHA-Sepharose was stored at 4°C as a suspension in water.

(c) Reaction of Aminoethylamino-Sepharose with 5'-carboxyfluorescein and 5'-carboxyeosin

Sepharose-6B (10ml) was activated with CNBr (50mg/ml) then reacted with 24 mmoles ethylenediamine hydrate. Half the aminoethylamino-Sepharose (AEA-Sepharose) was reacted with 0.05 mmoles 5'-carboxyfluorescein and half with 0.05 mmoles 5'-carboxyeosin as described above, and unreacted inhibitor was removed by the same washing procedure.

(3) Batch Method using Inhibitor-Substituted Sepharose

The substituted Sepharoses (1ml of settled gel) were stirred with 10ml enzyme solution (grass grub acetone powder, 1mg/ml water or pyrophosphate buffer pH 8.3) for 15min and the supernatants assayed after centrifugation. Elution from the gels was attempted with 5ml pyrophosphate buffer, pH 8.3, containing 2mM-GSH.

(4) Column chromatography using Inhibitor-substituted Sepharose

A small glass column (dimensions 0.5cm x 12cm) was fitted at each end with plastic stoppers through which number 12 stainless steel hypodermic needles passed. The column was packed at room temperature with the Sepharose derivative and equilibrated at 4°C with 0.01M-pyro-

phosphate buffer, pH 8.3. Enzyme solution (grass grub acetone powder , 20mg/ml buffer) was pipetted onto the top of the column and allowed to drain into the gel. Elution of GSH S-aryltransferase was attempted with a variety of solutions which were fed into the column through teflon tubing attached to the upper needle (details are given in the Results section). Fractions (usually 5 drops; approximately 0.2ml) were collected with the LKB UltroRac 7000 fraction collector.

RESULTS

STABILITY OF GSH S-ARYLTRANSFERASE

Grass grubs were homogenized in water and the stability of GSH S-aryltransferase in the pH 5-treated 13 000g supernatant was measured at 37°C and pH 5-9, using 5mM-GSH and 0.1mM-CDNB for the assays at the same temperature. Enzyme solution was diluted ten-fold with the appropriate buffer before incubation. The results (Figs. 1 & 2a) indicate that the enzyme is unstable over the entire pH range and that the instability increases with increasing pH. Reaction traces were linear for the first 20-30 seconds. Stability at pH 9 was improved by addition of GSH to the buffer (Fig. 2b).

Incubation of the 13 000g supernatant at pH 7 and 40-50°C resulted in loss of over half the activity in the two minutes required for temperature equilibration, and the remainder of the activity disappeared in the next 5-15 minutes. Protein was not precipitated during the heating.

Comparison of Frozen and Live Grass Grubs

The stability of the enzyme from frozen and live grubs was compared at 4°C and pH 5-9, using CDNB and DCNB as substrates. GSH (2mM) was included in one set of solutions from frozen grubs. Enzyme solution was diluted ten-fold before addition of GSH and pH adjustment (with 2M-NaOH) for CDNB measurements, but was incubated undiluted for DCNB studies. Activity was measured at pH 8.3, 37°C, using 2mM-GSH and either 0.1mM-CDNB or 0.45mM-DCNB. The effect on the reaction rates of GSH in the stored solutions was negligible. Figure 3 shows the loss of activity in solutions from frozen grubs for up to 50h after preparation. Graphs of log (activity remaining) against time (Fig. 4) are non-linear for the DCNB assays and the CDNB assays of solutions stored at pH 7-9, but are

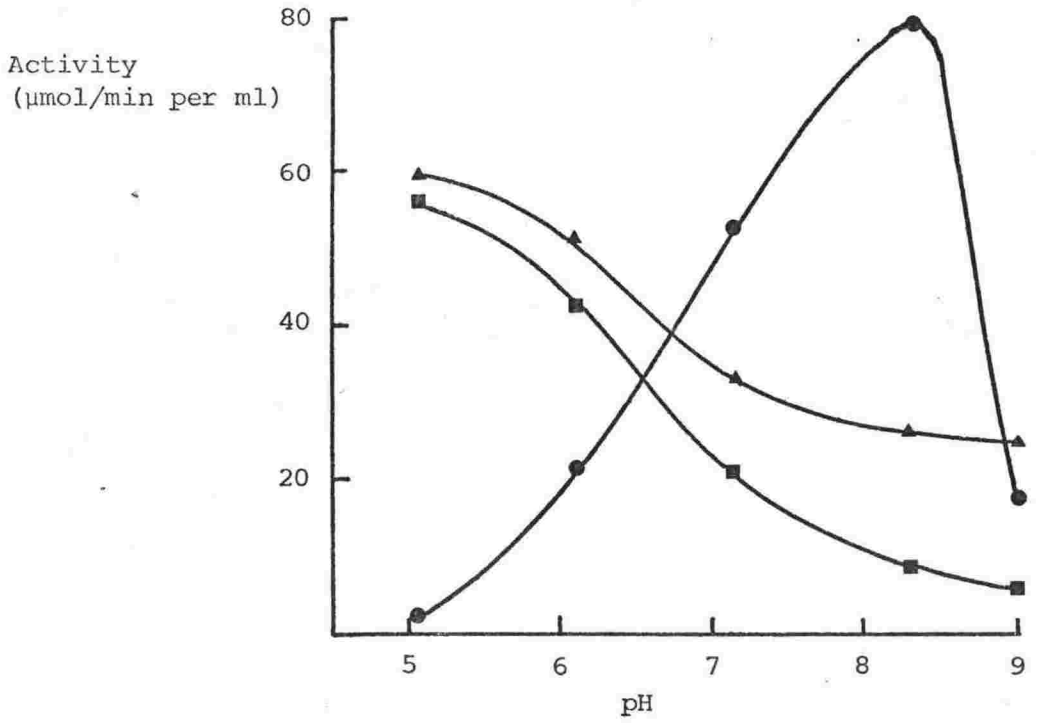


Fig. 1. pH stability at 37°C of GSH S-aryltransferase in pH 5-treated 13 000g supernatant from grass-grubs stored frozen. Assays at 37°C used 5mM-GSH and 0.1mM-CDNB.
● , solutions were assayed at the pH indicated;
▲& ■ , solutions were assayed at pH 8.3 after incubation at the indicated pH for 5 or 10 min respectively.

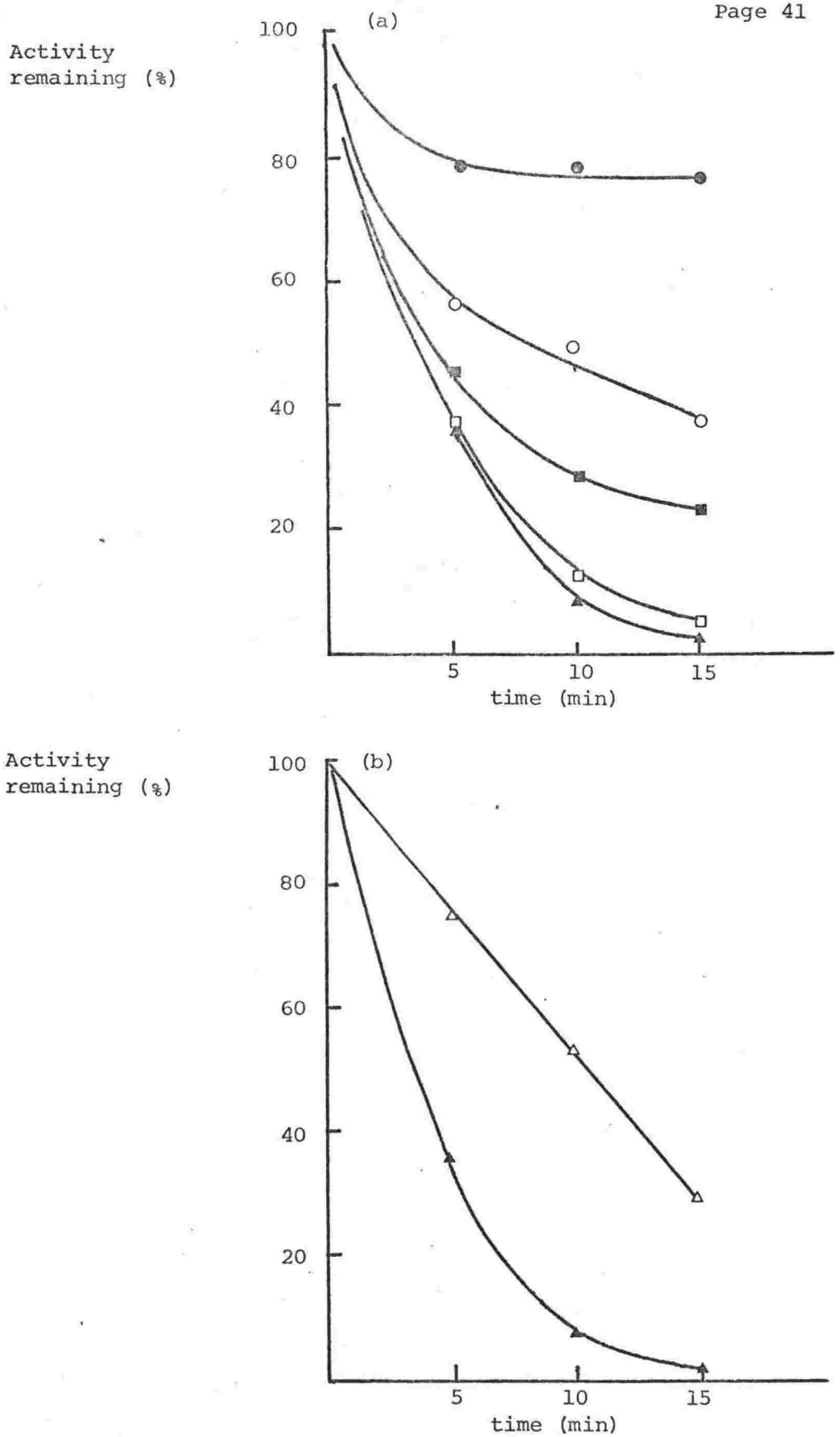


Fig. 2. Stability of GSH S-aryltransferase at 37°C and pH 5.1-9 in pH 5-treated 13 000g supernatant from grass-grubs stored frozen. Assays, at 37°C, and pH 8.3, used 5mM-GSH and 0.1mM-CDNB. Solutions were incubated at the following pH values:
(a): ● , 5.1; ○ , 6.1; ■ , 7.1; □ , 8.3; ▲ , 9.0.
(b): ▲ , 9.0; △ , 9.0 in the presence of 1mM-GSH.

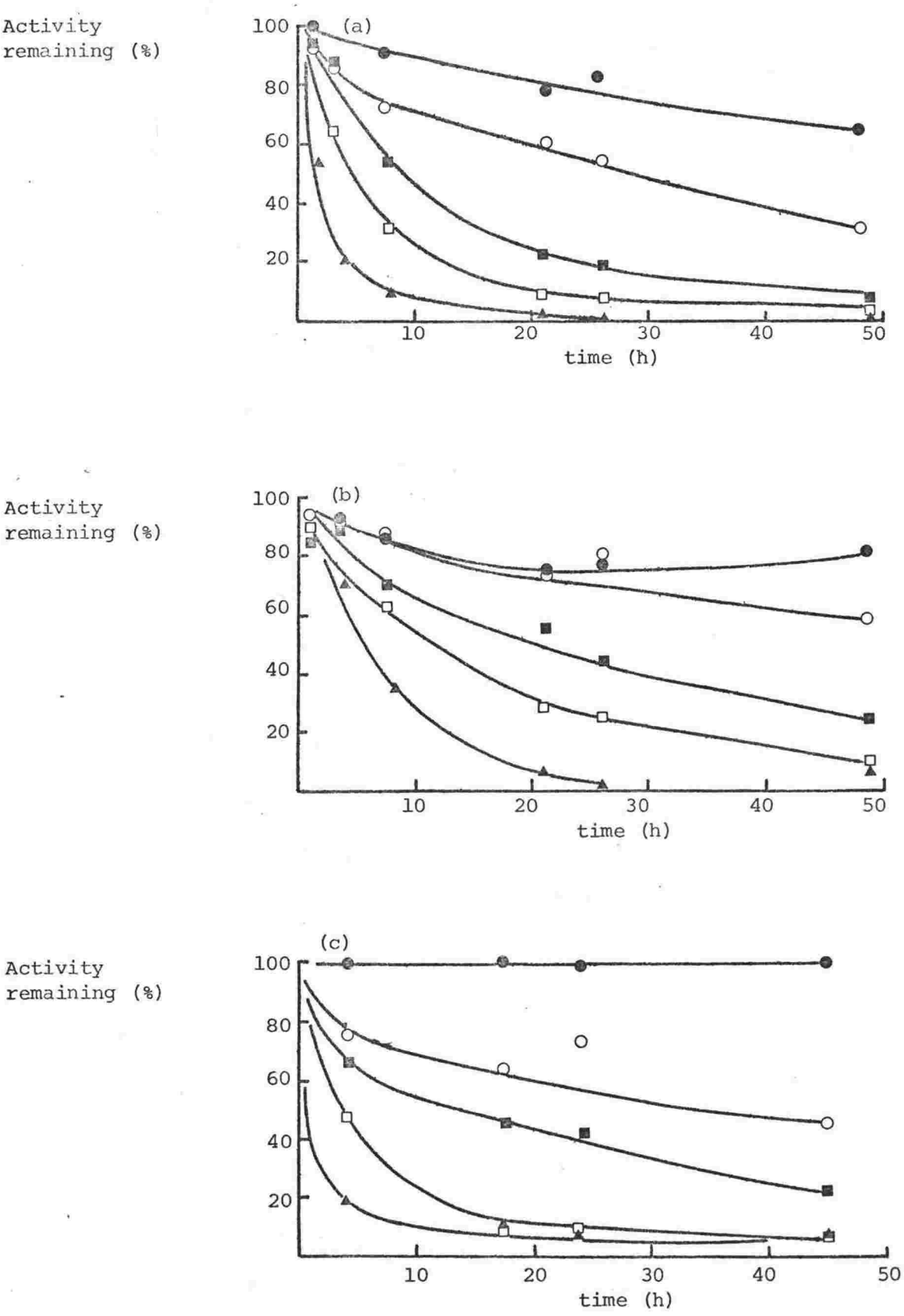


Fig. 3. Stability of GSH S-aryltransferase at 4°C and pH 5-9 in pH 5-treated 13 000g supernatant from grass-grubs stored frozen. Solutions were incubated at the following pH values:
● , 5; ○ , 6; ■ , 7; □ , 8; ▲ , 9. Assays at 37°C and pH 8.3, used 2mM-GSH and:
(a), 0.1mM-CDNB; (b), 0.1mM-CDNB with 2mM-GSH included in the incubations at 4°C; (c), 0.45mM-DCNB.

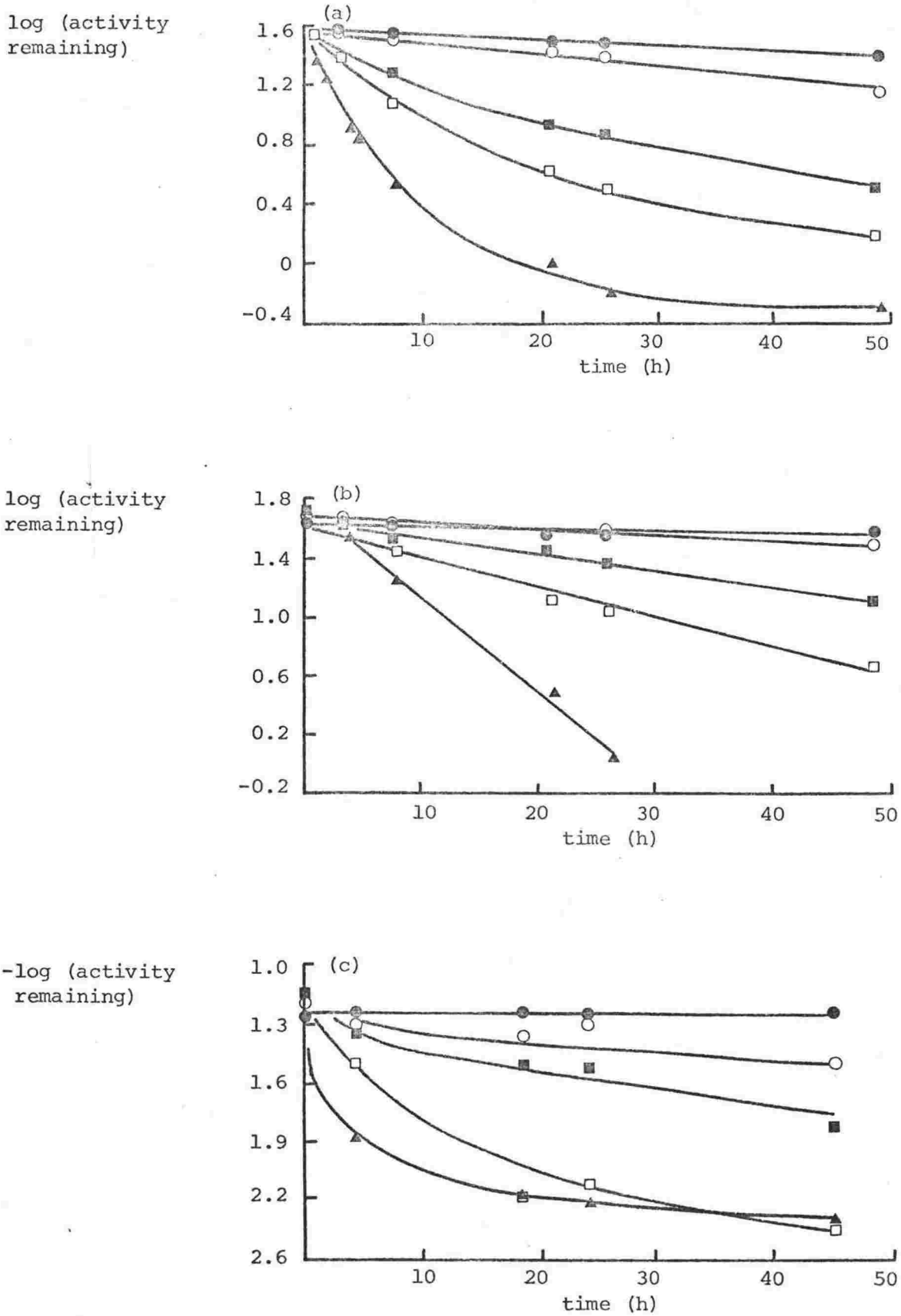


Fig. 4. Logarithmic decay curves for GSH S-aryltransferase at 4°C in pH 5-treated 13 000g supernatant from grass-grubs stored frozen. Solutions were incubated at the following pH values: ● , 5; ○ , 6; ■ , 7; □ , 8; ▲ , 9. Assays, at 37°C and pH 8.3, used 2mM-GSH and:
(a) 0.1mM-CDNB; (b) 0.1mM-CDNB with 2mM-GSH included in the incubations at 4°C; (c) 0.45mM-DCNB.

linear for CDNB assays of solutions stored at pH 5-6 and in the presence of GSH. Half lives for the loss of 50% of the total activity are plotted against pH in Fig. 5. Greater than 90% of the initial activity in the supernatant from live grubs remained after 45-50h at pH5-9.

Addition of up to 20mM-GSH to phosphate buffer, pH 6.9, before homogenization further stabilized the enzyme, but the effect was less marked at the higher concentrations (Fig. 6). Activity was not restored in inactivated solutions when concentrations of GSH which saturated the enzyme were used in the assays (Fig. 7).

The results of a typical experiment comparing stability of the enzyme from frozen and live grubs, and live grubs from which the hind gut contents had been squeezed out, are shown in Fig. 8. The enzyme was much more stable from live grubs than from those stored frozen, and stability in the live grubs was enhanced by removal of the hind gut contents. The rate of loss of activity was linear in solutions from live grubs. Although the total activity from live grubs was halved by removal of the gut contents (probably with some tissue), after three days at 4°C as much activity remained as in the homogenate of whole grubs.

Effect of Acetone Powder Preparation

Preparation of an acetone powder as a first purification step was investigated. The yield of powder was usually about 1% of the weight of grubs taken, and the specific activity about 1.9 U (µmol product formed/min) per mg protein when assayed under the standard conditions with CDNB. At pH 7 the enzyme was only slightly more stable than in the 13 000g supernatant at the same pH. The effect of homogenizing the grubs in acetone followed by rapid filtration and a second maceration in pH 6.9 phosphate buffer was also examined. Enzyme stability in the 13 000g supernatant was compared with stability in supernatants prepared in the normal way from frozen and live grubs (Fig. 9). The acetone

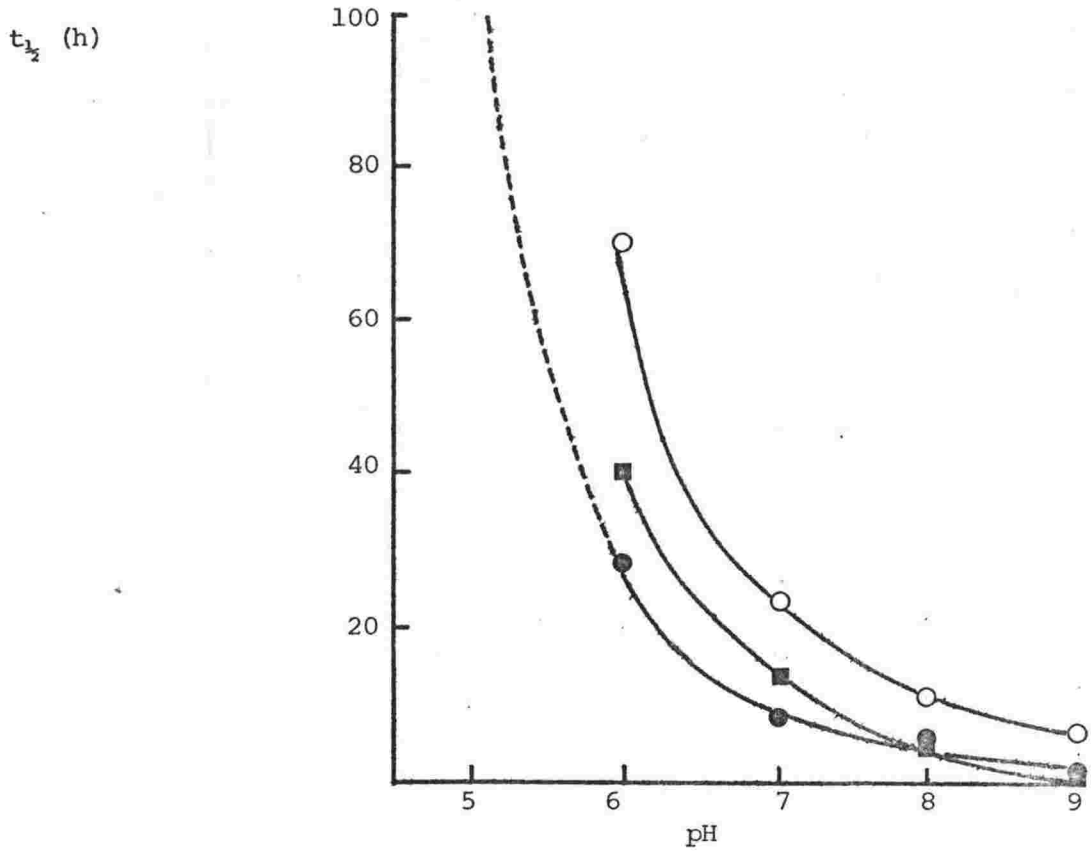


Fig. 5. Effect of pH on half-life of GSH S-aryltransferase activity at 4°C. Assays, at pH 8.3 and 37°C, used 2mM-GSH and:
● , 0.1mM-CDNB; O , 0.1mM-CDNB with 2mM-GSH in the incubations at 4°C; ■ , 0.45mM-DCNB.

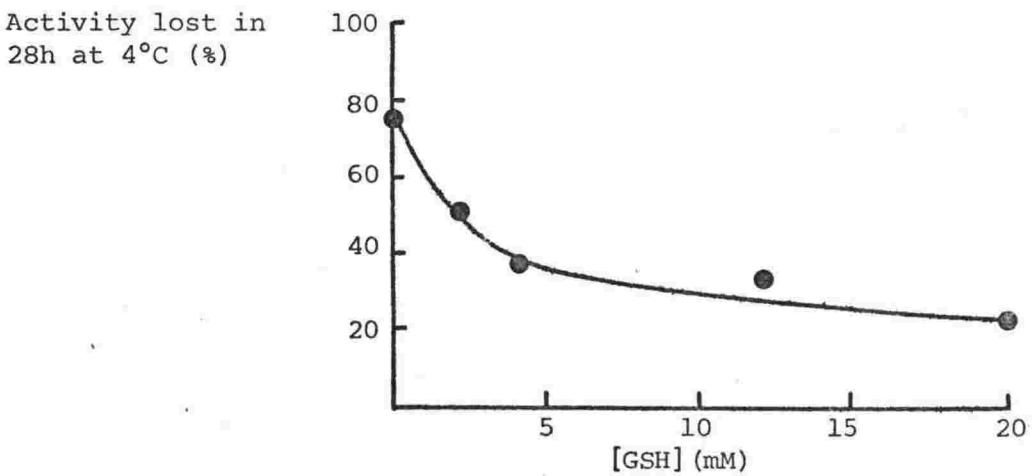


Fig. 6. Effect of including GSH in the homogenizing buffer on the stability of GSH S-aryltransferase at 4°C and pH 6.9.

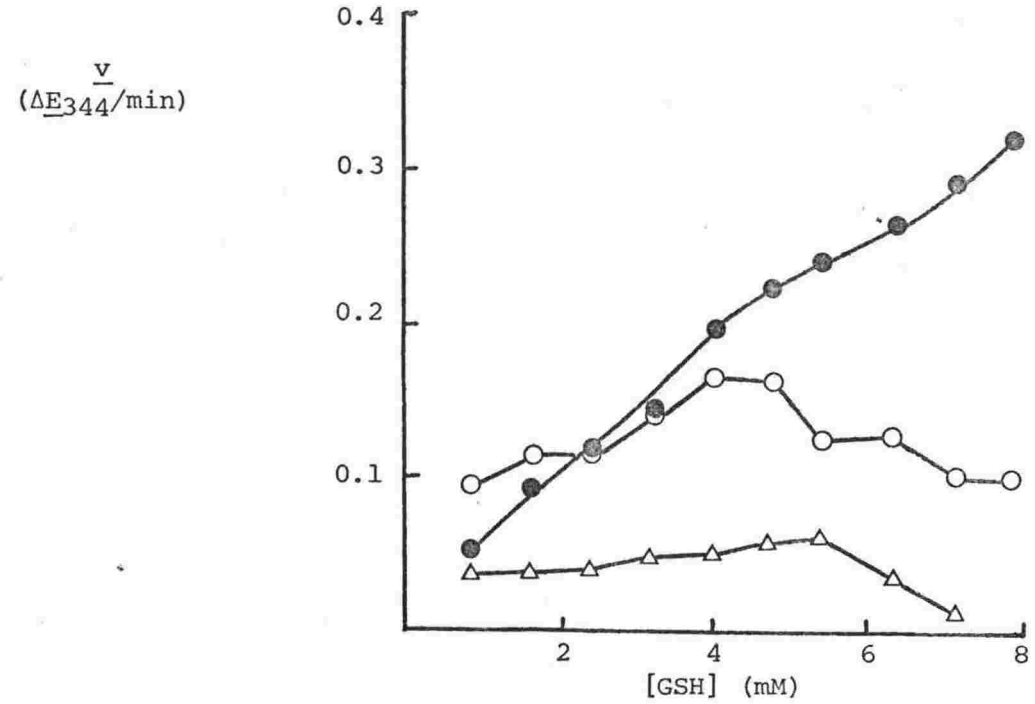


Fig. 7. Effect of GSH concentration on GSH S-aryltransferase activity in freshly prepared, and aged, homogenates.
● , non-enzymic reaction; ○ , initial enzyme activity;
△ , enzyme activity after 24h.

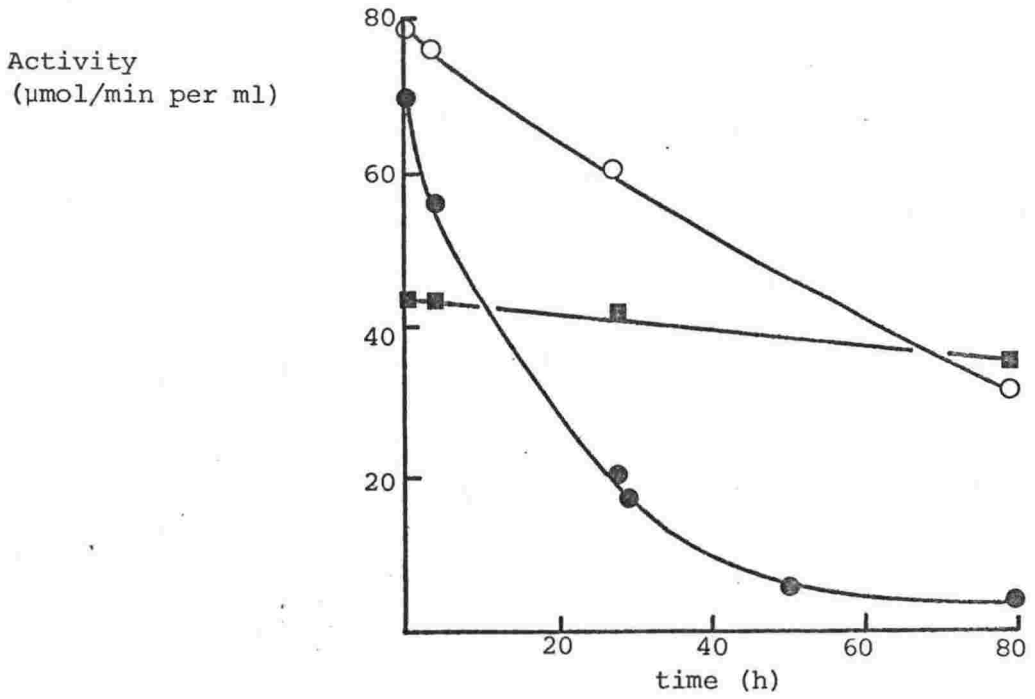


Fig. 8. Stability of GSH S-aryltransferase from live grass-grubs and grass-grubs that had been stored frozen. The 13 000g supernatant was prepared in phosphate buffer, pH 6.9, and stored at 4°C.
● , frozen grubs; ○ , live grubs; ■ , live grubs from which the hind gut contents had been removed.

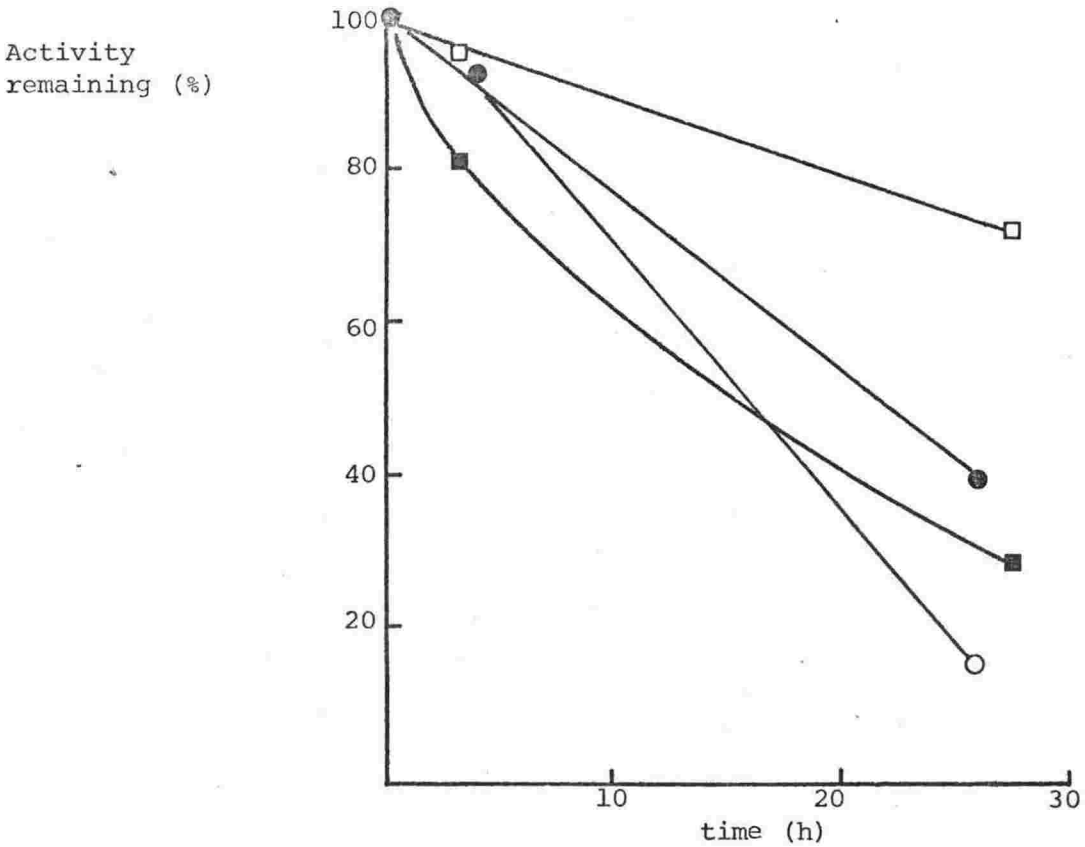


Fig. 9. Effect of an initial homogenization in acetone on the stability of GSH S-aryltransferase from grass-grubs stored frozen. Grubs were homogenized in acetone at -20°C (50g/100ml) then re-homogenized in phosphate buffer (pH 6.9, 100ml) after rapid filtration.

● , activity in the 13 000g supernatant; ○ , activity in dialysed 13 000g supernatant; ■ & □ , activity in the 13 000g supernatant prepared from frozen and live grubs, respectively, without the acetone treatment.

treatment improved stability, but not sufficiently to warrant use of the technique as a routine purification procedure, and dialysis against phosphate buffer to remove residual acetone resulted in further loss of activity.

Effect of Polyvinylpyrrolidone

Inclusion of a soluble polyvinylpyrrolidone (PVP; average molecular weight 12 000; May & Baker Ltd, Dagenham, England) in the buffer (phosphate, pH 6.9) before homogenization had, generally, little effect on the stability of GSH S-aryltransferase in the 13 000g supernatant with the exception of the 9% (w/v) solution in which stabilization was probably significant (Table 4), but all concentrations appeared to increase the total activity present initially.

Table 4. Effect of PVP on GSH S-aryltransferase stability at 4°C, pH6.9

Homogenizing Soln.		Initial Activity	% activity remaining after	
	%(w/v)PVP	U/ml	20h	44h
Water	0	55	35	9
P_i buffer	0	62	37	13
P_i buffer	1	64	38	13
P_i buffer	2	73	36	15
P_i buffer	3	73	44	17
P_i buffer	9	72	54	30

Addition of 10% (w/v) of the insoluble polyvinylpyrrolidone Bio-Clar^{TM}-G (BIORAD) to the supernatant and centrifugation after stirring for 15min failed to improve enzyme stability.

Effect of Ionic Strength

A 13 000g supernatant was prepared in phosphate buffer, pH 6.9, and diluted with water, buffer or buffer plus NaCl to give ionic strengths from 0.01 - 0.50M. Apart from the lowest ionic strength, at which the

enzyme appeared to be slightly more labile, a similar amount of activity was lost at each ionic strength in 21h at 4°C (Table 5).

Table 5. Effect of Ionic Strength on GSH S-aryltransferase stability at 4°C

Ionic Strength	Initial activity	Activity remaining after 21h
M	U/ml	%
0.01	61.5	15.3
0.10	60.9	28.6
0.15	57.5	25.9
0.20	61.2	23.9
0.30	61.2	22.9
0.40	55.2	24.3
0.50	59.5	28.4

Effect of Enzyme Inhibitors on GSH S-aryltransferase Stability

Incubation of 13 000g supernatant at pH 7 and 4°C with diisopropylphosphorofluoridate (DFP; stock solutions in ethanol) had negligible effect on the stability of the enzyme (Table 6). Dilution of the supernatant had little effect on the enzyme's stability. DFP had no measurable effect on the non-enzymic reaction between GSH and CDNB.

Table 6. Effect of DFP on GSH S-aryltransferase stability at 4°C

Dilution of 13 000g supernatant	% activity remaining after 22h at 4°C			
	DFP (M)			
	0	5.4×10^{-6}	5.4×10^{-7}	5.4×10^{-8}
0	51	51	45	49
1/100	55	54	51	52
1/500	59	53	54	54

The effects of heavy metal ion chelating agents, p-chloro mercuribenzoate (p-CMB), sodium dithionite, several thiols and the aromatic substrate CDNB on aryltransferase stability are shown in Table 7 and Figs. 10 and 11. Solutions were diluted ten-fold before an aliquot* was taken for assay and hence the concentration of the reagent in the assay was negligible, *(0.01 ml).

Table 7. Effect of heavy metal ion chelators, dithiothreitol (DTT), cysteine and GSH ion GSH S-aryltransferase stability at 4°C

Reagent*	Concn. (mM)	% activity remaining after 22 - 23h	
		13 000g supernatant	pH5-treated 13 000g supernatant
-	-	41	30
NaCN	10	34	33
NaCN	20	34	31
NaN_3	5	42	38
NaN_3	10	44	31
p-CMB	2.5	41	49
p-CMB	5	53	36
EDTA	2	35	32
EDTA	5	50	36
DTT	1	43	38
DTT	2	49	42
GSH	2	64	60
GSH	5	77	72
Cys	2	62	55
Cys	5	59	58

*reagents were added to the 13 000g supernatant.

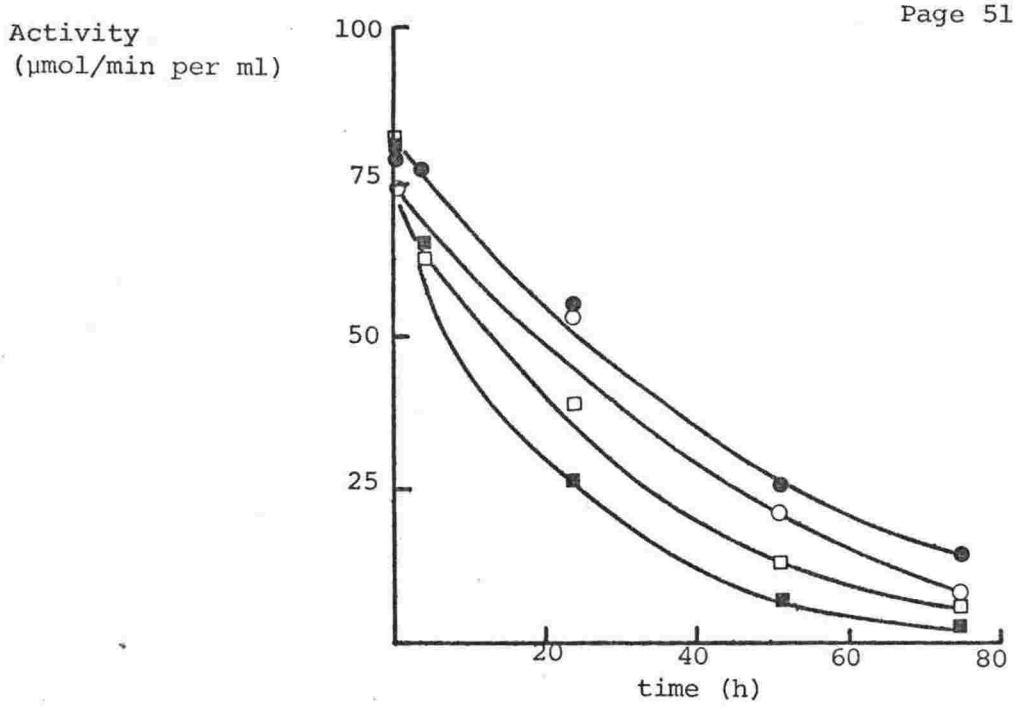


Fig. 10. Effect on GSH S-aryltransferase stability at 4°C of GSH (● , 2mM), GSH plus dithiothreitol (O , each 2mM), NaCN (■ , 10mM) and $Na_2S_2O_4$ (□ , 1mM). Reagents were added to the buffer (phosphate, pH 6.9) before homogenization and preparation of the 13 000g supernatant.

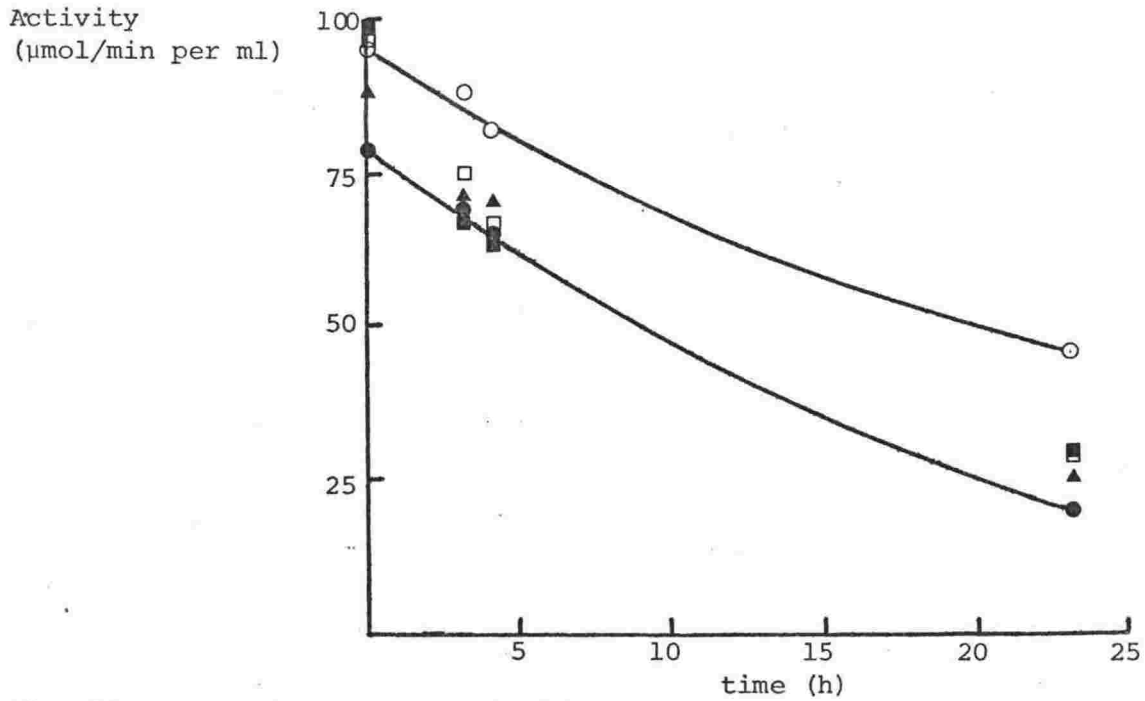


Fig. 11. Comparison of several thiols and CDNB as potential stabilizers of GSH S-aryltransferase at 4°C. Reagents were added to the buffer (phosphate, pH 6.9) before homogenization and preparation of the 13 000g supernatant.

● , control; O , 2mM-GSH; ■ , 2mM-Cys; □ , 2mM-DTT;
▲ , 2mM-mercaptoacetic acid.

Acid Phosphatase Measurements

Table 8. Acid Phosphatase Activity in Grass Grub Centrifugal Fractions

Fraction	Activity (μmol product / ml per h)					
	Water		0.25M-sucrose		0.44M-sucrose	
	Control	Triton-X	Control	Triton-X	Control	Triton-X
10 000g supernatant	3.2	3.8	3.5	3.8	3.8	4.2
23 000g supernatant	3.3	4.1	3.7	4.0	4.0	4.3
23 000g* ppt	-	-	-	-	-	-

*re-suspended in homogenizing solution.

ENZYME STABILIZATION AND PURIFICATION

AMMONIUM SULPHATE FRACTIONATION

Table 9. Ammonium Sulphate Fractionation of GSH S-aryltransferase at pH 6.9

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	110	82	9020	48.3	1.70	100	1
2.0M ppt.	50	25	1250	48.0	0.52	13.9	-
3.0M ppt.	25	126	3150	45.8	2.75	35.0	1.6
3.5M ppt.	10	141	1410	6.1	23.0	15.6	13.5
4.0M ppt.	10	151	1510	5.8	25.9	16.7	15.2
4.0M supernatant	1062	0	0	5.2	0		

The stability of the enzyme in each fraction is shown in Fig. 12. Most of the activity not recovered can be attributed to the instability of the enzyme. Eighty per cent of the protein in the 13 000g supernatant was recovered. Enzyme precipitated in the 0 - 2M fraction was more unstable than in the 13 000g supernatant, whereas the enzyme in the 3 - 3.5M and 3.5 - 4M fractions was stabilized considerably.

The effect of pH 5-precipitation of protein on the fractionation pattern is shown in Fig. 13. When much of the protein was first removed by the pH treatment a sharper fractionation of activity was obtained with a slight change in the peak of precipitation towards lower ionic strength. Both procedures improved the enzyme stability in the active fractions (Figs. 14a & b). The two most stable fractions from pH-treated supernatant contained more activity, but were slightly less stable, than the comparable fractions from untreated supernatant.

The stability, at pH 5.5 - 9.0, of the precipitates from ammonium sulphate fractionation at pH 6.9 is shown in Fig. 15. Loss of activity in the 13 000g supernatant and the very unstable 1.8M precipitate increased as pH increased, whereas the loss in the partially stabilized fractions was little affected by pH.

The pattern of fractionation at pH 5.9 and stability of the fractions at the same pH is shown in Fig. 16. The peak of precipitation occurred at about the same ammonium sulphate concentration as at pH 6.9. Slight differences in stability may be due to the differences in position of the fractions.

Stability of the fractions from ammonium sulphate precipitation in 0.4M-acetate buffer at pH 5.5 when the fractions were taken at different concentrations is shown in Figs. 17, 18, 19 and 20. The results show (Figs. 19 & 20) that if a fraction is taken at about 2.3 to 2.4M-$(NH_4)_2SO_4$, aryltransferase is stable in fractions above this concentration. The

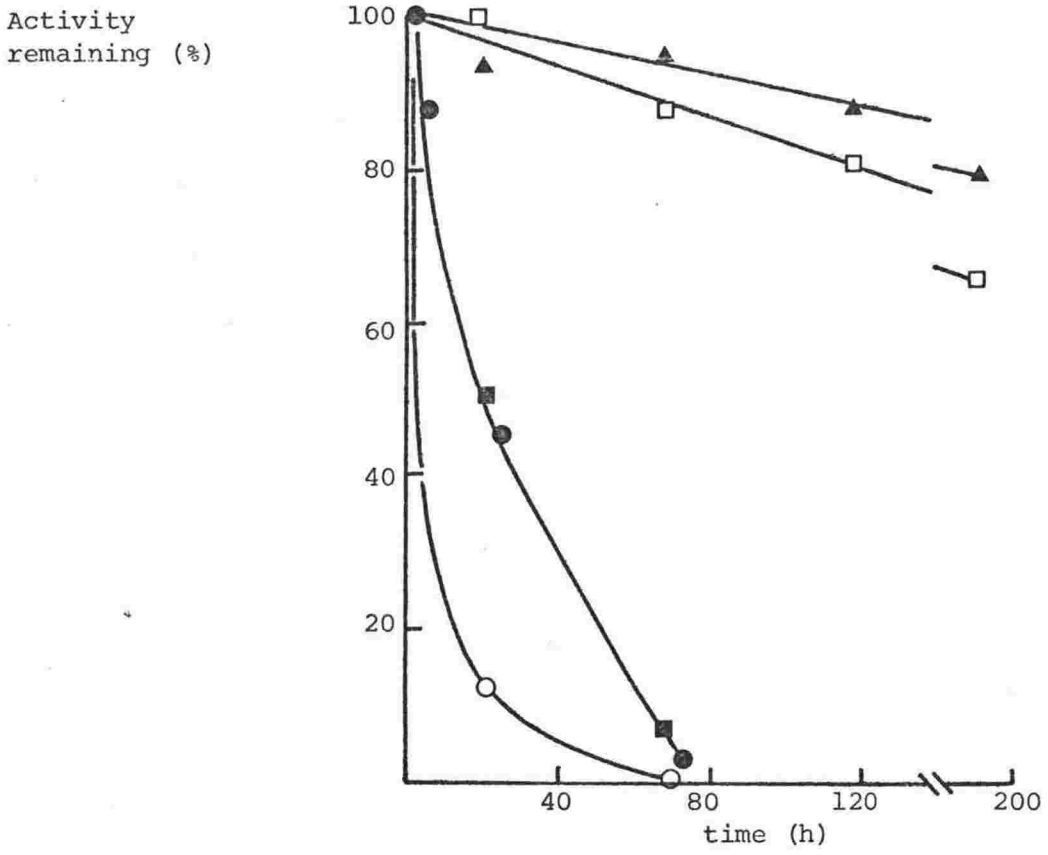


Fig. 12. Stability of GSH S-aryltransferase at 4°C in fractions from $(NH_4)_2SO_4$ treatment of the 13 000g supernatant.
● , 13 000g supernatant; ○ , 2M-$(NH_4)_2SO_4$ ppt.;
■ , 3M ppt.; □ , 3.5M ppt.; ▲ , 4M ppt.

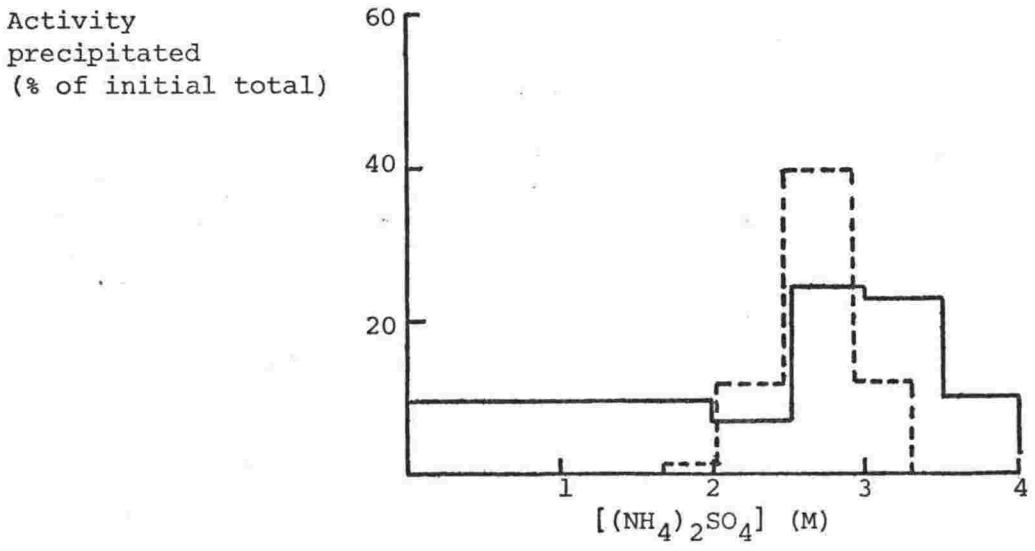
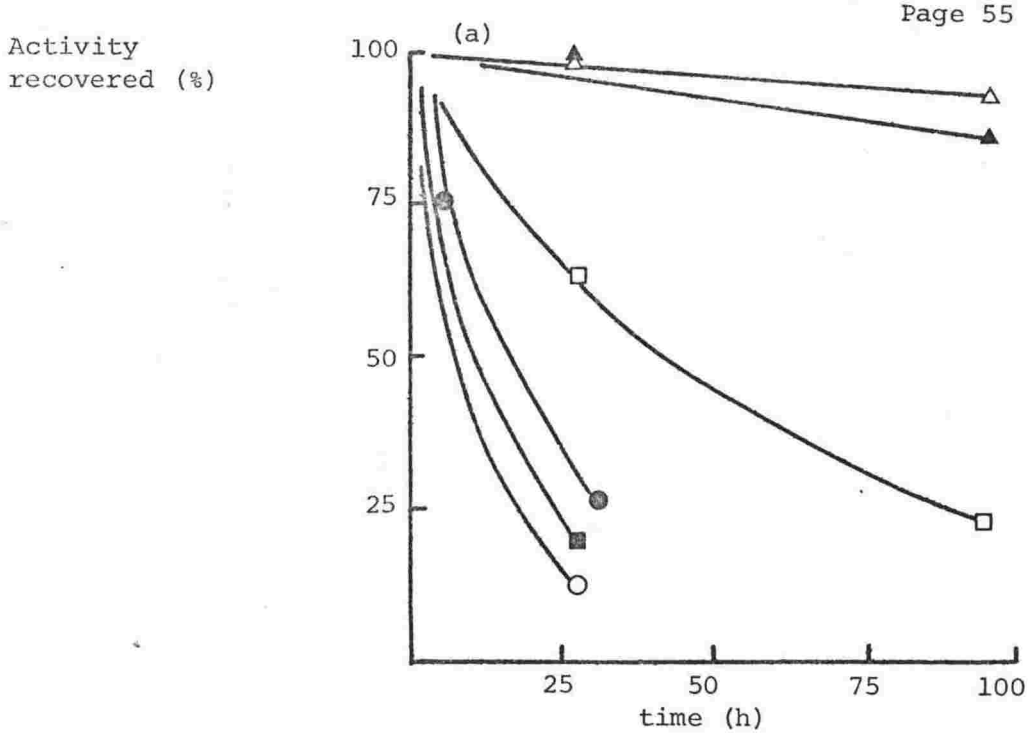


Fig. 13. $(NH_4)_2SO_4$ fractionation of the 13 000g supernatant. The supernatant was halved, and, after pH 5-treatment of one half, both solutions were fractionated at pH 6.9. —— , 13 000g supernatant;
---- , pH 5-treated supernatant.



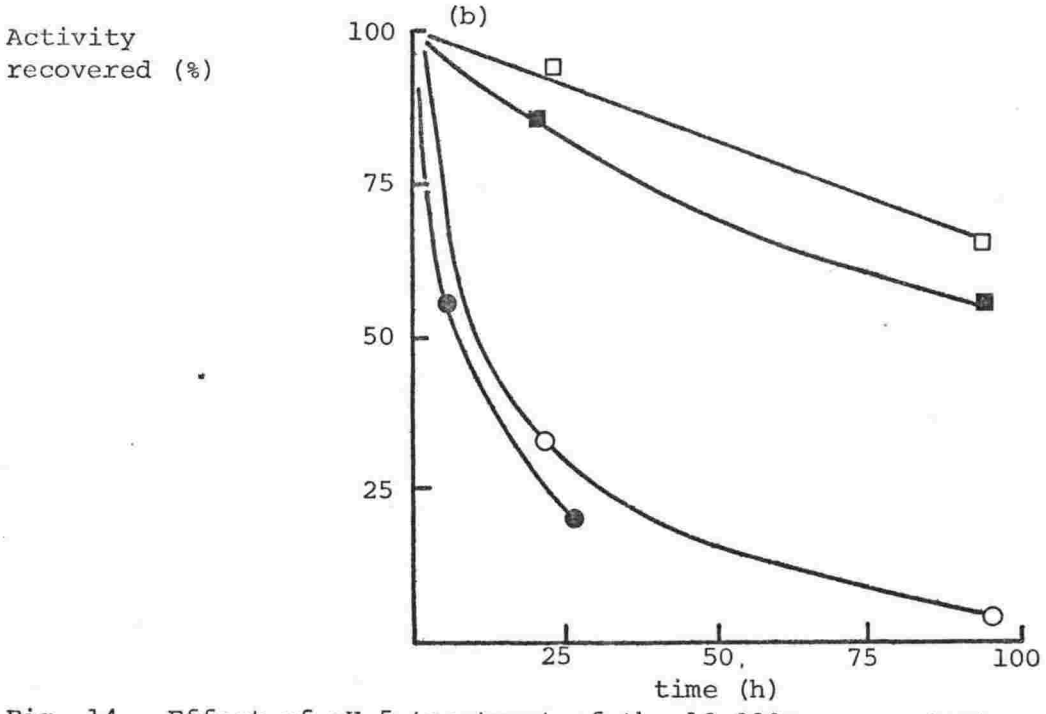


Fig. 14. Effect of pH 5-treatment of the 13 000g supernatant on the stability of GSH S-aryltransferase after $(NH_4)_2SO_4$ fractionation at 4°C and pH 6.9.

(a). 13 000g supernatant: ● , supernatant; ○, 2M-$(NH_4)_2SO_4$ ppt., ■, 2.5M ppt., □ , 3M ppt., ▲ , 3.5M ppt., △ , 4M ppt.

(b). pH 5-treated supernatant; ● , supernatant; ○ , 2.45M-$(NH_4)_2SO_4$ ppt.; ■ , 2.90M ppt.; □ , 3.30M ppt.

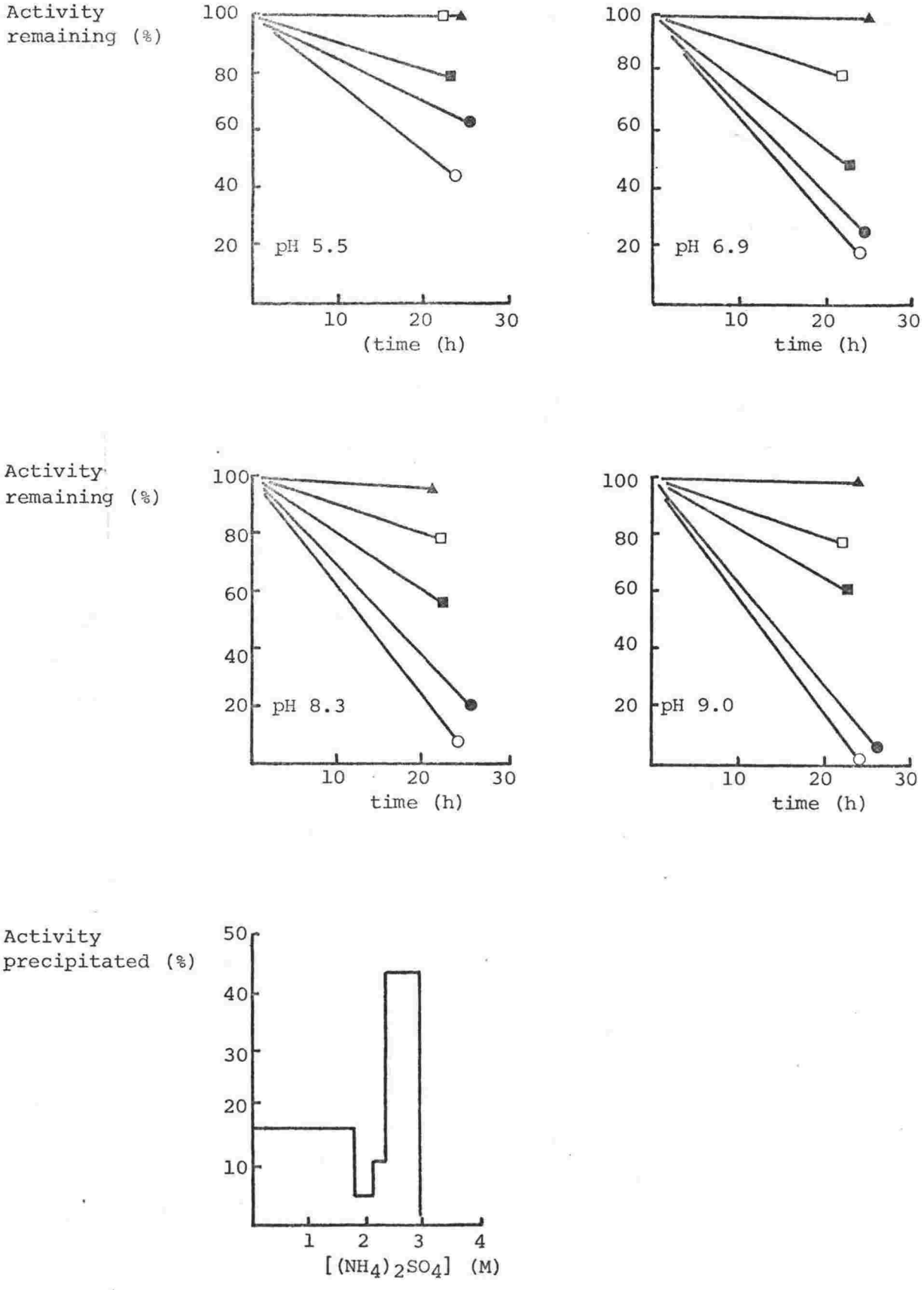


Fig. 15. Stability of GSH S-aryltransferase at 4°C and pH 5.5-9.0 in fractions from $(NH_4)_2SO_4$ treatment of grass-grub 13 000g supernatant at pH 6.9, and the pattern of precipitation of activity.
● , 13 000g supernatant; ○ , 1.8M-$(NH_4)_2SO_4$ ppt.;
■ , 2.1M ppt.; □ , 2.35M ppt.; ▲ , 2.94M ppt.

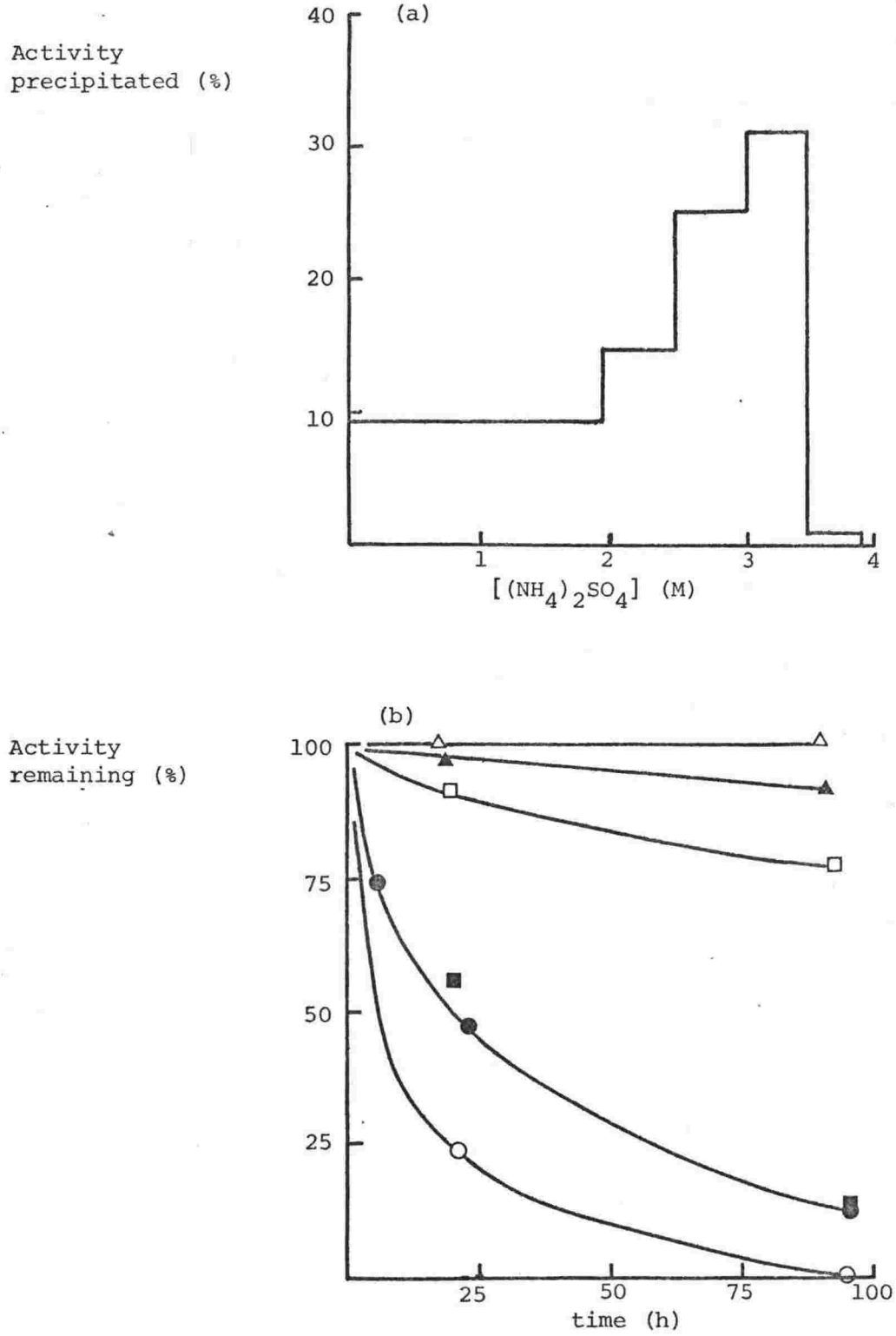


Fig. 16. (a) $(NH_4)_2SO_4$ precipitation of GSH S-aryltransferase activity at 4°C and pH 5.9.

(b) Stability of the redissolved precipitates at 4°C and pH 5.9. ● , 13 000g supernatant; ○ , 1.9M-$(NH_4)_2SO_4$ ppt.; ■ , 2.47M ppt.; □ , 2.97M ppt.; ▲ , 3.45M ppt.; △ , 3.9M ppt.

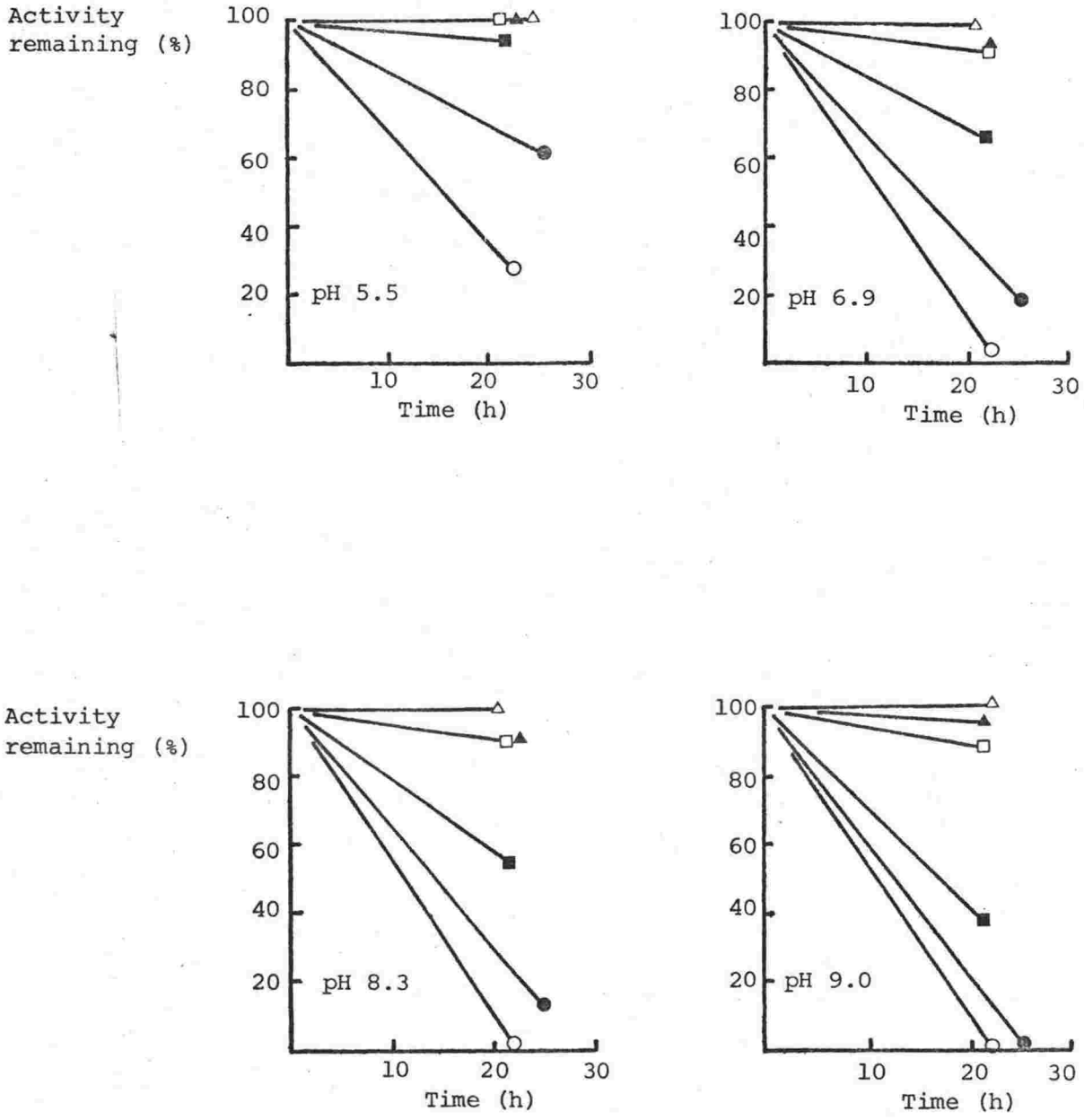


Fig. 17. Stability of GSH S-aryltransferase at 4°C and pH 5.5-9.0 in fractions from $(NH_4)_2SO_4$ treatment of grass-grub 13 000g supernatant at pH 5.5.

● , 13 000g supernatant; ○ , 1.8M-$(NH_4)_2SO_4$ ppt., ■ , 2.2M ppt., □ , 2.5M ppt.; ▲ , 2.9M ppt.; △ , 3.4M ppt.

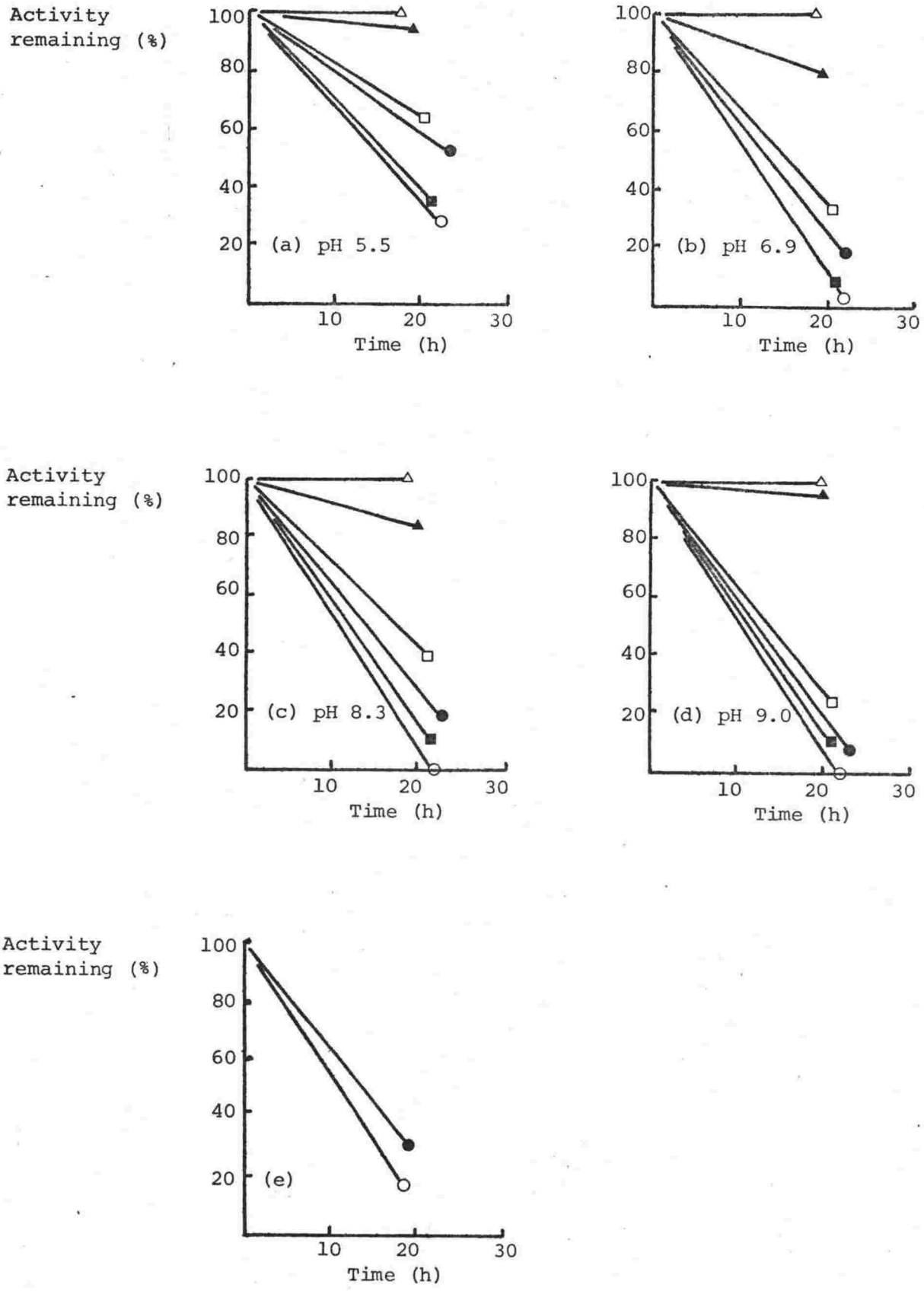


Fig. 18. Stability of GSH S-aryltransferase at 4°C and pH 5.5-9.0 in fractions from $(NH_4)_2SO_4$ treatment of grass-grub 13 000g supernatant at pH 5.5.
(a) - (d): ● , 13 000g supernatant; ○ , 1.8M-$(NH_4)_2SO_4$ ppt.; ■ , 2.06M ppt.; □ , 2.28M ppt.; ▲ , 2.9M ppt.; △ , 3.5M ppt.
(e): ● , 1.8M plus 2.9M ppts. at pH 6.9; ○ , same, at pH 8.3.

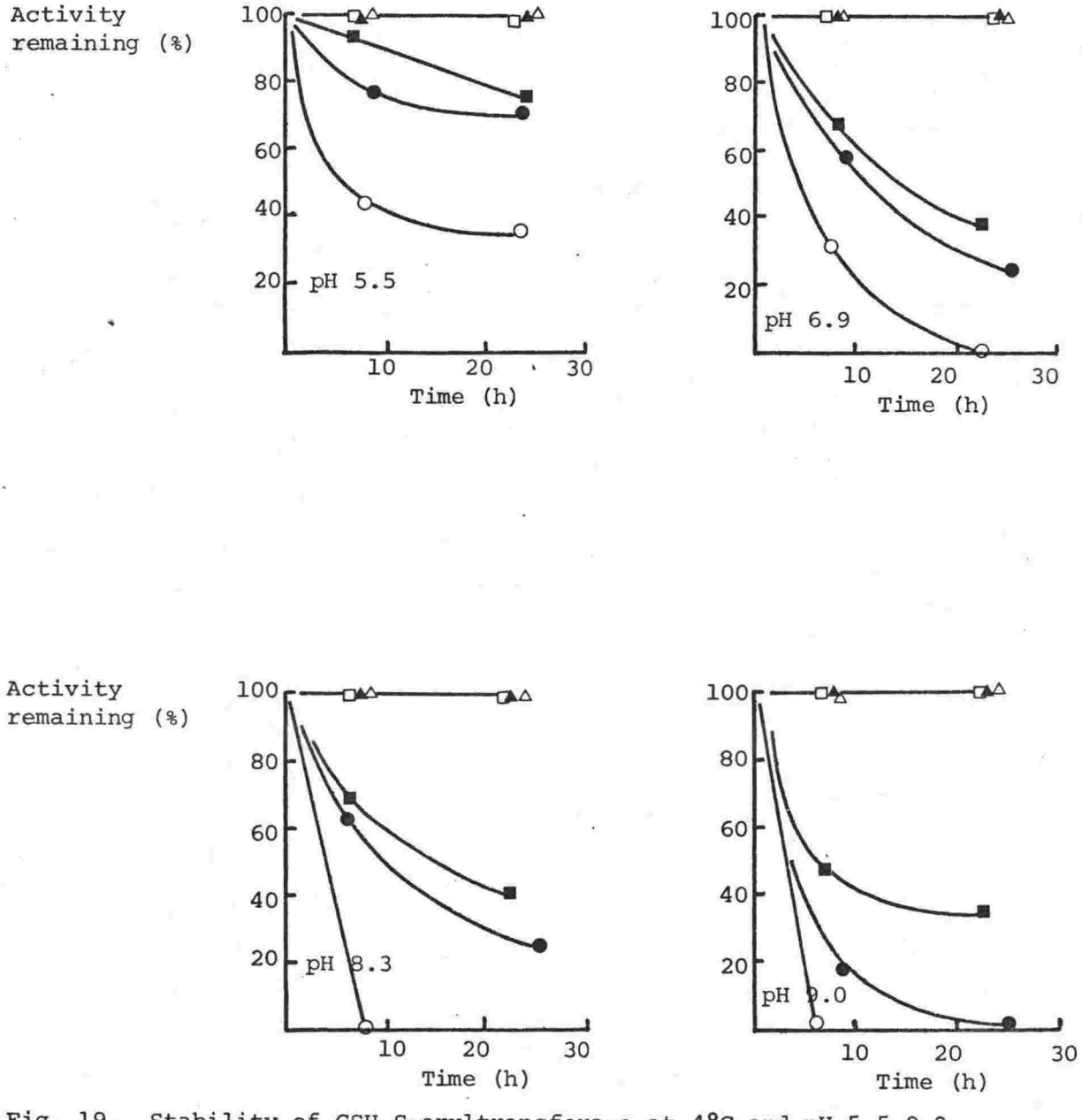


Fig. 19. Stability of GSH S-aryltransferase at 4°C and pH 5.5-9.0 in fractions from $(NH_4)_2SO_4$ treatment of grass-grub 13 000g supernatant at pH 5.5.

● , 13 000g supernatant; ○ , 1.8M-$(NH_4)_2SO_4$ ppt.; ■ , 2.34M ppt.; □ , 2.64M ppt.; ▲ , 3.3M ppt.; △ , 3.9M ppt.

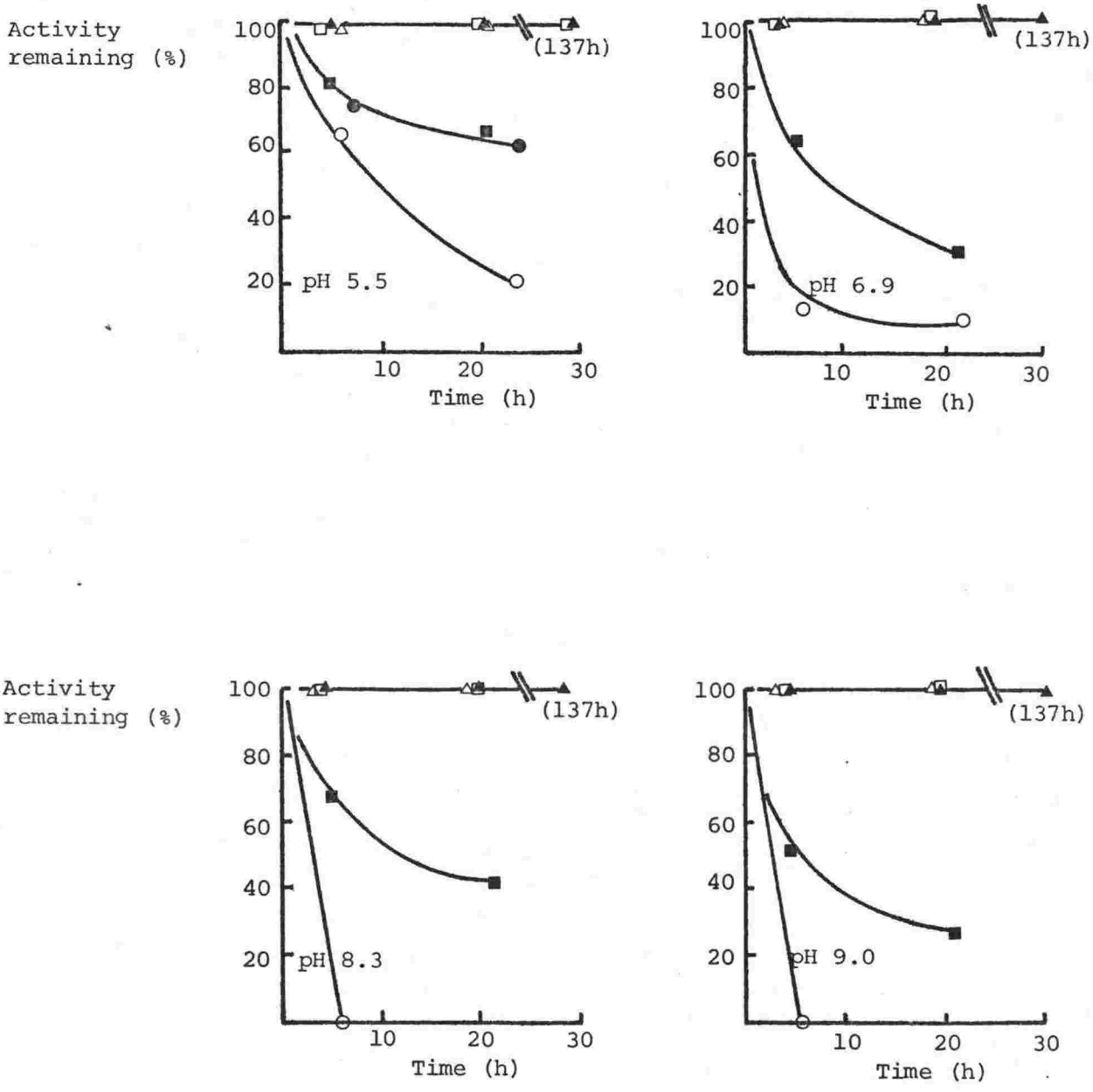


Fig. 20. Stability of GSH S-aryltransferase at 4°C and pH 5.5-9.0 in fractions from $(NH_4)_2SO_4$ treatment of grass-grub 13 000g supernatant at pH 5.5.

● , 13 000g supernatant; O , 1.8M-$(NH_4)_2SO_4$ ppt.; ■ , 2.4M ppt; □ , 2.9M ppt.; ▲ , 3.4M ppt.; △ , 3.8M ppt.

inclusion of extra fractions before 2.4M-$(NH_4)_2SO_4$ (Figs. 17 & 18) had little effect on stability in the active fractions. The recovery of activity in each fraction is shown in Tables 10, 11, 12 and 13, which correspond to Figs. 17, 18, 19 & 20 respectively.

Table 10. Ammonium Sulphate Fractionation of GSH S-aryltransferase at pH 5.5

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Yield (%)
13 000g supernatant	100	49.6	4960	100
1.8M-$(NH_4)_2SO_4$ ppt.	50	8.3	415	8.4
2.2M-$(NH_4)_2SO_4$ ppt.	25	41.5	1038	20.9
2.5M-$(NH_4)_2SO_4$ ppt.	25	45.8	1145	23.1
2.9M-$(NH_4)_2SO_4$ ppt.	10	163	1630	32.9
3.4M-$(NH_4)_2SO_4$ ppt.	10	22.9	229	4.6

Table 11. Ammonium Sulphate Fractionation of GSH S-aryltransferase at pH 5.5

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Yield (%)
13 000g supernatant	100	72.7	7270	100
1.80M-$(NH_4)_2SO_4$ ppt.	50	6.8	340	4.7
2.06M-$(NH_4)_2SO_4$ ppt.	10	11.1	111	1.5
2.28M-$(NH_4)_2SO_4$ ppt.	25	16.3	408	5.6
2.90M-$(NH_4)_2SO_4$ ppt.	25	135	3375	46.5
3.50M-$(NH_4)_2SO_4$ ppt.	10	135	1350	18.6
3.50M-$(NH_4)_2SO_4$ supernatant	100	0.6	60	0.8

Table 12. Ammonium Sulphate Fractionation of GSH S-aryltransferase at pH 5.5

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	112	49.8	5580	16	3.1	100	1
1.80M-$(NH_4)_2SO_4$ ppt.	50	2.5	125	8.2	0.3	2.2	
2.34M-$(NH_4)_2SO_4$ ppt.	25	44.1	1103	21.8	2.0	19.8	
2.64M-$(NH_4)_2SO_4$ ppt.	25	104	2600	9.3	11.2	46.6	3.6
3.30M-$(NH_4)_2SO_4$ ppt.	10	70	700	3.3	21.2	12.6	6.8
3.90M-$(NH_4)_2SO_4$ ppt.	10	3.5	35	2.3	1.5	0.6	

Table 13. Ammonium Sulphate Fractionation of GSH S-aryltransferase at pH 5.5

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	103	50	5150	24.3	2.06	100	1
1.80M-$(NH_4)_2SO_4$ ppt.	50	3	150	9.0	0.30	2.9	
2.40M-$(NH_4)_2SO_4$ ppt.	25	26	650	22.8	1.14	11.6	
2.90M-$(NH_4)_2SO_4$ ppt.	10	149	1490	19.5	7.60	28.9	3.7
3.40M-$(NH_4)_2SO_4$ ppt.	10	193	1930	8.4	23.0	37.5	11.2
3.80M-$(NH_4)_2SO_4$ ppt.	10	7	70	4.2	1.70	1.4	

Incubation of the enzyme in the 2.9M precipitate (from the experiment described in Table 11 and Fig. 18) with an equal volume of the 1.8M precipitate from the same experiment markedly decreased the stability of the active fraction (Fig. 18e).

Dialysis of the ammonium sulphate stabilized enzyme against water or dilute buffer resulted in further loss of activity. The amount lost was not consistent from one preparation to another and varied between 20 - 50%.

For studies of the ammonium sulphate-stabilized enzyme the active fraction was routinely collected between 2.3-3.3M-$(NH_4)_2SO_4$.

Pilot Scale Second Ammonium Sulphate Fractionation

The active fraction from the first ammonium sulphate precipitation was dialysed against water, one half was fractionated at pH 5 and the other at pH 8 after adjustment with 2M-NaOH. Results are shown in Tables 14a and 14b.

Table 14a. Second Ammonium Sulphate Fractionation; pH 5

Fraction	Vol. (ml)	Concn. (U/ml)	Total U*	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
first $(NH_4)_2SO_4$ precipitation	10	286	2860	19.5	14.7	100	1
1.4-1.9M-$(NH_4)_2SO_4$ ppt.	10	28.6	286	2.55	11.2	10	
1.9-2.4M-$(NH_4)_2SO_4$ ppt.	10	95.8	958	5.52	17.4	33.5	1.2
2.4-2.9M-$(NH_4)_2SO_4$ ppt.	10	114.5	1145	4.36	26.3	40	1.8
2.9-3.5M-$(NH_4)_2SO_4$ ppt.	10	4.2	42	0.57	7.3	1.5	

*total recovery in the precipitates = 85%

Table 14b. Second Ammonium Sulphate Fractionation; pH 8

Fraction	Vol. (ml)	Concn. (U/ml)	Total U*	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
first $(NH_4)_2SO_4$ precipitation	10	286	2860	19.5	14.7	100	1
1.4-1.9M-$(NH_4)_2SO_4$ ppt.	10	3.72	37.2	1.10	3.4	0.9	
1.9-2.4M-$(NH_4)_2SO_4$ ppt.	10	24.3	243	4.13	5.9	8.5	
2.4-2.9M-$(NH_4)_2SO_4$ ppt.	20	63.0	1260	3.28	19.2	44	1.3
2.9-3.4M-$(NH_4)_2SO_4$ ppt.	10	83.8	838	2.58	32.5	29	2.1

*total recovery in the precipitates = 83%

ISOELECTRIC FOCUSING

Analytical scale isoelectric focusing of the ammonium sulphate fraction showed the presence of two major peaks of GSH S-aryltransferase with isoelectric points (pI) of 4.75 and 8.7 (Fig. 21). Shoulders on the peaks suggest the presence of other enzymes. Two major aryltransferases with pI 4.65 and 8.9 again were found when a larger amount of another preparation was focused (Fig. 22). 1.8% of the pI 4.65 activity and 4.7% of the pI 8.9 activity was lost when stored at the isoelectric point at 4°C for 5 days. Total recovery of activity in both experiments was about 80% and approximately equal amounts of activity were in the two peaks.

Isoelectric focusing at pH 7-10 and collection of smaller fractions appeared to resolve aryltransferases with pI values of 6.6, 7.6 and 8.05 in addition to the major enzyme with pI 8.65 (Fig. 23). Enzyme solution was incorporated into the dense and light gradient solutions so that a uniform concentration of enzyme initially was present throughout the column. Recovery of activity (70%) was only slightly less than in

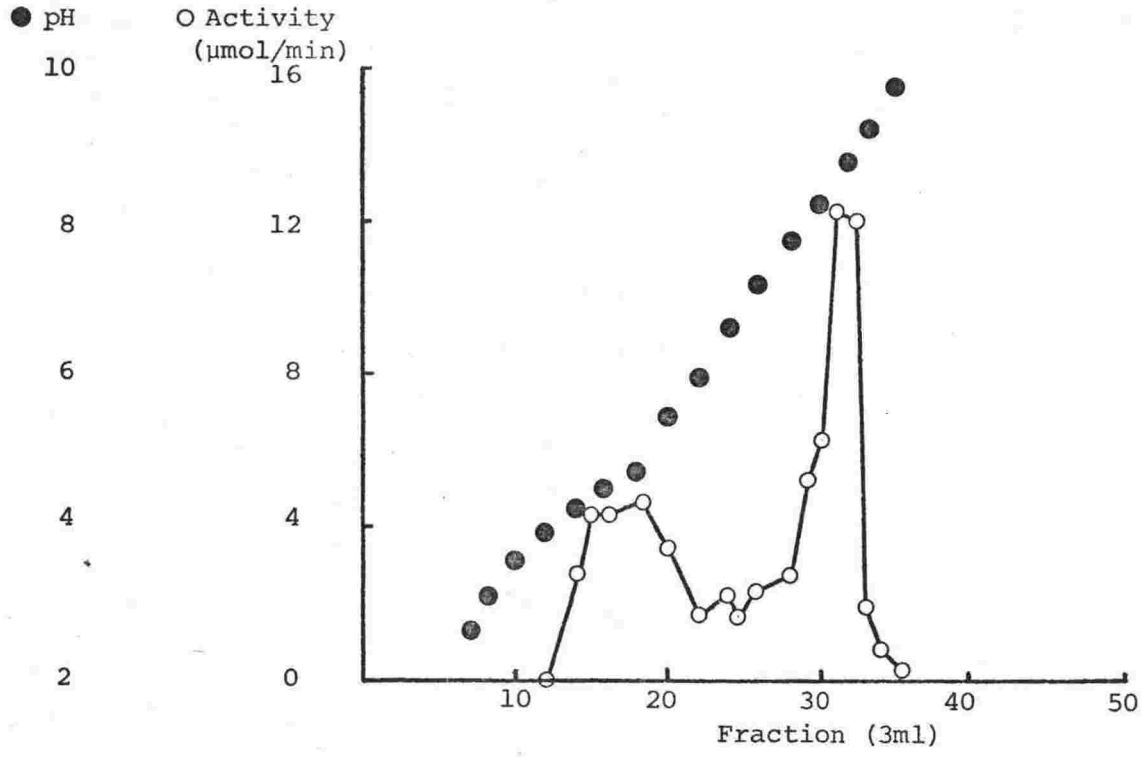


Fig. 21. pH 3-10 electrofocusing of grass-grub 2.3-2.9M-$(NH_4)_2SO_4$ ppt. Duration of focusing was 64h; total recovery was 80% of the 129U applied.

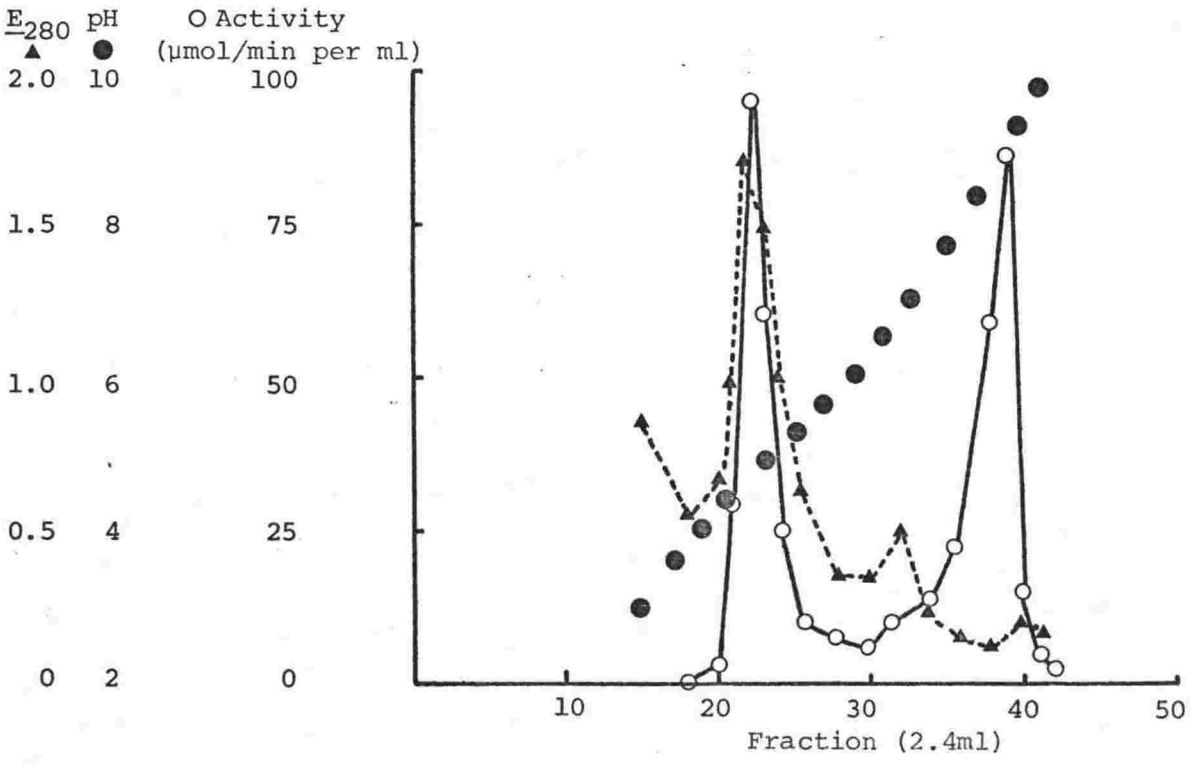


Fig. 22. pH 3-10 electrofocusing of grass-grub 2.3-2.9M-$(NH_4)_2SO_4$ ppt. Duration of focusing was 60h; total recovery was 72% of the 850U applied.

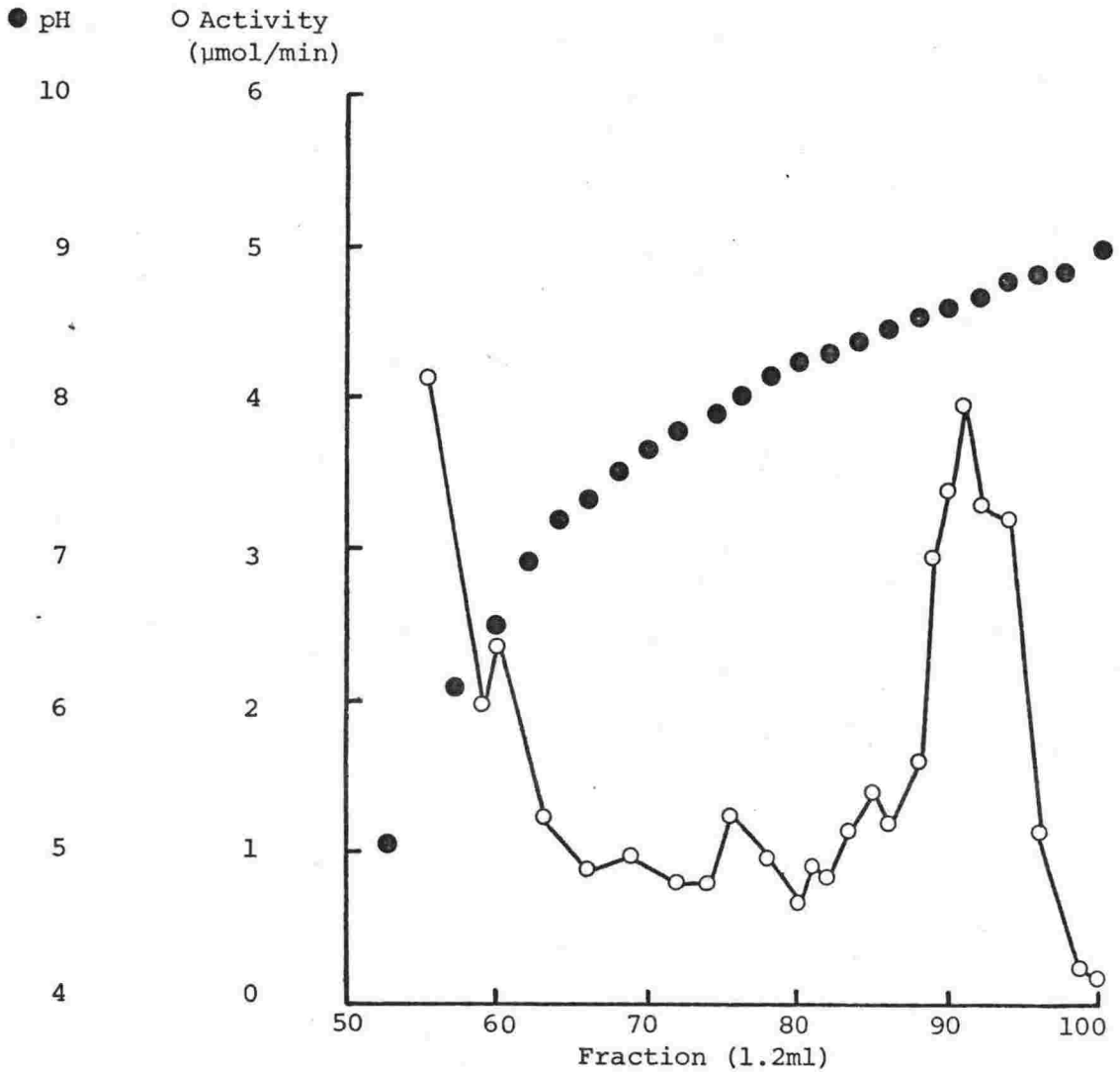


Fig. 23. pH 7-10 electrofocusing of grass-grub 2.3-2.9M-$(NH_4)_2SO_4$ ppt. Duration of focusing was 87h; total recovery was 50% of the 150U applied.

experiments in which the enzyme was placed in the middle of the density gradient, away from the pH extremes at the electrodes.

Isoelectric focusing at pH 3-10 of the ammonium sulphate fraction from live grass grubs is shown in Fig. 24. The result confirms the pattern obtained under similar conditions using deep-frozen grubs, but 50% of the activity recovered was as the pI 8.8 enzyme and only 31% as the pI 4.75 enzyme. Only 4% of the activity in the ammonium sulphate fraction was lost during dialysis against 0.01,-acetate buffer, pH 5.5, for 20h.

The result of pH 3-10 isoelectric focusing of the total 13 000g supernatant from a homogenate of one live grub in 10ml water is shown in Fig. 25. The pI 8.60 peak comprised 61% of the 19.3U recovered and the pI 4.65 peak, 26%.

ANION EXCHANGE CHROMATOGRAPHY

DEAE-Cellulose - Effect of Ionic Strength on elution of GSH S-aryltransferase

Ion exchange chromatography on DEAE-cellulose at pH 6.3 gave three peaks of GSH S-aryltransferase (Fig. 26). Activity peaks coincided when the assays were performed with CDNB and DCNB (Fig. 27), and the purification of activity with the two substrates was identical, within experimental error, in each enzyme peak. However, the ratio of specific activity for the two substrates was different for each peak (Table 15).

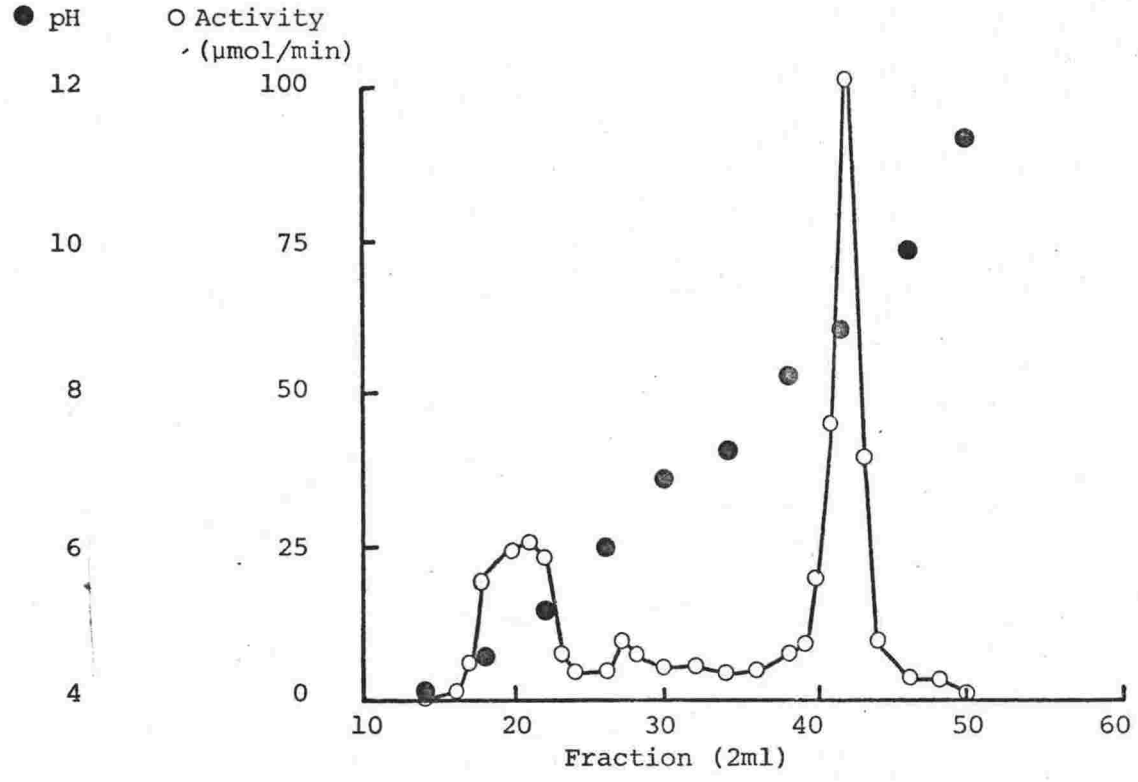


Fig. 24. pH 3-10 electrofocusing of 2.2-3.0M-$(NH_4)_2SO_4$ ppt. from live grass-grubs. Duration of focusing was 89h. Total recovery was 73% of the 625U applied.

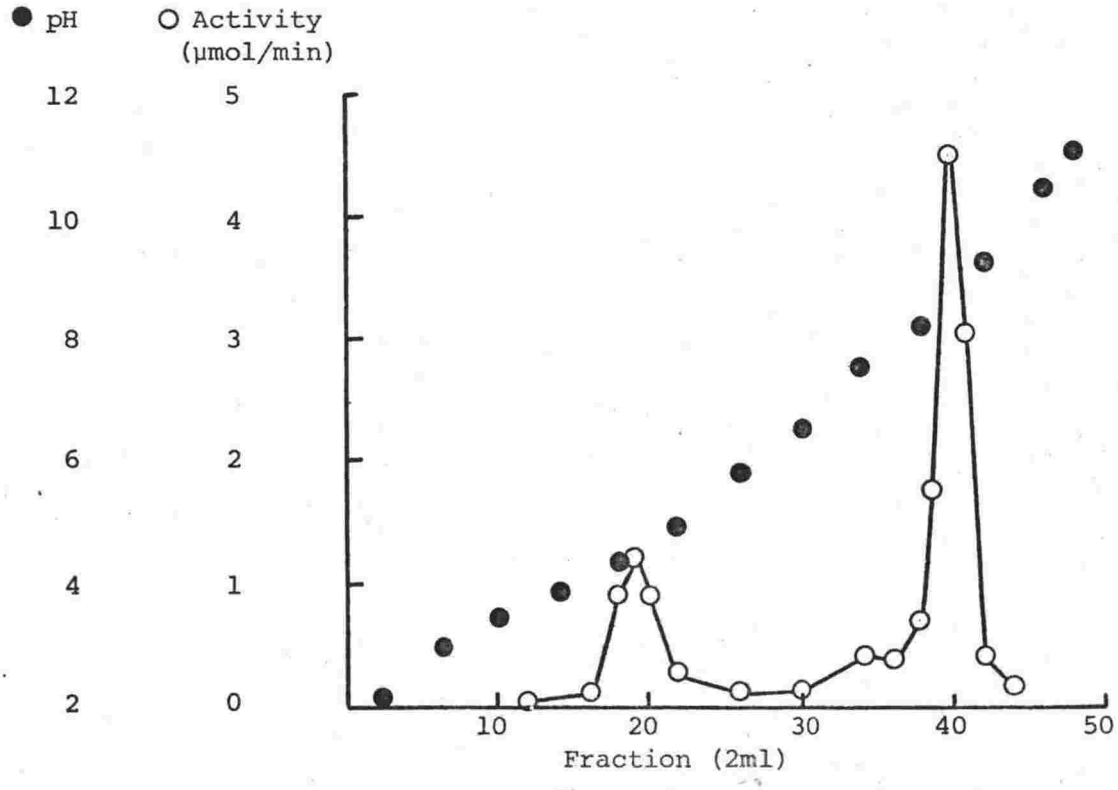


Fig. 25. pH 3-10 electrofocusing of the total 13 000g supernatant from one live grass-grub. Duration of focusing was 88h. Total activity recovered was 20U.

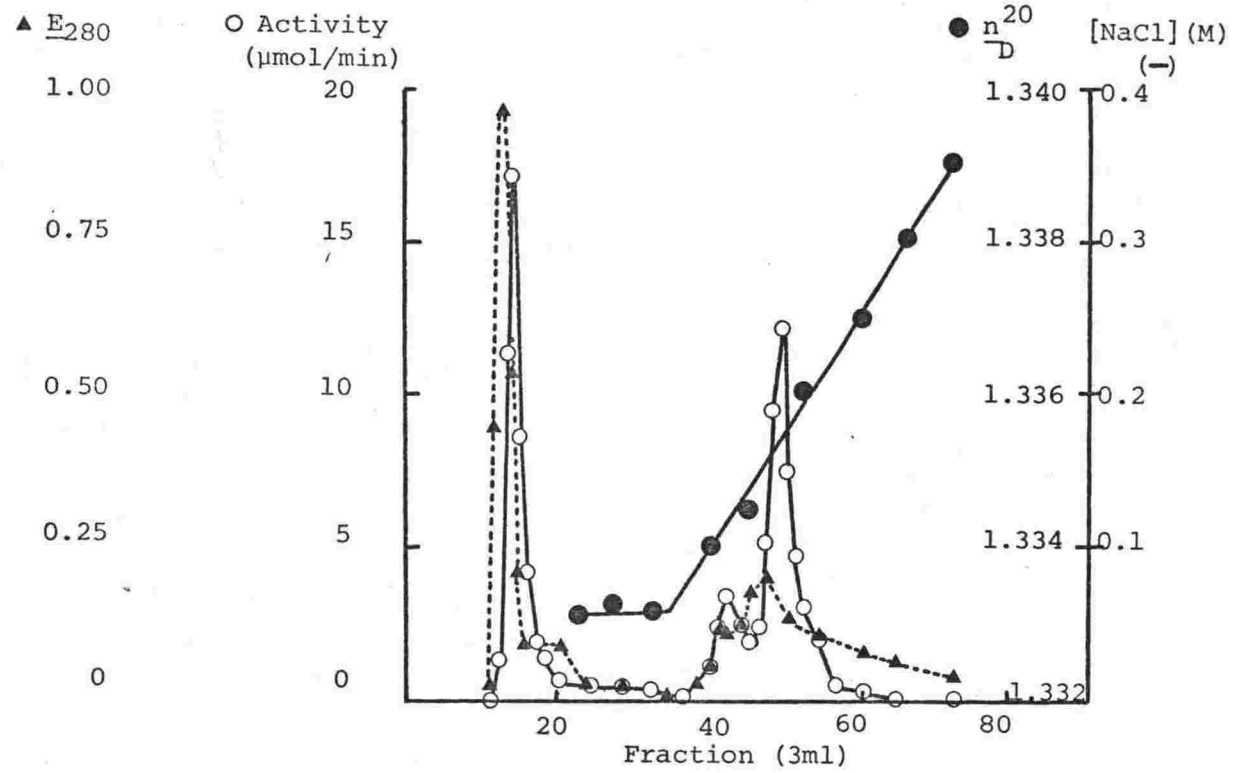


Fig. 26. DEAE-cellulose chromatography of grass-grub 2.24-3.03M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C. Buffer was 0.01M-ethylenediamine/HCl; elution of the gradient (0.01-1M-NaCl, 200ml) was determined by refractive index measurements. Sample size was 4.5ml.

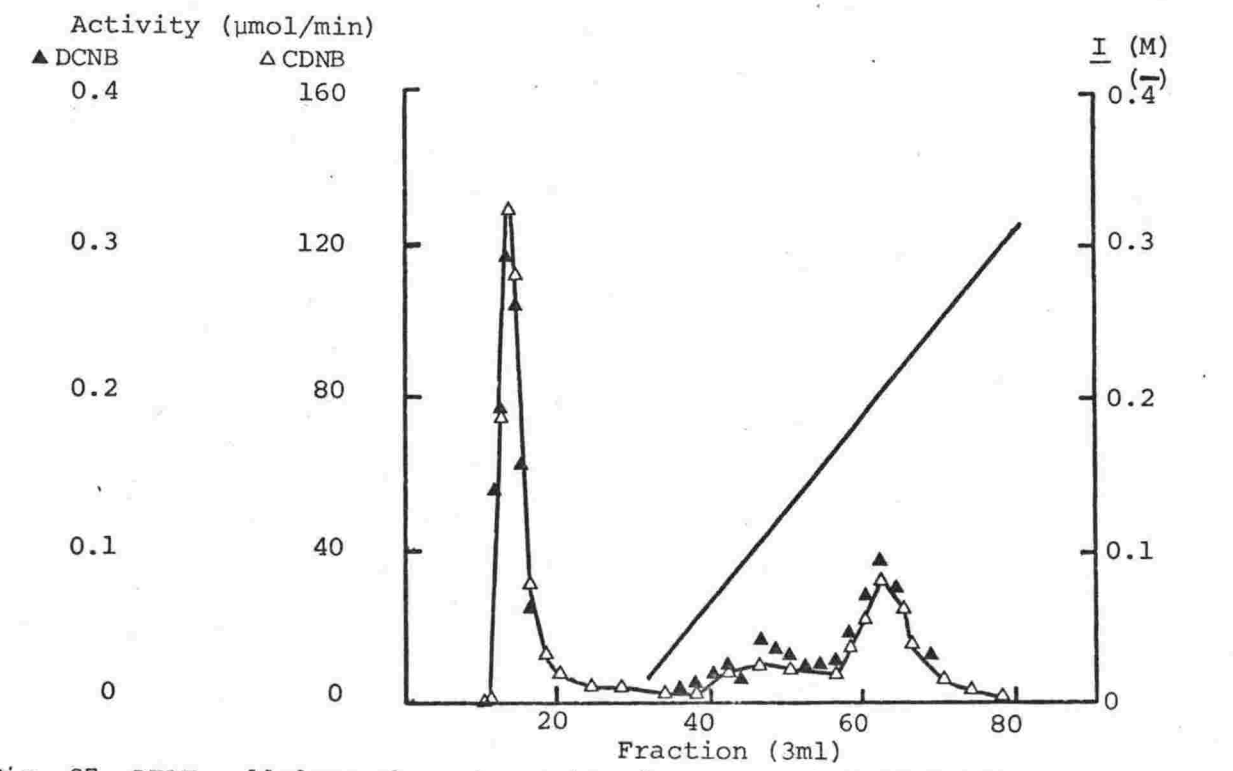


Fig. 27. DEAE-cellulose chromatography of grass-grub 2.24-3.03M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C, showing elution of activity with substrates CDNB and DCNB. The gradient was 0.01-0.51M-NaCl, 250ml; sample size was 3.7ml.

Table 15. Relative purification of GSH S-aryltransferase activity with CDNB and DCNB by DEAE-cellulose ion exchange chromatography*

Fraction no.	Ionic Strength at peak (M)	Specific Activity (CDNB) / Specific Activity (DCNB)
12		392
13	0.01	448
14		426
46		251
48	0.11	292
50		266
61		331
62	0.20	342
63		320

*results refer to Fig. 25

The three peaks of activity were obtained when the ionic strength gradient was started immediately after application of the enzyme sample (Fig. 28); when the column was washed with starting buffer for two or four times the void volume before commencement of the gradient (e.g. Figs. 26 & 27; Fig. 29); when the ionic strength gradient was varied from 0.01 - 0.31M to 0.01 - 1.01M and when solutions of different specific activity and total activity were applied. Table 16 summarizes the results of five experiments that incorporated starting buffer development in the procedure.

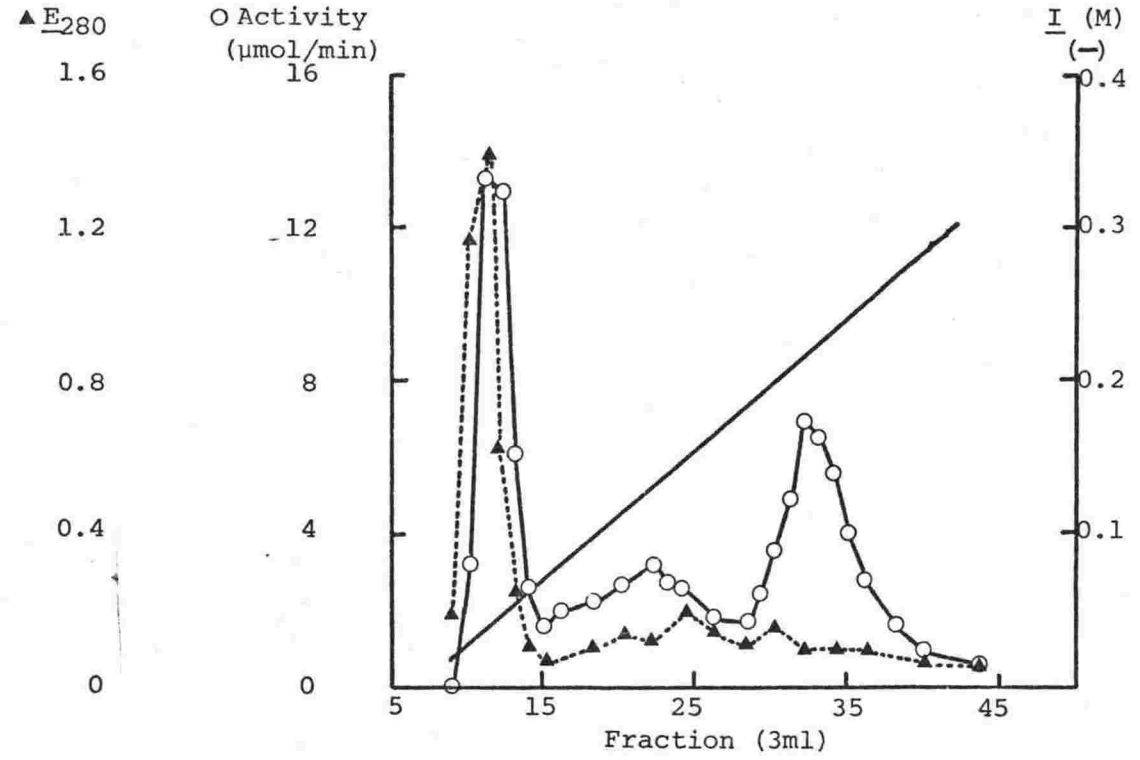


Fig. 28. DEAE-cellulose chromatography of grass-grub 2.24-3.03M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C, without initial starting buffer development. The gradient was 0.01-0.71M-NaCl, 200ml; sample size was 5 ml.

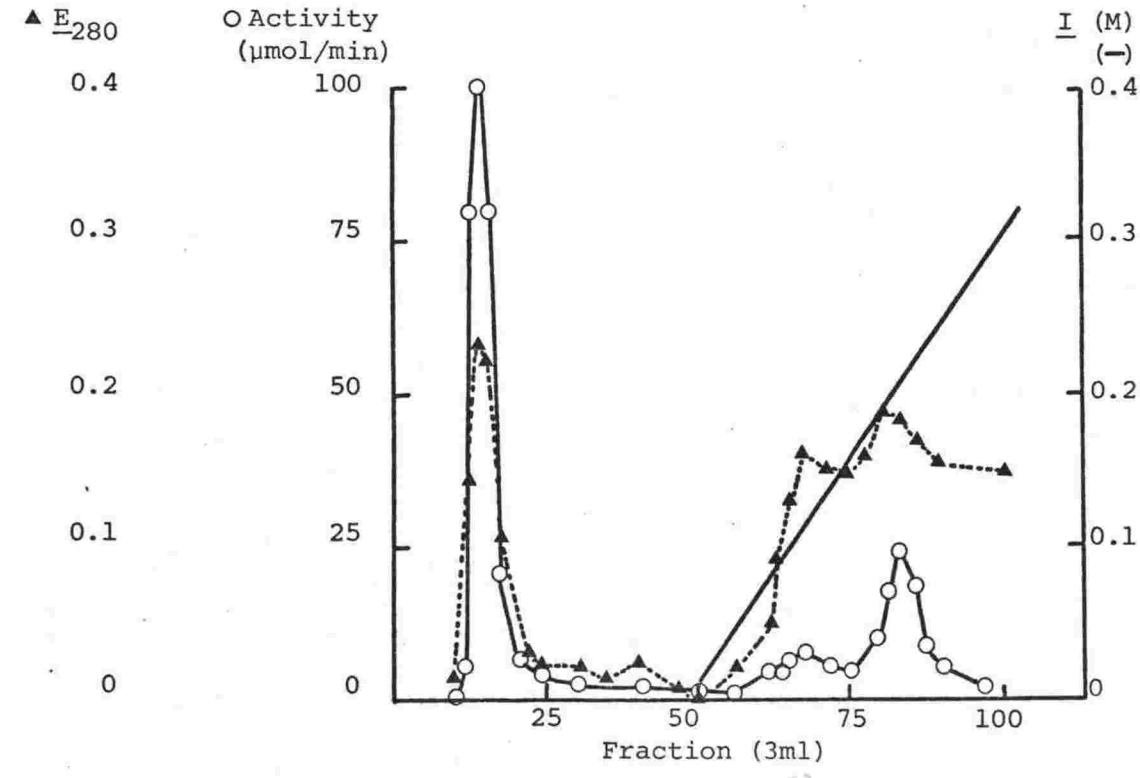


Fig. 29. DEAE-cellulose chromatography of grass-grub 2.34-3.13M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C. Starting buffer development was continued for 40 fractions then a 0.50M-NaCl gradient (250ml) was applied. Sample size was 3.0ml.

Table 16. Behaviour of GSH S-aryltransferases on DEAE-cellulose at pH 6.3

Ionic strength at enzyme peak (M)	% of recovered activity in peak*	Increase in specific activity in the peak fraction**
0.01	39-52 (48)	1.1 - 4.7
0.11	10-14 (12)	0.5 - 1.3
0.20	30-40 (35)	1.5 - 3.2

*mean in parentheses
**assayed with CDNB

Total enzyme recovery varied from 65 - 80% between experiments. Purification varied from one preparation to another, but was consistent (± 0.2 in the specific activity ratio) in repeated experiments with the same preparation.

The results shown in Fig. 29 suggest that the activity peak at $\underline{I}$ = 0.11M may comprise two enzymes, one being eluted at $\underline{I}$ = 0.08M and the other at $\underline{I}$ = 0.11M. Figure 30a shows the result of isoelectric focusing of fractions 60-70 (inclusive) from Fig. 29. Fortyeight per cent of the recovered activity was in a peak at pH 5.8, 31% in a peak at pH 9 and the remainder in shoulders at pH 4.75, 4.90 and 8.20, and in small peaks at pH 6.70 and 7.55. Re-plotting of Fig. 30a as the first derivative curve (Fig. 30b) demonstrates better the presence of the shoulders and small peaks.

DEAE-Cellulose - attempts at specific elution of GSH-dependent transferases by GSH

Several attempts were made to elute specifically GSH S-transferase activity from the DEAE-cellulose column using a GSH gradient. Only the first enzyme peak ($\underline{I}$ = 0.01M) was obtained when a 0 - 10mM-GSH gradient in 0.01M-ethylenediamine buffer was applied after starting buffer development. The first two peaks (normally eluted at $\underline{I}$ = 0.01M and 0.11M) were obtained when the column was eluted with 0.01M-ethylene

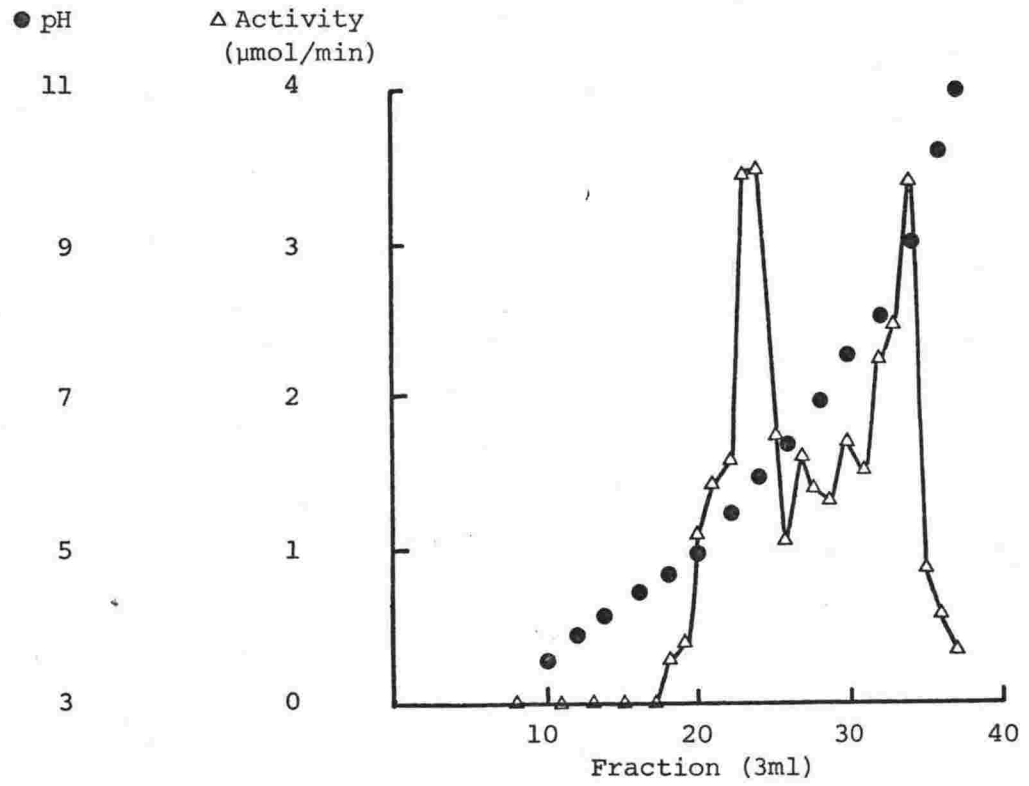


Fig. 30a. pH 3-10 electrofocusing of fractions 60-70 (inclusive) from the experiment shown in Fig. 29. The pooled fractions were dialysed against distilled water before refocusing. Duration of focusing was 142h. 50% of the activity (measured before dialysis) was recovered.

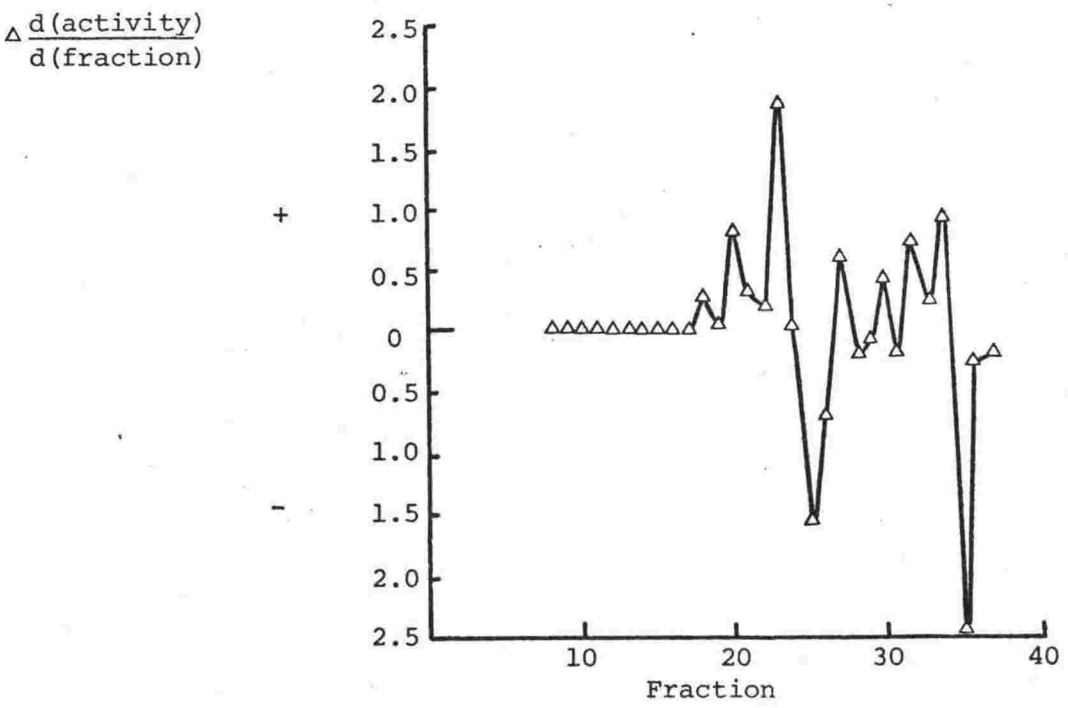


Fig. 30b. First derivative plot of the data in Fig. 30a.

diamine buffer followed by $\underline{I}$ = 0.13M buffer (0.01M-ethylenediamine buffer, 0.12M-NaCl), but a 0-10mM-GSH gradient in the 0.13M buffer did not elute the third peak. When 10mM-GSH was included in the reservoir vessel for a 0.01 - 0.51M ionic strength gradient the elution pattern (Fig. 31) was identical to that in the absence of GSH (cf. Fig.29), but a better separation of the $\underline{I}$ = 0.11M peak into two peaks ($\underline{I}$ = 0.085M and 0.115M at the peaks) was achieved. Similar distribution of activity within the peaks was also obtained - the first peak contained 50% of the recovered activity, the second (fractions 55 - 74), 12%, and the third, 37% (cf. results in Table 16).

Ion Exchange Chromatography of GSH S-aryltransferases from live Grass Grubs

DEAE-cellulose

DEAE-cellulose chromatography of the 2.2-3.3M-$(NH_4)_2SO_4$ fraction from live grubs gave only two aryltransferase peaks (Fig. 32). The peak eluted by the starting buffer comprised 71% of the recovered activity and the other at $\underline{I}$ = 0.19M, 26%. The results of pH 3-10 isoelectric focusing of each peak are shown in Figs. 33a & b. The first DEAE-cellulose enzyme focused as a single enzyme with pI=8.7; 90% of the activity applied to the focusing column was recovered. The second DEAE-cellulose peak gave two peaks upon isoelectric focusing; total recovery of activity was 65%; 68% of this was in the pI 4.8 peak and 26% in the pI 8.65 peak.

TEAE-cellulose

TEAE-cellulose chromatography at pH 6.3 of the ammonium sulphate fraction from live grubs gave the same result as with DEAE-cellulose - peaks of activity emerged at the void volume of the column and at $\underline{I}$ = 0.20M.

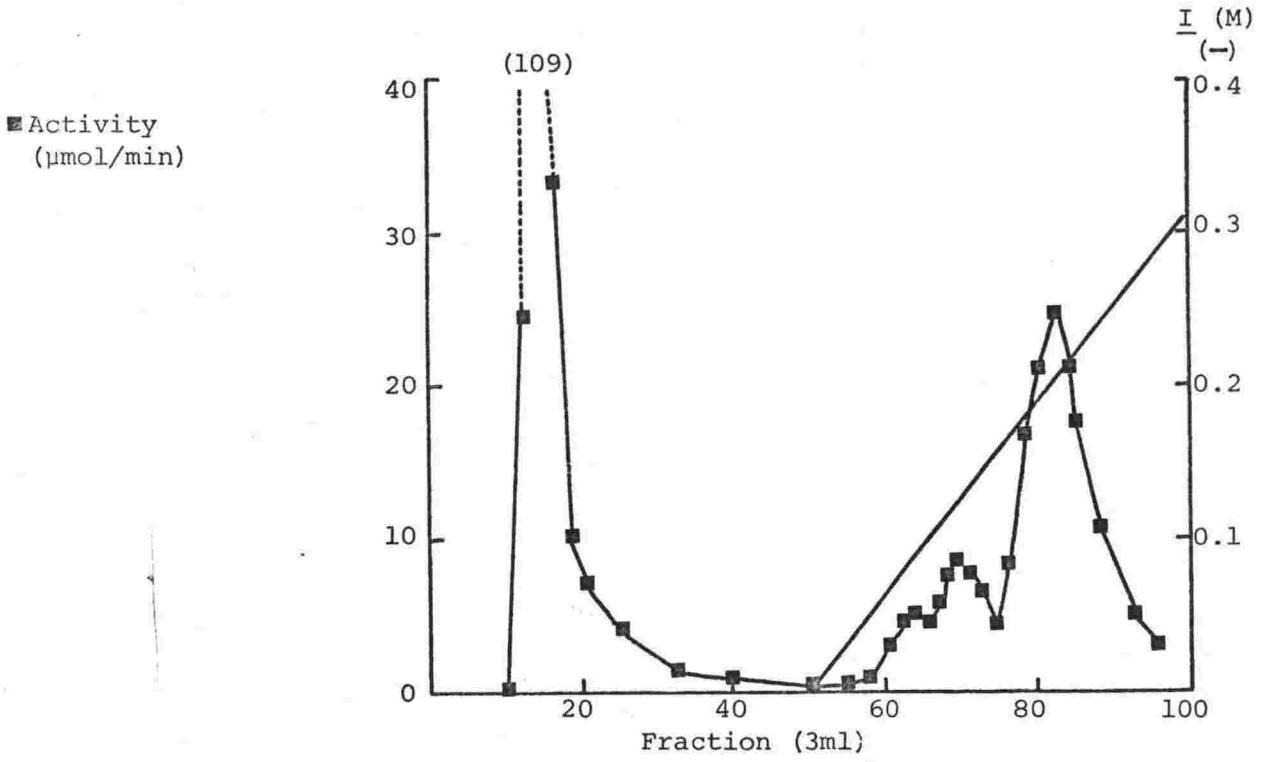


Fig. 31. Attempted substrate-specific elution of GSH S-aryltransferase from DEAE-cellulose at pH 6.3 and 5°C. Starting buffer development was continued for 40 fractions before application of the NaCl gradient (0.50M, total vol. 250ml) containing GSH (10mM). Sample was 3ml of 2.3-3.1M-$(NH_4)_2SO_4$ fraction.

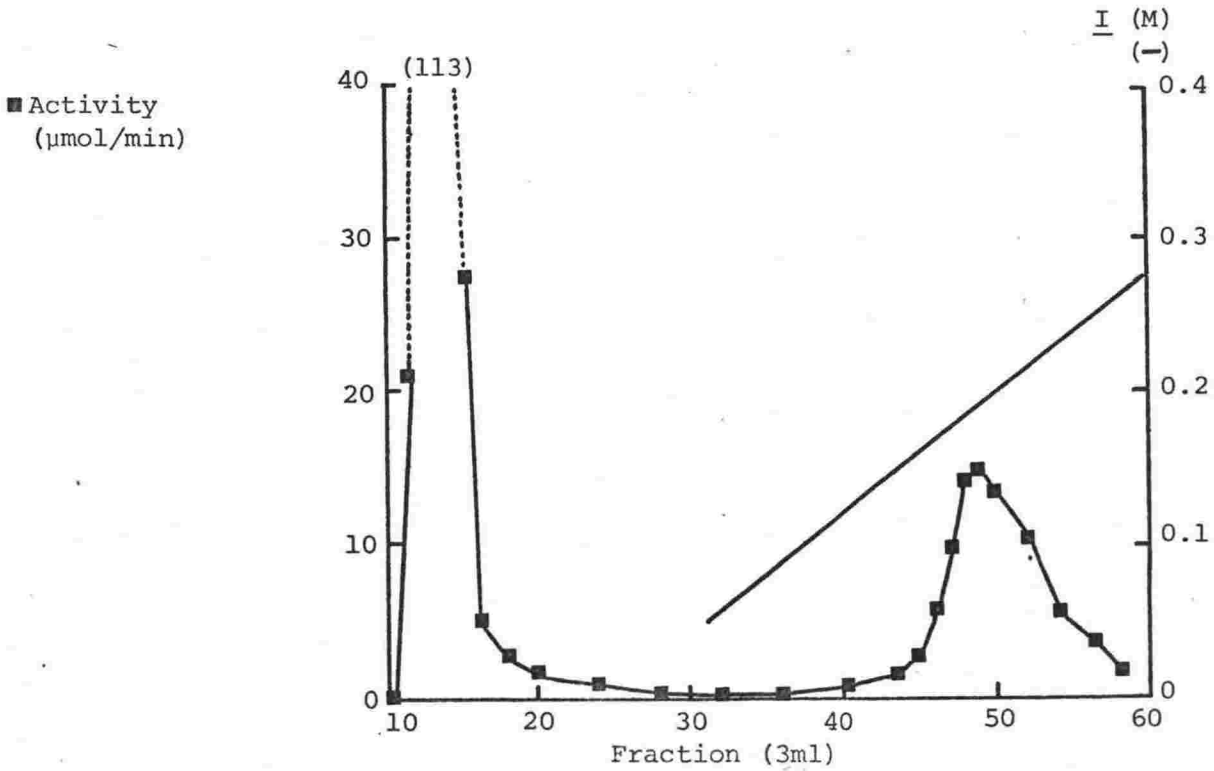


Fig. 32. DEAE-cellulose chromatography of 2.2-3.3M-$(NH_4)_2SO_4$ fraction, from live grass-grubs, at pH 6.3 and 5°C. Twenty fractions of starting buffer (I=0.05M) were applied, followed by 200ml of a 0.50M NaCl gradient. Sample size was 5 ml.

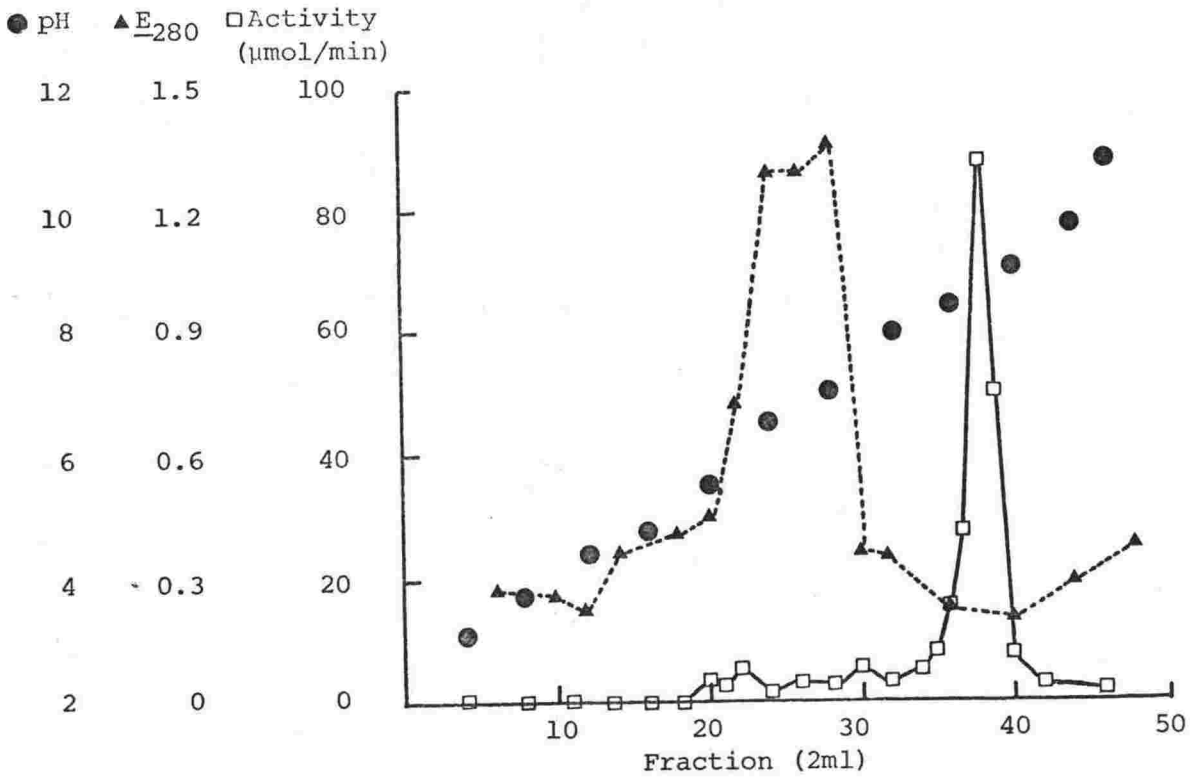


Fig. 33a. pH 3-10 electrofocusing of the first peak from DEAE-cellulose chromatography. Fractions 11-16 from the experiment shown in Fig. 32 were pooled and dialysed against distilled water before focusing; 5% of the activity was lost during dialysis. Duration of focusing was 90h; total activity recovered was 90%.

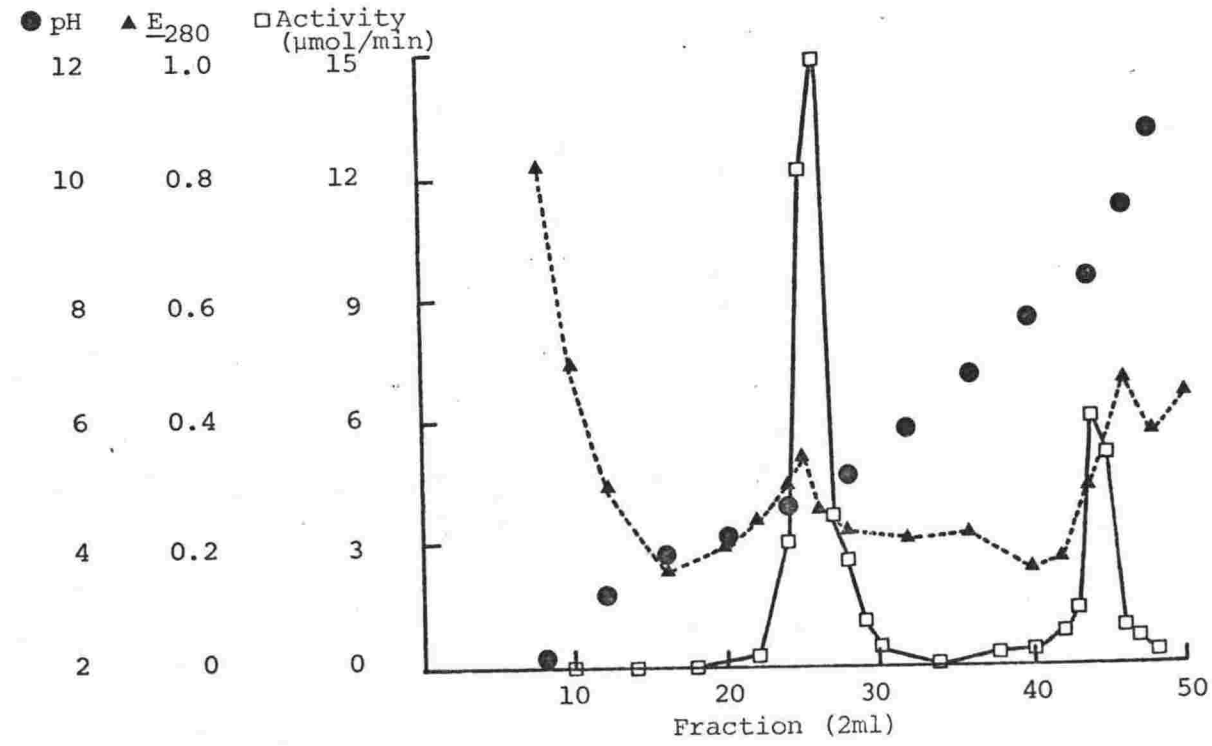


Fig. 33b. pH 3-10 electrofocusing of the second peak from DEAE-cellulose chromatography. Fractions 46-55 from the experiment shown in Fig. 32 were pooled and dialysed before focusing; 18% of the activity was lost during dialysis. Duration of focusing was 137h; total activity recovered was 65%.

CATION EXCHANGE CHROMATOGRAPHY

CM-cellulose

CM-cellulose chromatography at pH 6.3 of the ammonium sulphate fraction from frozen grubs gave two peaks of aryltransferase activity (Fig. 34). About 80% of the activity recovered was in the peak at the void volume of the column and only 13% in a peak at $\underline{I}$ = 0.06M. The same preparation gave 3-4 peaks of activity on DEAE-cellulose (cf. Figs. 27, 29 & 31).

SE-cellulose

SE-cellulose chromatography at pH 6.3 of the same preparation as used with CM-cellulose (cf. Fig. 34) also gave only two peaks of activity (Fig. 35); 60% of the activity recovered was in the first peak and 38% in the second. The expected tighter binding of the second peak to SE-cellulose, compared with CM-cellulose, was shown by its elution at $\underline{I}$ = 0.13M.

The result of elution from SE-cellulose using a stepwise pH gradient, at constant ionic strength, is shown in Fig. 36. Two major peaks of activity were obtained. If the two small peaks are assumed to arise from the tailing of the previous larger peak, the first peak (pH 5-6 boundary) comprises 57% and the second peak (pH 7-8 boundary) 43%, of the recovered activity. Elution using a pH 6.9-9 gradient gave different proportions of activity in the two peaks (Fig. 37) - the increase in the first peak was equivalent to the decrease in the second.

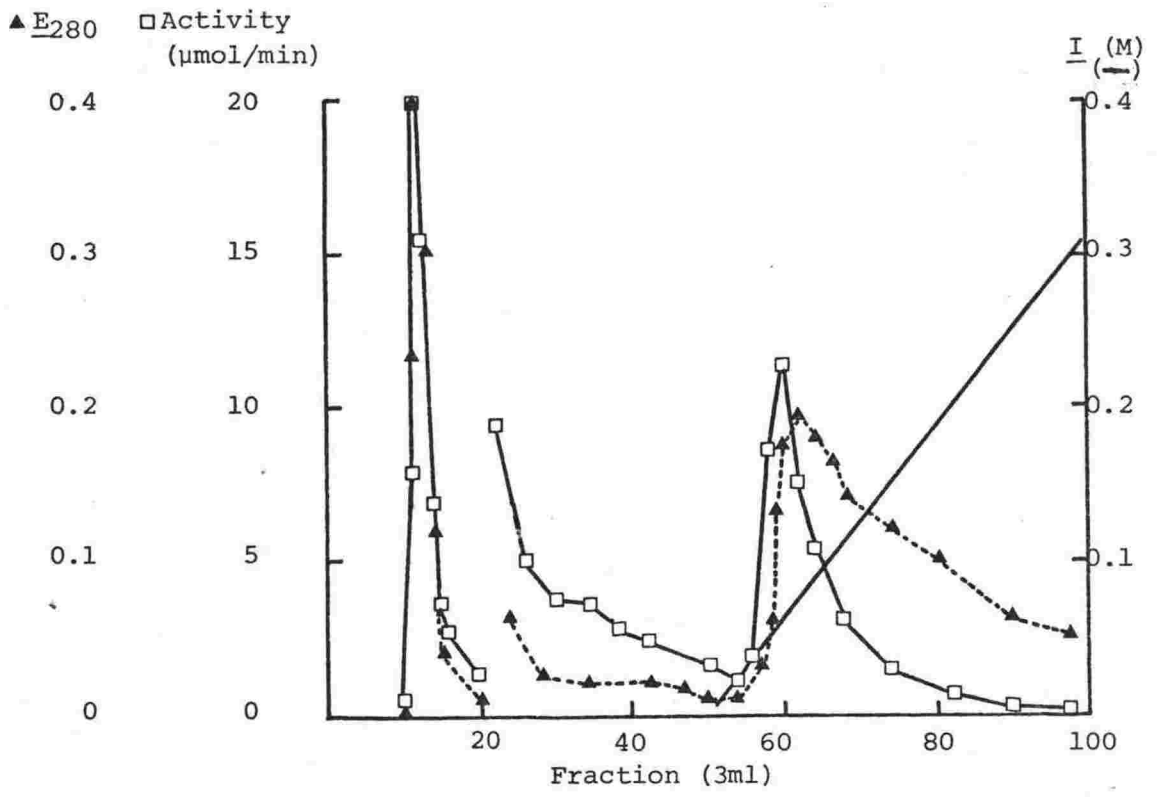


Fig. 34. CM-cellulose chromatography of grass-grub 2.34-3.13M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C. Starting buffer development (phosphate, I=0.01M) was continued for 40 fractions followed by 250ml of a 0.50M NaCl gradient. Sample size was 3.0ml. Activity and absorbance values for fractions 11 - 20 (inclusive) are for 10-fold diluted solutions.

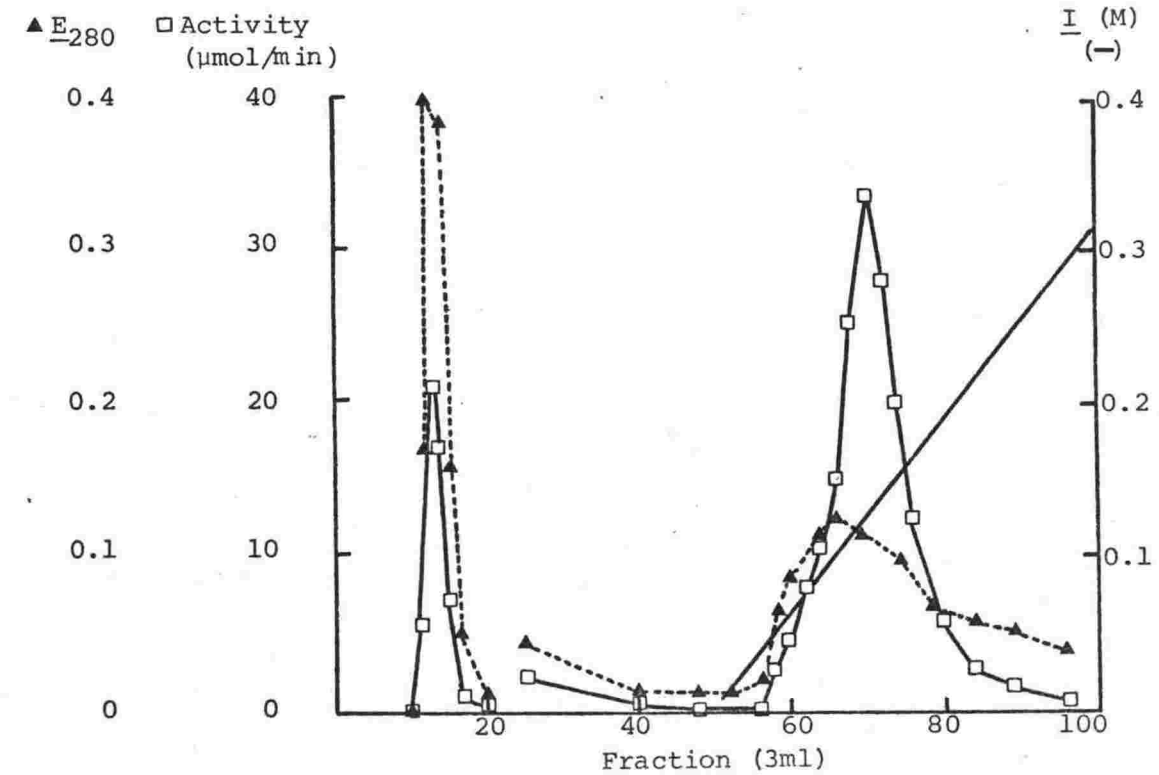


Fig. 35. SE-cellulose chromatography of grass-grub 2.34-3.13M-$(NH_4)_2SO_4$ ppt. at pH 6.3 and 5°C. Conditions were the same as in the experiment shown in Fig. 34. Activity and absorbance values for fractions 12-20 (inclusive) are for 10-fold diluted solutions.

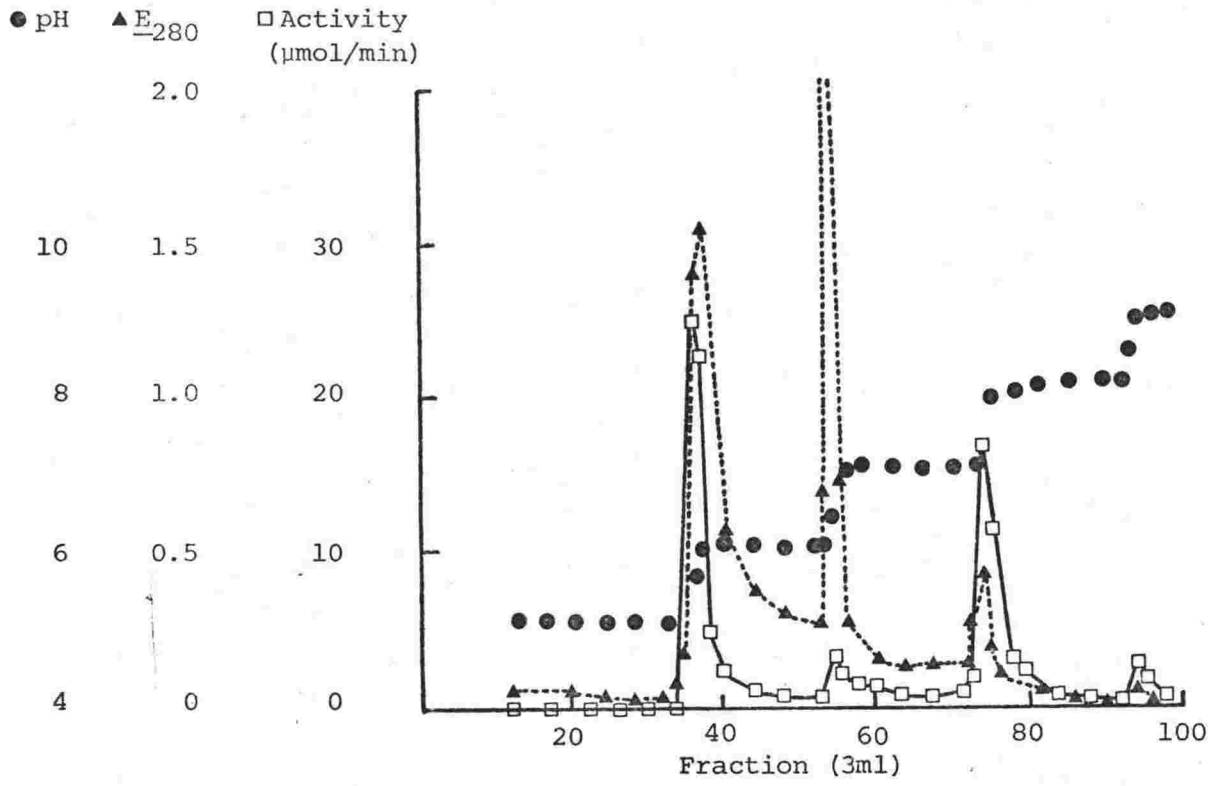


Fig. 36. SE-cellulose chromatography of 2.2-3.0M-$(NH_4)_2SO_4$ ppt. from live grass-grubs at 5°C using a stepwise pH gradient. Twenty fractions of each of the following buffers was applied: $\underline{I}$ = 0.01M acetate, pH 5.0; $\underline{I}$ = 0.10M phosphate, pH 6.05; $\underline{I}$ = 0.10M phosphate, pH 7.1; $\underline{I}$ = 0.1M pyrophosphate, pH 8.3; $\underline{I}$ = 0.1M pyrophosphate, pH 9.3. Sample size was 2.5ml.

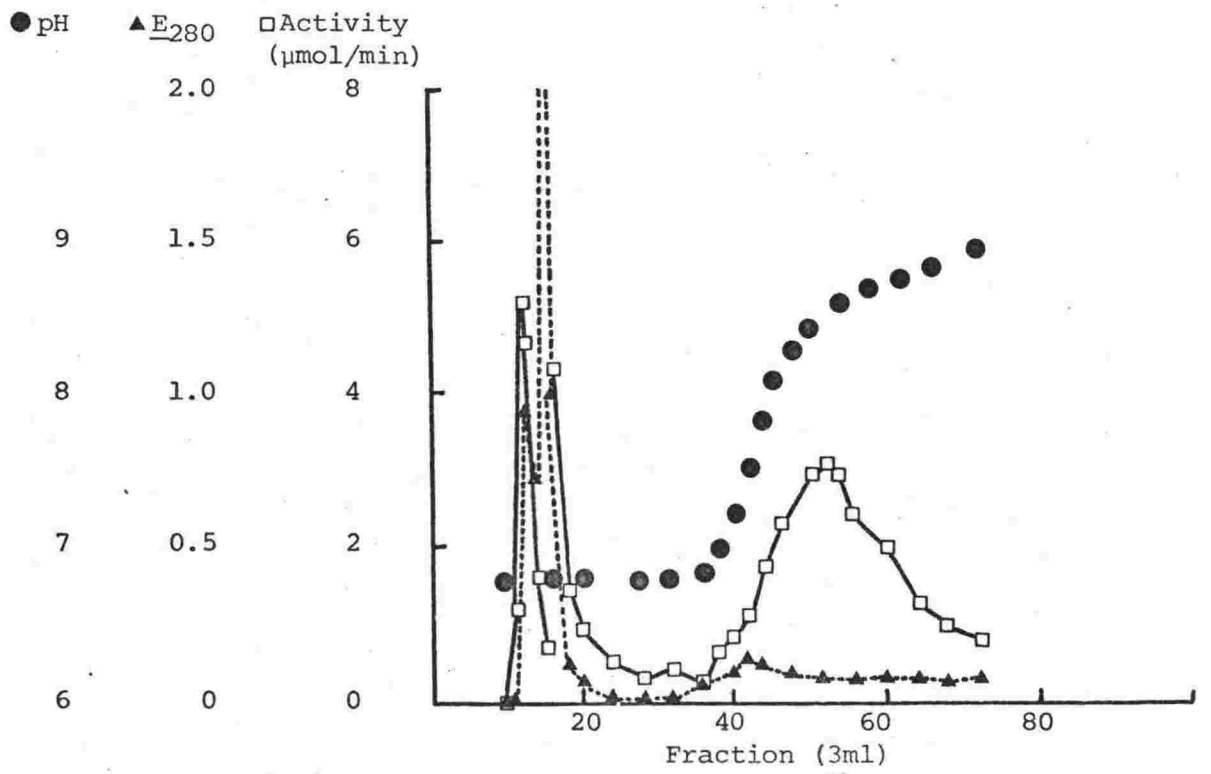


Fig. 37. SE-cellulose chromatography of 2.2-3.0M-$(NH_4)_2SO_4$ ppt. from live grass-grubs at 5°C using a continuous pH gradient. Starting buffer ($\underline{I}$ = 0.10M phosphate, pH 6.9) was applied for 20 fractions followed by the gradient produced from 100ml starting buffer and 100ml Tris.HCl ($\underline{I}$ = 0.1M, pH 9.0). Sample size was 2.4ml. Activity values for fractions 12-15 are for 10-fold dilutions.

PROPERTIES OF GSH S-ARYLTRANSFERASES PURIFIED BY ISOELECTRIC FOCUSING

pH Stability

The more sensitive assay with CDNB as second substrate was used.

(i) pI 4.6 and pI 8.7 GSH S-aryltransferases at 4°C

Each enzyme was diluted ten-fold with buffer of pH 5,6,7,8 or 9 to give a concentration of about 1U/ml and stored at 4°C. Over 48h, 5-10% activity was lost with each enzyme but the loss was independent of pH and would not be significant during the course of an experiment lasting 3 - 4h.

(ii) pI 8.7 GSH S-aryltransferase at 37°C

Stability was measured at pH 8.3 in buffer alone and in the presence of each substrate. Cells containing the required reagents were equilibrated in the KINTRAC cell basket, enzyme was added and after timed intervals activity was assayed by addition of substrate(s). No activity was lost in 15min, relative to the control at 4°C, when enzyme was incubated in buffer or buffer plus 2mM-GSH. A slight decrease (5%) was observed in the presence of 0.1mM- or 0.2mM-CDNB, but this was only slightly greater than the precision of the assays.

At pH 9.7, in the presence of 2mM-GSH activity was constant for 10min then decreased by 5 - 10% in the next 10 minutes.

Effect of Ionic Strength on Reaction Rate

The activity of the pI 8.7 enzyme was measured under the usual conditions with CDNB, except that the ionic strength was varied from 0.1 - 1.5M by either varying the $Na_4P_2O_7$ concentration or using 0.05M-$Na_4P_2O_7$ buffer and adding NaCl. Variation of ionic strength by either means had no effect on the non-enzymic or the enzyme-catalysed reaction.

Optimum pH for Enzyme catalysed reactions

Enzymes were purified by ammonium sulphate fractionation, pH 3-10 isoelectric focusing and re-focusing of the main peaks at pH 4-6 and 7-10. Assays at pH values from 5.25 - 9.60 established that the optimum pH for each enzyme was between pH 8.30 and 8.95. Further measurements at pH 7.6 - 9.5 using additional pH values showed optimum activity for each enzyme at pH 8.6 (Fig. 38). The total activity in the pI 5.2 and pI 8.0 peaks was low compared with the two major enzymes and contamination of these smaller peaks was probable.

Molecular Weight Measurements

The results of BioGel P150 chromatography of the pI 4.6 and pI 8.7 enzymes are shown in Fig. 39a. Only one peak of activity was found for each enzyme over the range 20 000 - 80 000. The molecular weight of each enzyme, from interpolation of the standard curve, was between 35 000 - 40 000 (Fig. 39b).

Comparison of 1 - halogeno-2,4-dinitrobenzenes as substrates for GSH S-aryltransferases

Activity of the pI 4.6 and pI 8.7 enzymes was assayed with each aromatic substrate at a concentration of 0.1mM under the standard conditions for CDNB. Results are presented in Table 17.

Table 17. Comparison of 1-halogeno-2,4-dinitrobenzenes as substrates for GSH S-aryltransferases

Aromatic Substrate	Activity* with pI 4.6 enzyme	Activity* with pI 8.7 enzyme	Non-enzymic reaction (ΔE_{344}/min)
CDNB	1.00	1.00	0.116
BDNB	1.42	1.45	0.178
IDNB	0.94	1.17	0.158

*relative to CDNB = 1.00

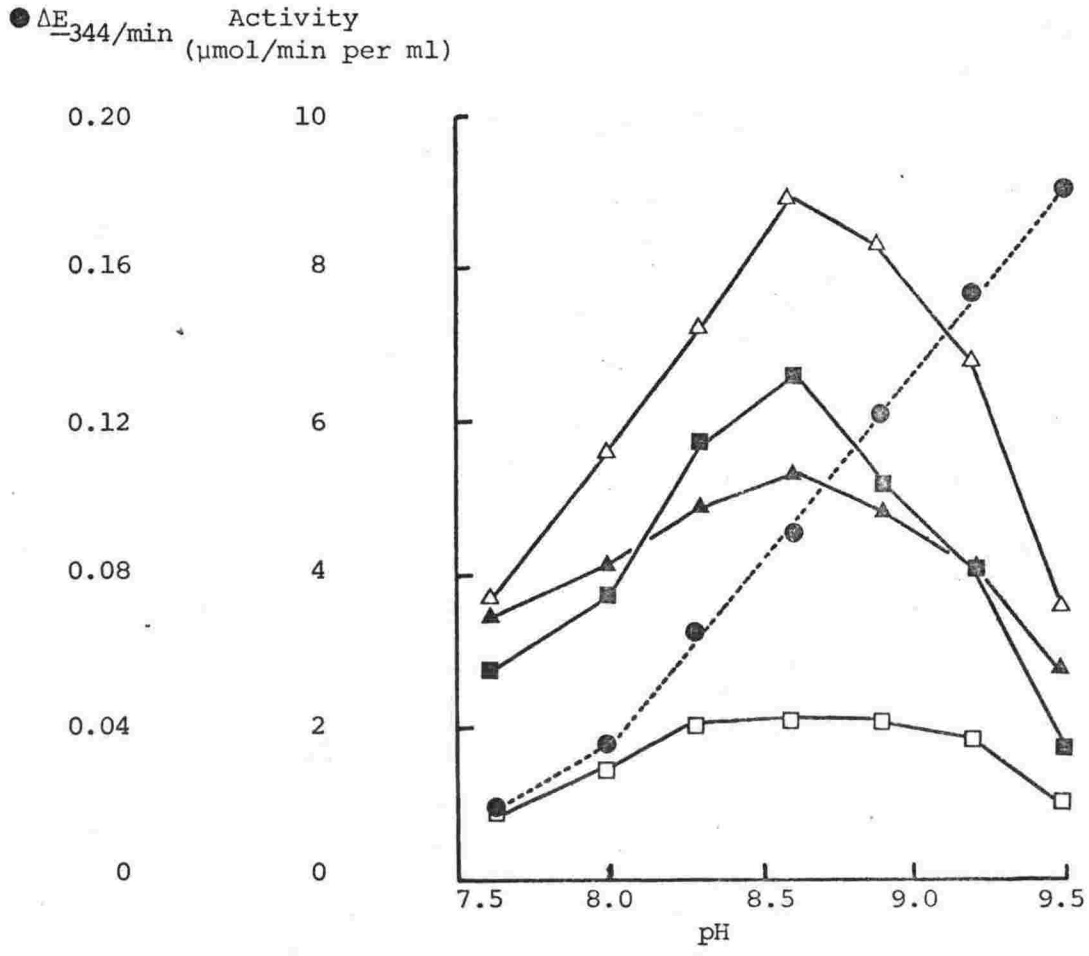


Fig. 38. Measurement of optimum pH for activity of GSH S-aryltransferases. Buffers were: I = 0.1M phosphate, pH 7.6; 0.1M-pyrophosphate, pH 8.0-9.5. Assays at 37°C used 1.0mM-GSH and 0.1mM-CDNB.

● , non-enzymic reaction; △ , pI 4.6 enzyme;
■ , pI 8.7 enzyme; ▲ , pI 5.2 enzyme; □ , pI 8.05 enzyme.

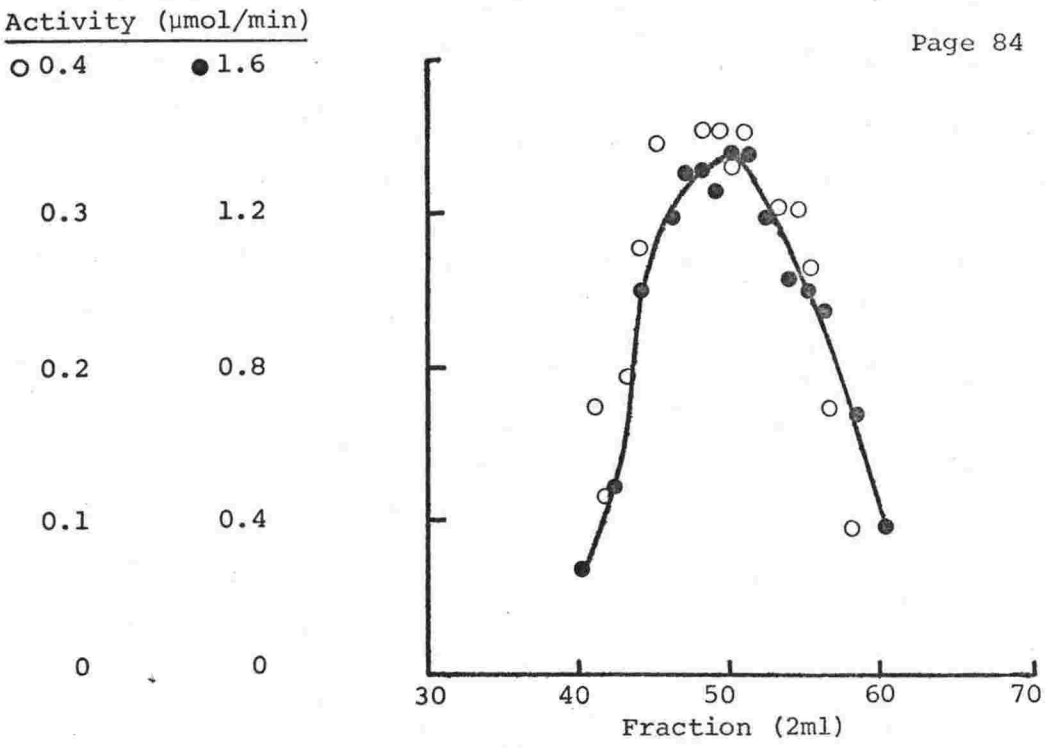


Fig. 39a. BioGel P150 chromatography of grass-grub GSH S-aryltransferases purified by electrofocusing.
○ , pI 4.6 enzyme; ● , pI 8.7 enzyme.

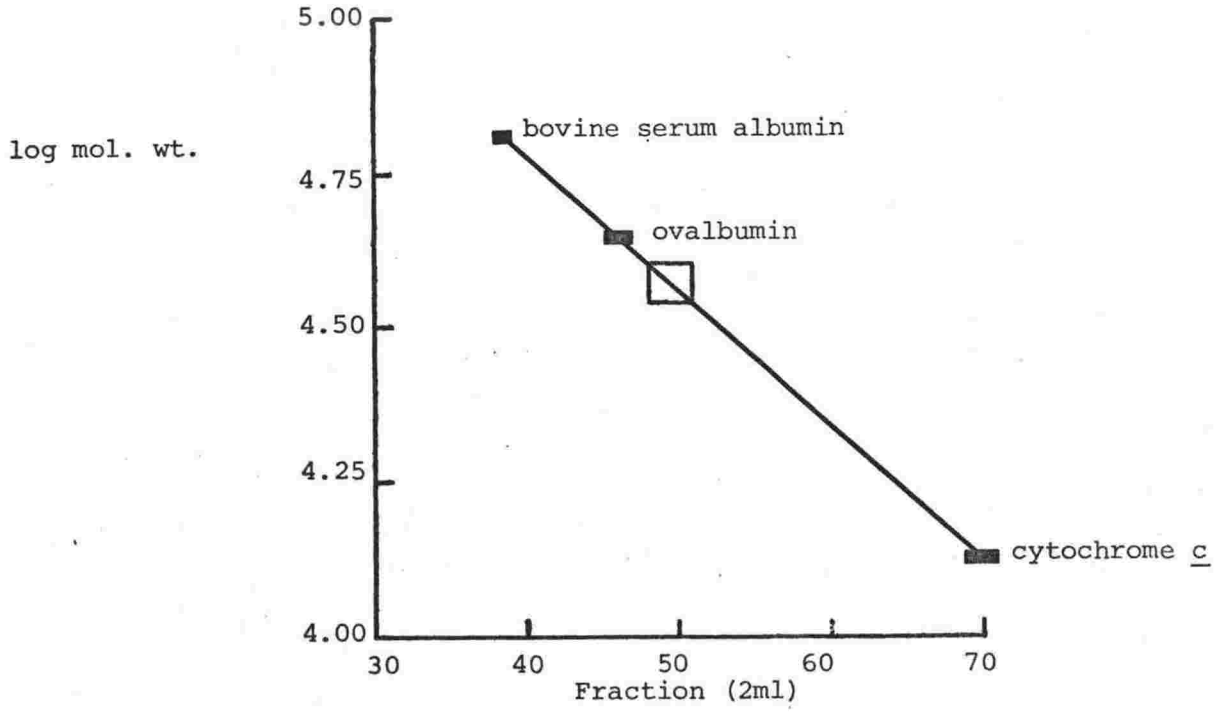


Fig. 39b. Calibration curve for calculation of molecular weight by chromatography on BioGel P150.

Activation energy of enzymic and non-enzymic reactions

Reaction rates at pH 8.3 and 15-40°C were measured using 2mM-GSH and 0.1mM-CDNB or 0.45mM-DCNB. The enzyme concentration for DCNB measurements was ten times greater than for CDNB. The combined results of two experiments are presented in Fig. 40. The apparent activation energies (see Appendix II), calculated from the slopes of the lines fitted by the method of least squares, are given in Table 18.

Table 18. Apparent activation energies for reaction of GSH with CDNB and DCNB

	E			
	CDNB		DCNB	
	(kcal/mole)	(kJ/mole)	(kcal/mole)	(kJ/mole)
pI 4.6 enzyme	7.83	32.76	8.16	34.14
pI 8.7 enzyme	7.10	29.71	10.99	45.98
Non-enzymic reaction	15.25	63.81		

COMPARATIVE STUDIES OF GSH S-TRANSFERASES

Ammonium Sulphate Fractionation

The 13 000g supernatant from a homogenate of 400g grass grubs in 800 ml acetate buffer, pH 5.5, was fractionated with $(NH_4)_2SO_4$. Each fraction was assayed with CDNB, DCNB, methyl parathion (MeP), and crotonyl-N-acetylthioethanolamine (CrSE) (Table 19).

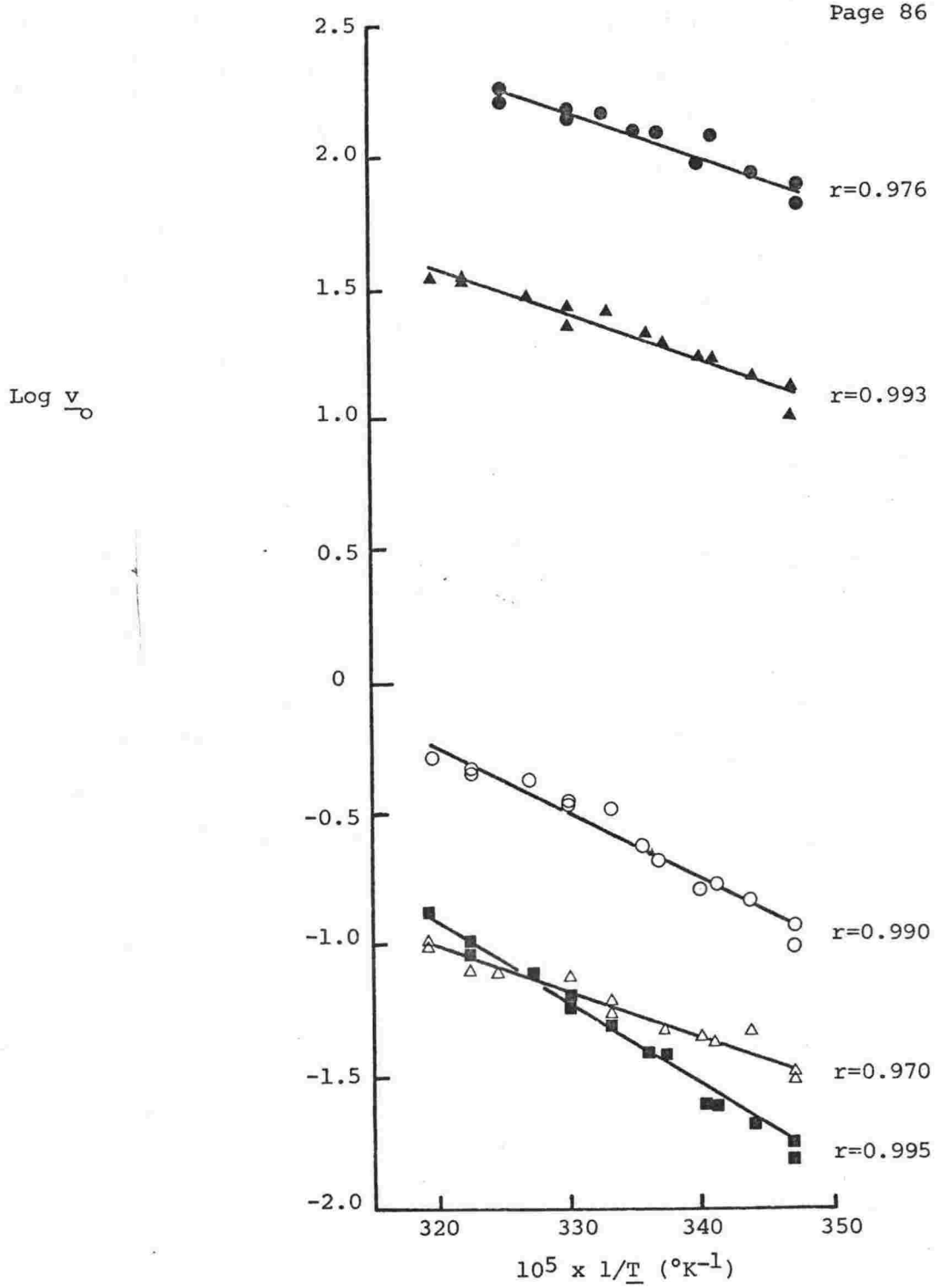


Fig. 40. Arrhenius plots for GSH S-aryltransferases at pH 8.30. Assays used 2mM-GSH and either 0.1mM-CDNB or 0.45mM-DCNB.
■ , non-enzymic reaction (CDNB); ▲ , pI 4.6 enzyme (CDNB);
△ , pI 4.6 enzyme (DCNB); ● , pI 8.7 enzyme (CDNB);
O , pI 8.7 enzyme (DCNB). r is the correlation coefficient from the least squares fit.

Table 19. Ammonium Sulphate Fractionation of Grass Grub 13 000g Supernatant - Comparative Studies

Fraction	Recovery of Activity (%)				Specific Activity (µmol/min per mg)			
	CDNB	DCNB	CrSE	MeP*	CDNB	DCNB	CrSE	MeP
13 000g supernatant	100	100	100	100	0.93	0.0063	0.001	-
2.07M ppt.	11.3	6.8	0	0	0.28	0.0011	0	0
2.34M ppt.	10.7	12.5	4.3	9.1	0.56	0.0044	0.0003	-
3.13M ppt	53.5	72.0	60.3	81.0	5.16	0.0466	0.007	0.0001
3.50M ppt.	7.7	9.5	19.5	9.9	3.5	0.0289	0.007	0.00007
3.50M supernatant	10.9	20.1	0	0	0.19	0.0039	0	0

*percentages for alkyltransferase assays are based on total recovered activity as activity in the 13 000g supernatant was not detectable.

Stability of the various GSH S-transferases upon dialysis against water is shown in Table 20.

Table 20. Stability to dialysis of GSH S-transferases in the active ammonium sulphate fraction

Fraction	Total Activity (µmol per min)				Specific Activity (µmol/min per mg)			
	CDNB	DCNB	CrSE	MeP	CDNB	DCNB	CrSE	MeP
3.13M ppt. before dialysis	37 350	112.5	16.9	0.175	5.16	0.0466	0.007	0.0001
3.13M ppt. after dialysis*	30 858	85.8	20.2	0.307	9.73	0.0813	0.027	0.0003

*protein precipitated during dialysis was removed by centrifugation

DEAE-cellulose Ion Exchange

Dialysed 3.13M-$(NH_4)_2SO_4$ fraction was chromatographed. Results are plotted in Fig. 41. Recoveries of activity in each peak are given in Table 21.

Table 21. Recovery of GSH S-transferase activity in DEAE-cellulose peaks

Fractions	Ionic Strength at Peak (M)	Recovery of Activity (% of total applied)	
		CDNB	CrSE
12 - 16	0.01	26.8	25.0
65 - 70	0.11	3.3	10.1
80 - 84	0.20	8.4	0

Isoelectric Focusing

Dialysed 3.13M-$(NH_4)_2SO_4$ fraction (50 ml) was used as the light gradient solution for loading the column. pH 3-10 Ampholines were used. Results are shown in Fig. 42 and the recoveries of activity in each peak in Table 22.

Table 22. Recovery of GSH S-transferase activity in peaks from pH 3-10 Isoelectric focusing

Fractions	pH range	Recovery of Activity (% of total applied)			
		CDNB	DCNB	CrSE	MeP
14 - 17	4.36 - 4.63	16.2	17.0	11.8	18.5
19 - 22	5.00 - 5.45	4.1	4.4	28.2	26.6
30 - 33	7.90 - 9.58	16.2	14.3	2.5	0.8

The remainder of fractions 14 - 24 inclusive (20 ml) from pH 3-10 isoelectric focusing (Fig. 42) was diluted to 42 ml with dense gradient solution, loaded on to the column as the dense solutions in all but the first three fractions and re-focused over the range pH 4-6. Results are shown in Fig. 43. Recoveries and specific activities

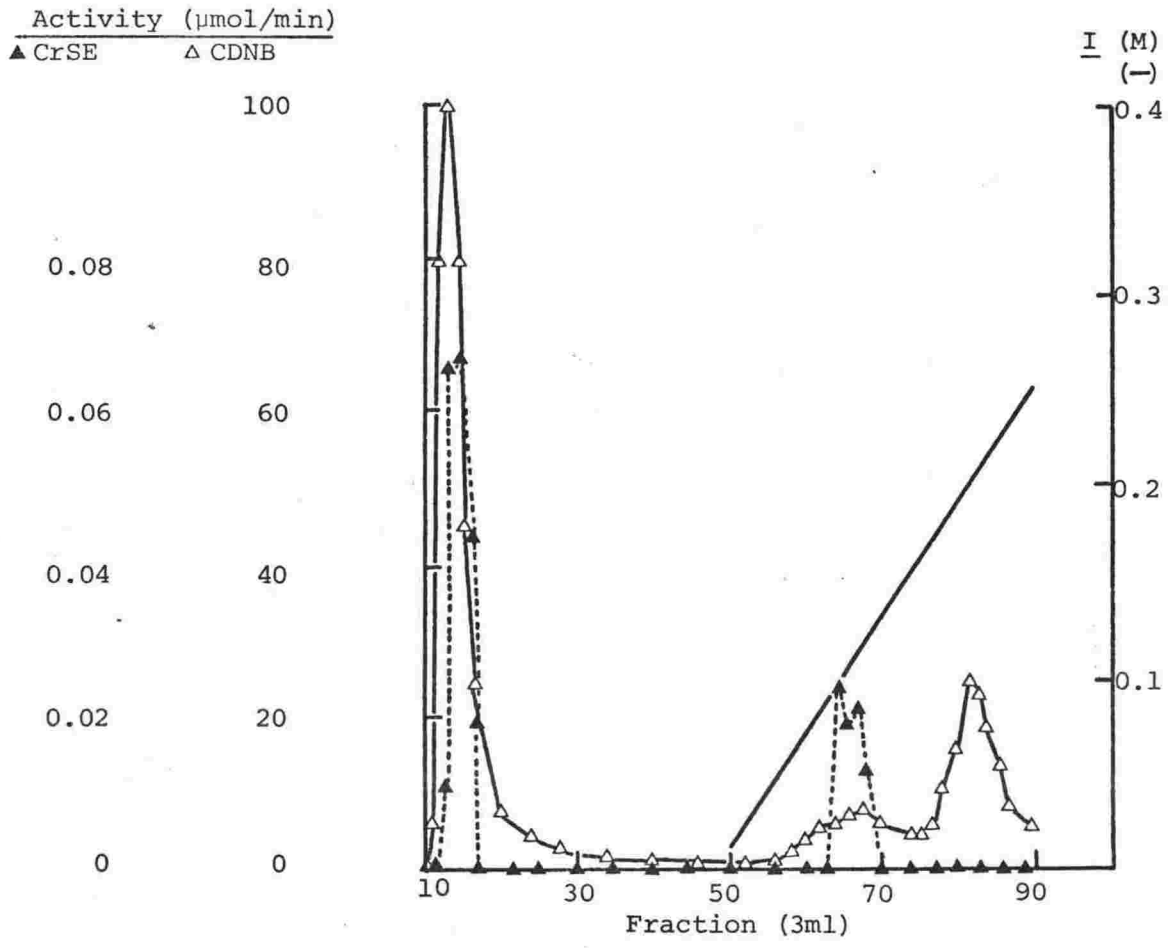


Fig. 41. DEAE-cellulose chromatography of GSH S-transferases, from grass-grub 2.34-3.13M-$(NH_4)_2SO_4$ ppt., at pH 6.3 and 5°C. Starting buffer development was continued for 40 fractions then a 0.50M-NaCl gradient was applied. Sample size was 3.0ml. Alkyltransferase-catalysed reaction rates were immeasurably low in all fractions.

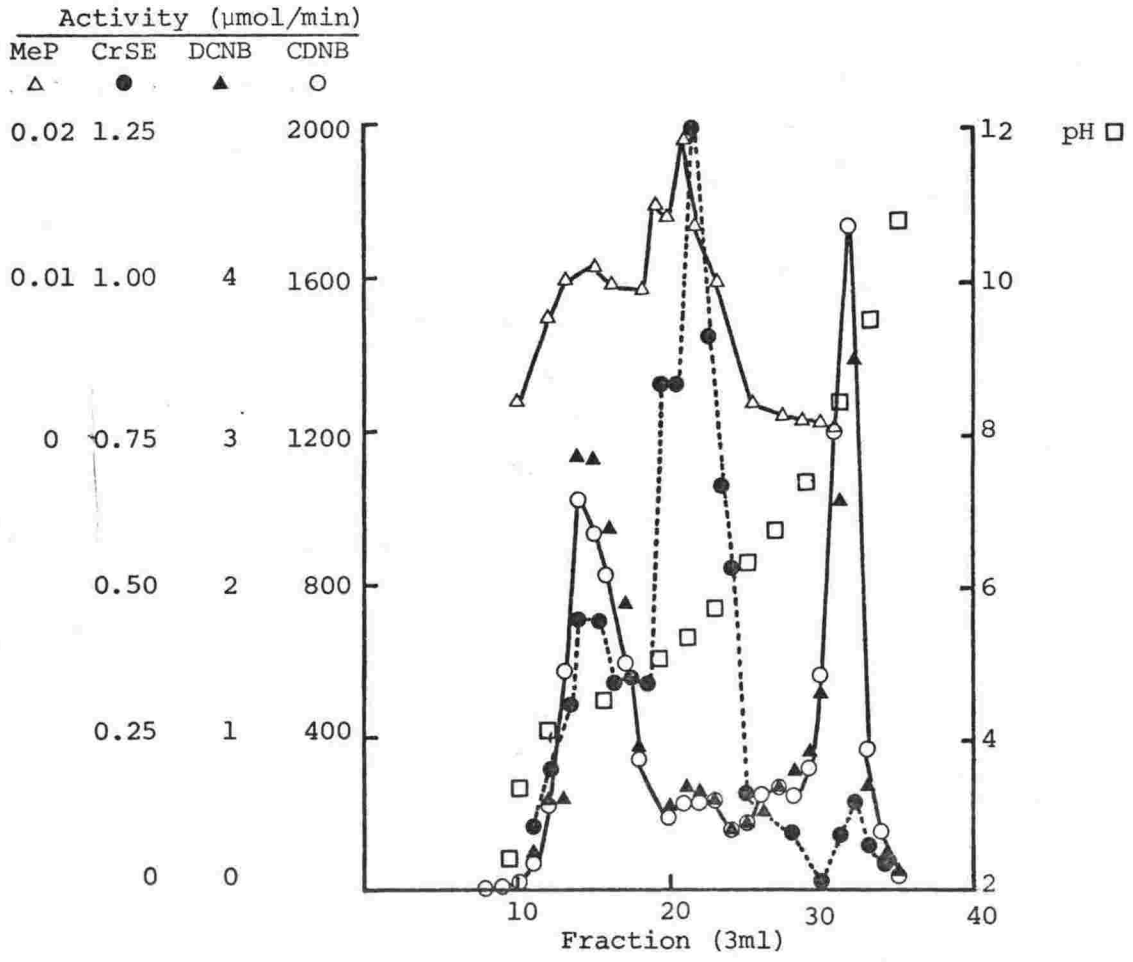


Fig. 42. pH 3-10 electrofocusing of GSH S-transferases from grass-grub 2.34-3.13M-$(NH_4)_2SO_4$ ppt. Duration of focusing was 110h. Recoveries of activity are given in Table 22.

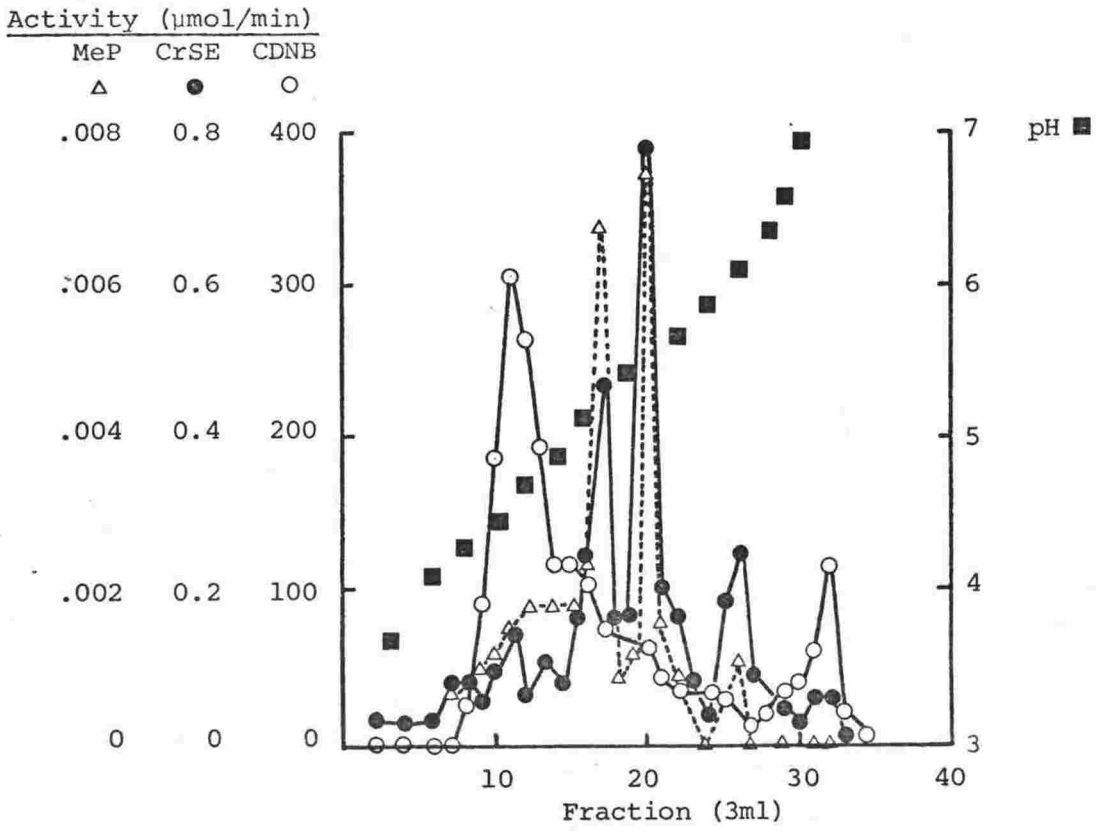


Fig. 43. pH 4-6 electrofocusing of fractions 14-24 (inclusive) from the experiment shown in Fig. 42. Duration of focusing was 89h. Recoveries of activity are given in Table 23.

in each peak are given in Table 23.

Table 23. Recovery of GSH S-transferase activity in peaks from pH 4-6 Isoelectric Focusing

Fractions	pH range	Recovery of Activity (% of total applied)			Specific Activity of most active fraction (μmol/min per mg)		
		CDNB	CrSE	MeP	CDNB	CrSE	MeP
10 - 13	4.47 - 4.80	38.2	14.0	12.2	34.8	0.017	0.0002
16 - 18	5.15 - 5.35	8.6	29.7	19.4	9.8	0.075	0.0015
19 - 21	5.42 - 5.60	6.1	39.0	19.4	11.6	0.138	0.0018
25 - 27	6.00 - 6.22	3.0	17.7	3.2	14.2	0.129	0.0008
31 - 33	7.55 - 9.92	7.9	4.3	0	26.4	0.013	0

PROCEDURES FOR PURIFICATION OF GSH S-ARYLTRANSFERASES

(a) Small Scale

(i) GSH S-aryltransferases from 50g grass grubs were partially purified by ammonium sulphate fractionation, DEAE-cellulose ion exchange chromatography and isoelectric focusing. The results are given in Table 24.

Table 24. Small Scale purification of GSH S-aryltransferases

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	115	53	6 100	29	1.83	100	1
2.3 - 3.1M-$(NH_4)_2SO_4$ ppt.							
(a) before dialysis	10	418	4 180	32	13.0	69	7.1
(b) after dialysis	62	57	3 534	4.47	12.8	58	7.0
DEAE-cellulose, pH 6.3*							
peak at I=0.04M	71	14.3	1 015	1.85	7.7	17	4.2
peak at I=0.20M	65	6.98	454	0.54	12.9	7.5	7.1
Isoelectric Focusing pH 7-10							
pI 8.7 enzyme (dialysed)	31	9.6	300	0.069	139	4.9	76

*starting buffer was I=0.04M-ethylenediamine/HCl, pH 6.3

(ii) The specific activity of the pI 8.7 enzyme was greatly increased when the procedure involved ammonium sulphate fractionation, pH 3-10 isoelectric focusing and re-focusing of the pI 4.6 and pI 8.7 enzymes at pH 4-6 and pH 7-10 respectively.

Table 25 gives the results obtained from 100g grass grubs.

Table 25. Small scale purification of GSH S-aryltransferases using wide and narrow pH range isoelectric focusing

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	230	62	14 260	26.5	2.34	100	1
2.2-3.2M-$(NH_4)_2SO_4$ ppt.							
(a) before dialysis	25	405	10 125	23.4	17.3	71	7.4
(b) after dialysis	31	274	8 500	16.5	16.6	60	7.1
Isoelectric Focusing pH 3-10							
(a) dialysed pI 4.6 enzyme	79	22.0	1 740			12.2	
(b) dialysed pI 8.7 enzyme	41	31.5	1 295			9.1	
Isoelectric Focusing pH 4-6							
pI 4.6 enzyme							
(a) before dialysis	20	30.8	616			4.3	
(b) after dialysis	30	9.72	292	0.65	15.0	2.1	6.4
Isoelectric Focusing pH 7-10							
pI 8.7 enzyme							
(a) before dialysis	10	72.1	721			5.1	
(b) after dialysis	22	33.6	740	0.0835	402	5.2	172

(b) Large Scale

Table 26. Large Scale purification of GSH S-aryltransferases

Fraction	Vol. (ml)	Concn. (U/ml)	Total U	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification
13 000g supernatant	4 700	58	272 000	29	2.0	100	1
2.3-3.2M-$(NH_4)_2SO_4$ ppt. pH 5							
a) suspension	500	303	151 500	44	6.9	56	3.5
b) dialysed	250	286	71 500	19.5	14.7	26	7.4
2.4-3.4M-$(NH_4)_2SO_4$ ppt. pH 8							
a) before dialysis	30	1 845	55 350	76	24.3	20	12.2
b) after dialysis	94	405	38 000	13.5	30.0	14	15.0
Isoelectric Focusing pH 3-10							
pI 8.7 enzyme (dialysed)	101	54.4	5 500	0.38	143	2.0 (20)*	71.5
Isoelectric Focusing pH 4-6							
pI 4.6 enzyme (dialysed)	200	15.8	3 160	1.8	8.8	1.2 (11)*	4.4

*expressed as percentage of total activity loaded in the two focusings at pH 3-10.

AFFINITY CHROMATOGRAPHY

The volume of Sepharose (3 ml) recovered after coupling of inhibitor indicated shrinkage of about 40% during either the reaction or washing procedures.

Batch Method

Table 27. Batch Treatment of GSH S-aryltransferase solutions with inhibitor-substituted Sepharoses

Ligand	% remaining in supernatant after 15 min				Activity Removed (U/ml gel)		Activity recovered in GSH wash (Total U)*	
	Activity		Protein					
	H_2O	PP_i	H_2O	PP_i	H_2O	PP_i	H_2O	PP_i
5'-CE-AHA-	60	61	89	87	10.9	10.6	1.4	1.7
5'-CE-AEA-	56	50	85	86	10.0	11.5	1.7	1.9
5'-CF-AEA-	75	82			6.0	4.2		

*corrected for activity in the supernatant that was carried over from the initial treatment.

Column Chromatography

Results are presented in Figs. 44 and 45.

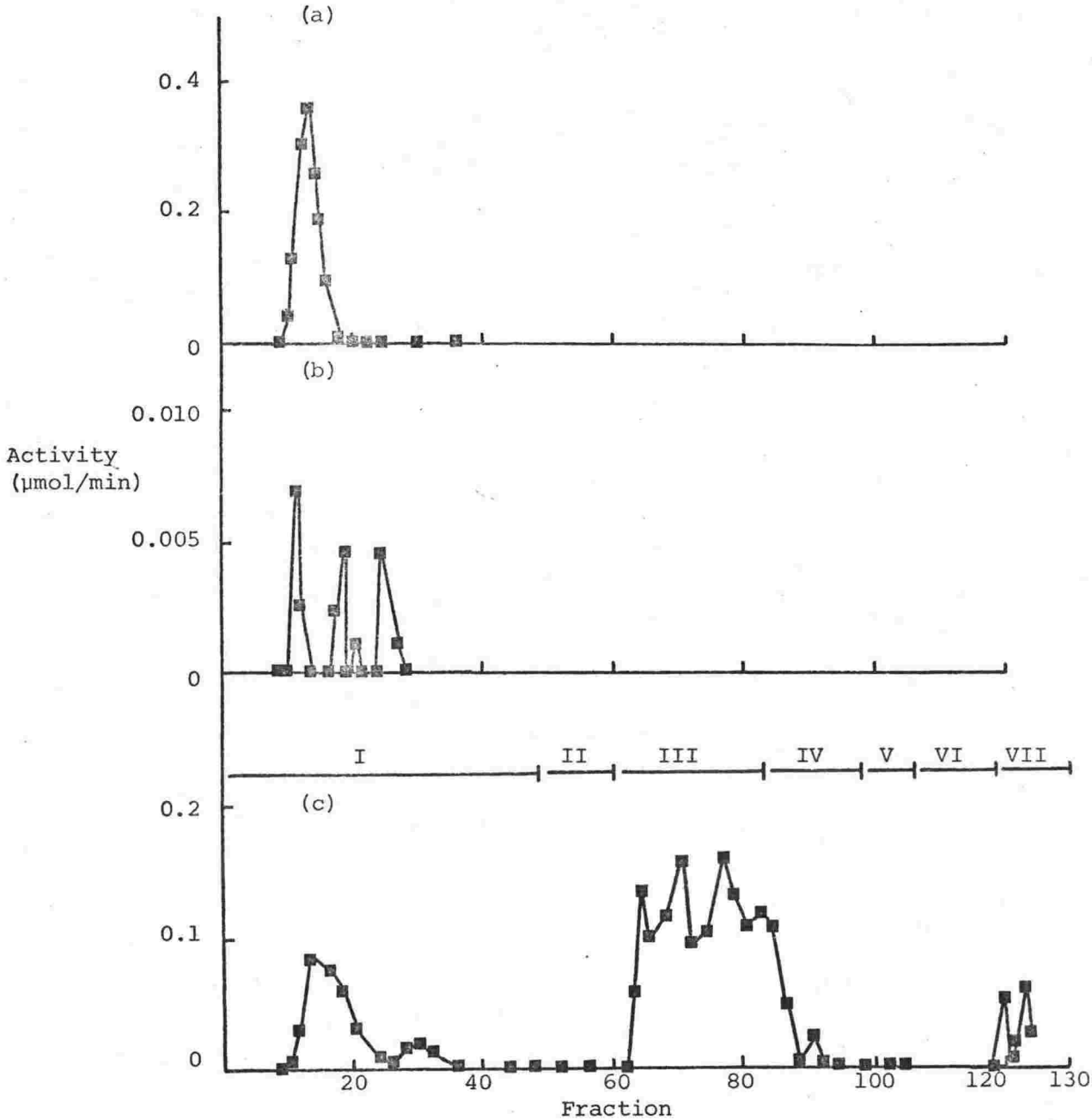


Fig. 44. Affinity chromatography of GSH S-aryltransferase using 5'-carboxyeosin-substituted Sepharose.
(a). Unsubstituted Sepharose: Sample size was 0.05ml ≡ 1mg acetone powder; eluent was 0.01M-pyrophosphate, pH 8.3; fraction size was 5 drops (0.2ml). Recovery was 100%.
(b). 5'-CE-AHA-Sepharose: Conditions as in (a). Recovery was <2% of that on unsubstituted Sepharose.
(c). 5'-CE-AHA-Sepharose: Sample size was 0.5ml ≡ 10mg acetone powder; fraction size was 5 drops up to fraction 34, thereafter 25 drops. Total recovery was 11%. Eluents were as follows:
I:0.01M-PP_i pH 8.3; II:0.01M-PP_i, pH 9.5; III:0.1M-PP_i + 4mM-GSH, pH 8.3; IV:2M-NaCl, pH 8.3; V:0.1M-acetate, pH 4.0; VI:0.1M-acetate + 5% ethanol, pH 4.0; VII:0.01M-PP_i + 120mM-GSH, pH 8.3.

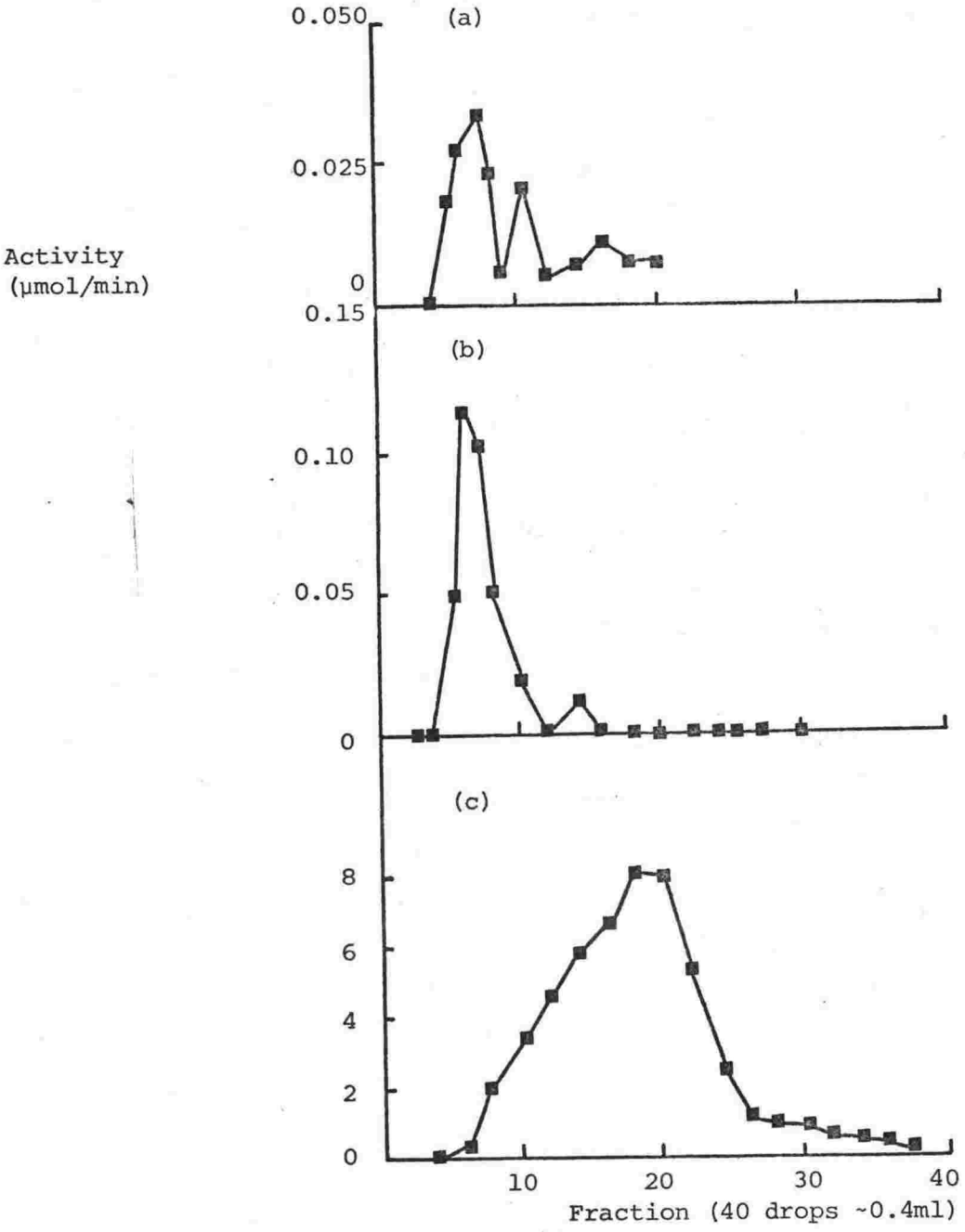
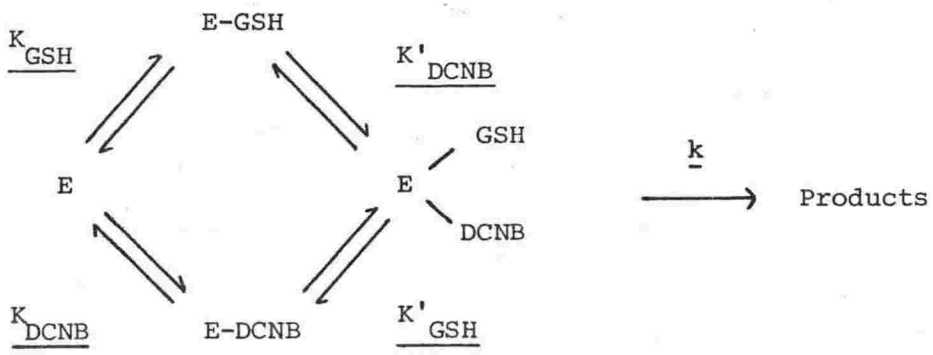


Fig. 45. Affinity chromatography of GSH S-aryltransferase using substituted AEA-Sepharose derivatives.
All experiments used 0.01M-PP_i, pH 8.3, as buffer.
(a). 5'-CE-AEA-Sepharose: Sample size was 0.10ml ≡ 2mg acetone powder (6.3U). Recovery was 2.6%.
(b). 5'-CF-AEA-Sepharose: Same conditions as (a). Recovery was 7.9%.
(c). 5'-CF-AEA-Sepharose: Sample size was 4.9ml ≡ 48mg acetone powder (192U). Recovery was 28%.

KINETIC STUDIES

The equilibria and associated substrate dissociation constants for GSH S-aryltransferase have been defined by Clark et al (1967) and are shown here in Scheme I with DCNB as aromatic substrate.



Scheme I Reaction sequence for addition of substrates to GSH S-aryltransferase.

Graphical analysis of initial kinetic runs (Figs.46-50) by the method of Hanes (1932) gave linear plots and suggested that rapid equilibrium kinetics could be applied to the system. Results were then processed on an IBM 1130 computer using the program of Cleland (1967) and the equation appropriate for a bireactant mechanism, namely

$$\underline{v} = \frac{\underline{V_m}\ ab}{ab + b\underline{K_a} + a\underline{K_b} + \underline{K_{ia}}\ \underline{K_b}} \qquad (1)$$

This equation is compatible with the mechanism in Scheme I with

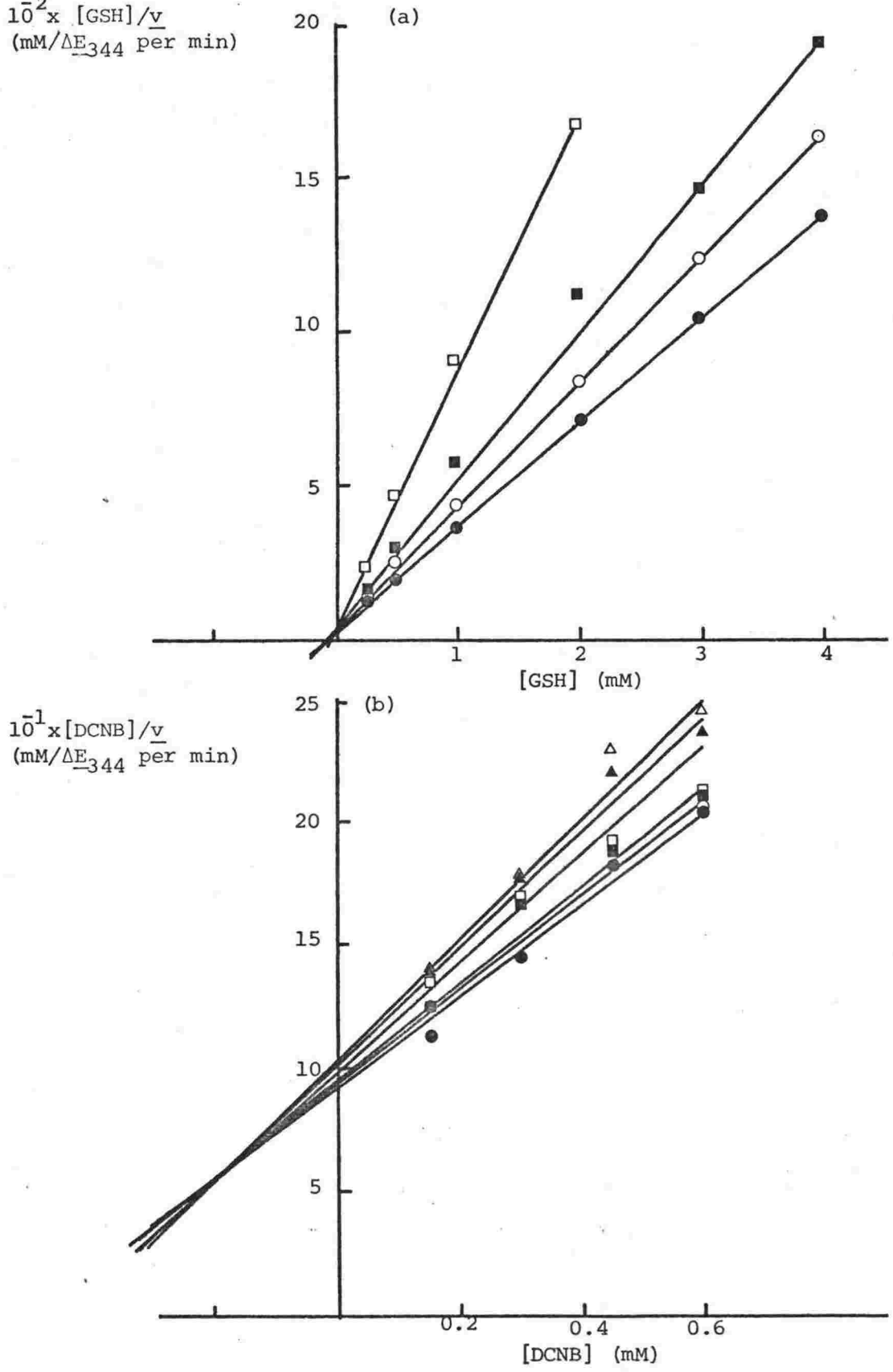


Fig. 46. [S]/v against [S] plots for pI 8.7 GSH S-aryltransferase at pH 8.7, 15°C.

In (a), DCNB concentrations were: ● , 0.60mM; ○ , 0.45mM; ■ ,0.30mM; □ , 0.15mM.

In (b), GSH concentrations were: ● , 4mM; ○ , 3mM; ■ , 2mM; □ , 1mM; ▲ , 0.50mM; △ , 0.25mM.

Lines were fitted by eye using the coordinates of the intersection point calculated from the computed substrate dissociation constants.

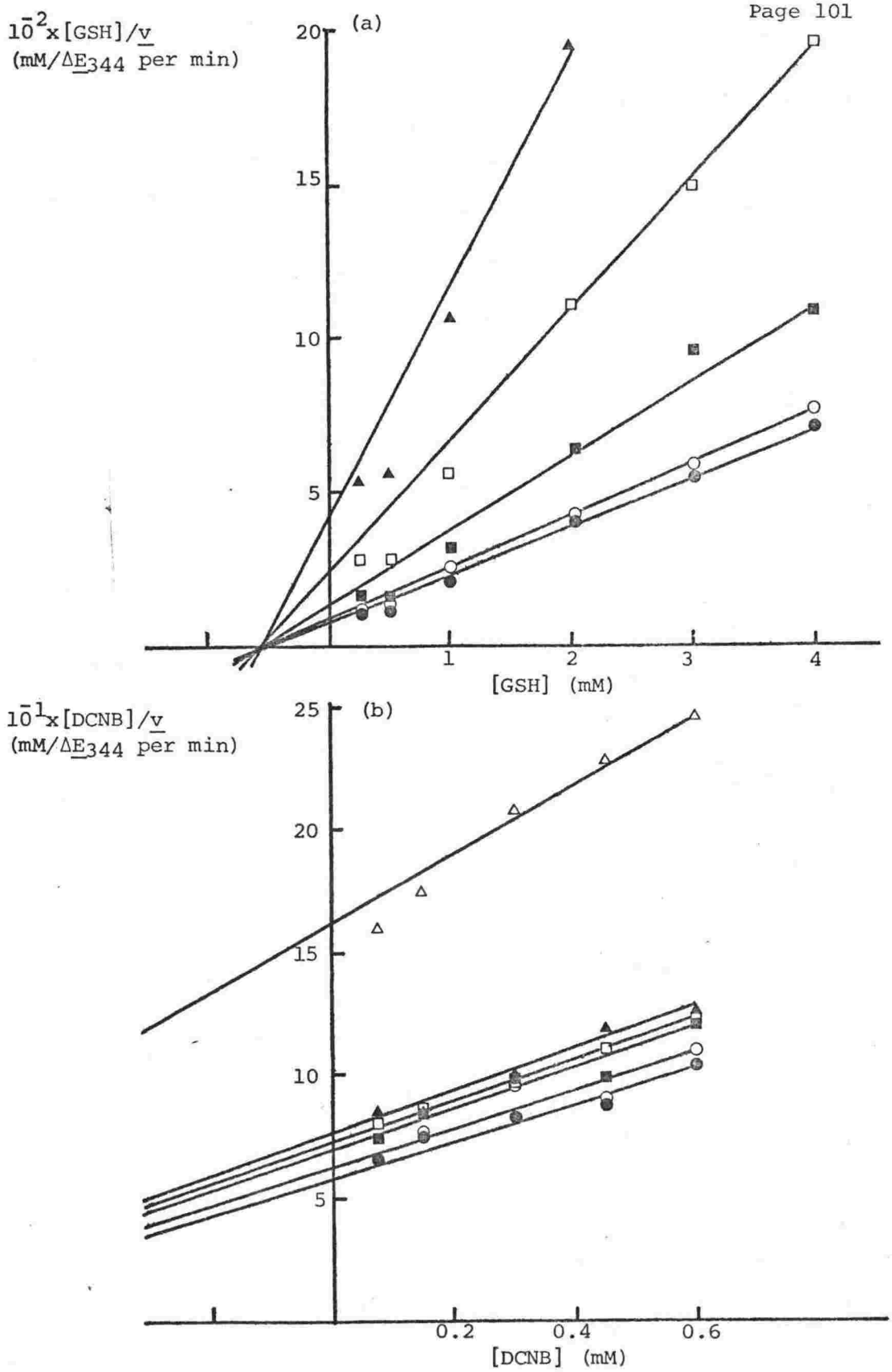


Fig. 47. [S]/v against [S] plots for pI 8.7 GSH S-aryltransferase at pH 8.7, 22°C.

In (a), DCNB concentrations were: ● , 0.60mM, ○ , 0.45mM; ■ , 0.30mM; □ , 0.15mM; ▲ , 0.075mM.

In (b), GSH concentrations were: ● , 4mM; ○ , 3mM; ■ , 2mM; □ , 1mM; ▲ , 0.50mM; △ , 0.25mM.

Lines were fitted by eye using the coordinates of the intersection point calculated from the computed substrate dissociation constants. In (b), lines intersect at (-1.6, -6.0).

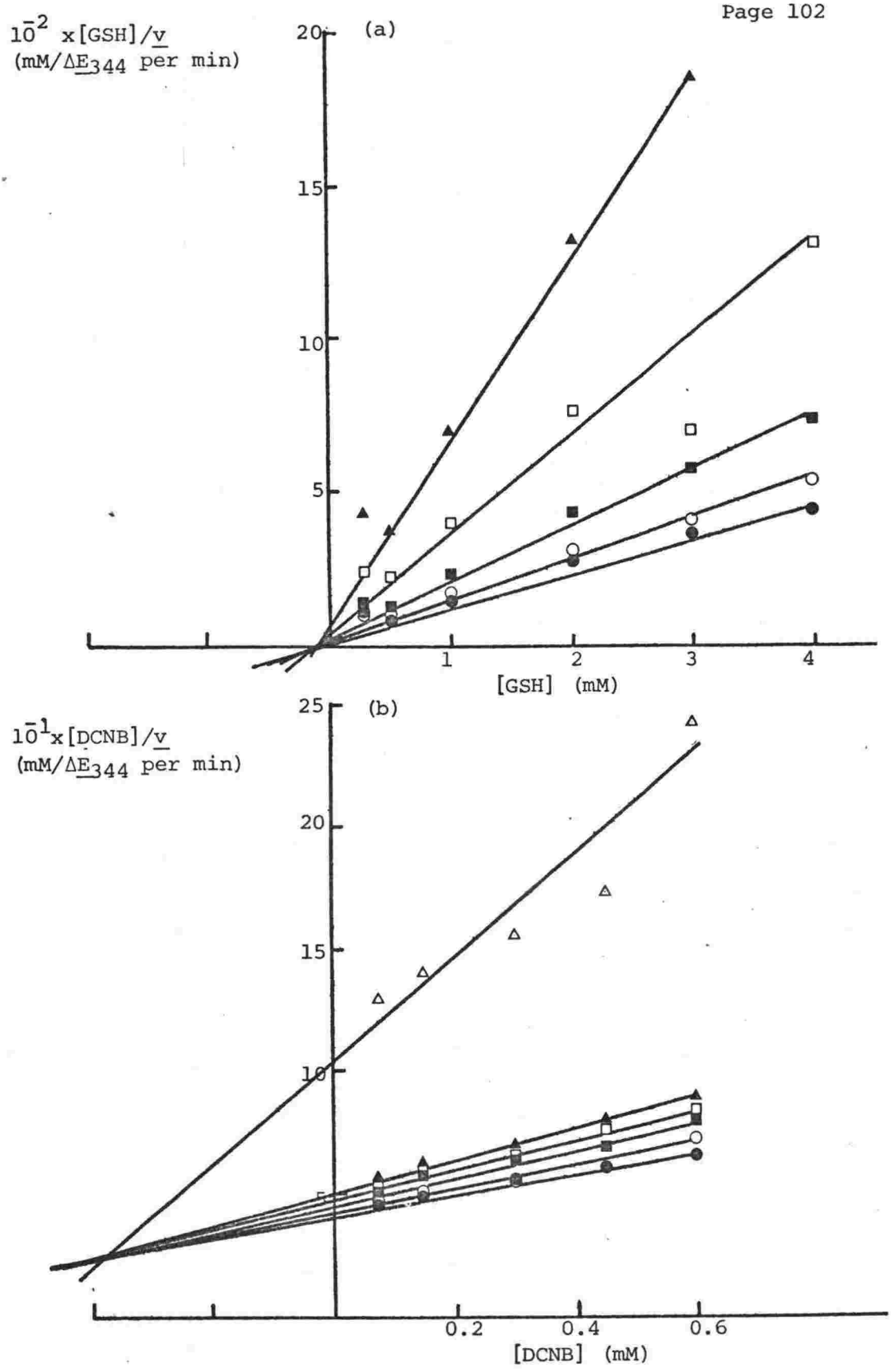


Fig. 48. [S]/v against [S] plots for pI 8.7 GSH S-aryltransferase at pH 8.7, 30°C.

In (a), DCNB concentrations were: ● , 0.60mM; ○ , 0.45mM; ■ , 0.30mM; □ , 0.15mM; ▲ , 0.075mM.

In (b), GSH concentrations were: ● , 4mM; ○ , 3mM; ■ , 2mM; □ , 1mM; ▲ , 0.50mM; △ , 0.25mM.

Lines were fitted by eye using the coordinates of the intersection point calculated from the computed substrate dissociation constants.

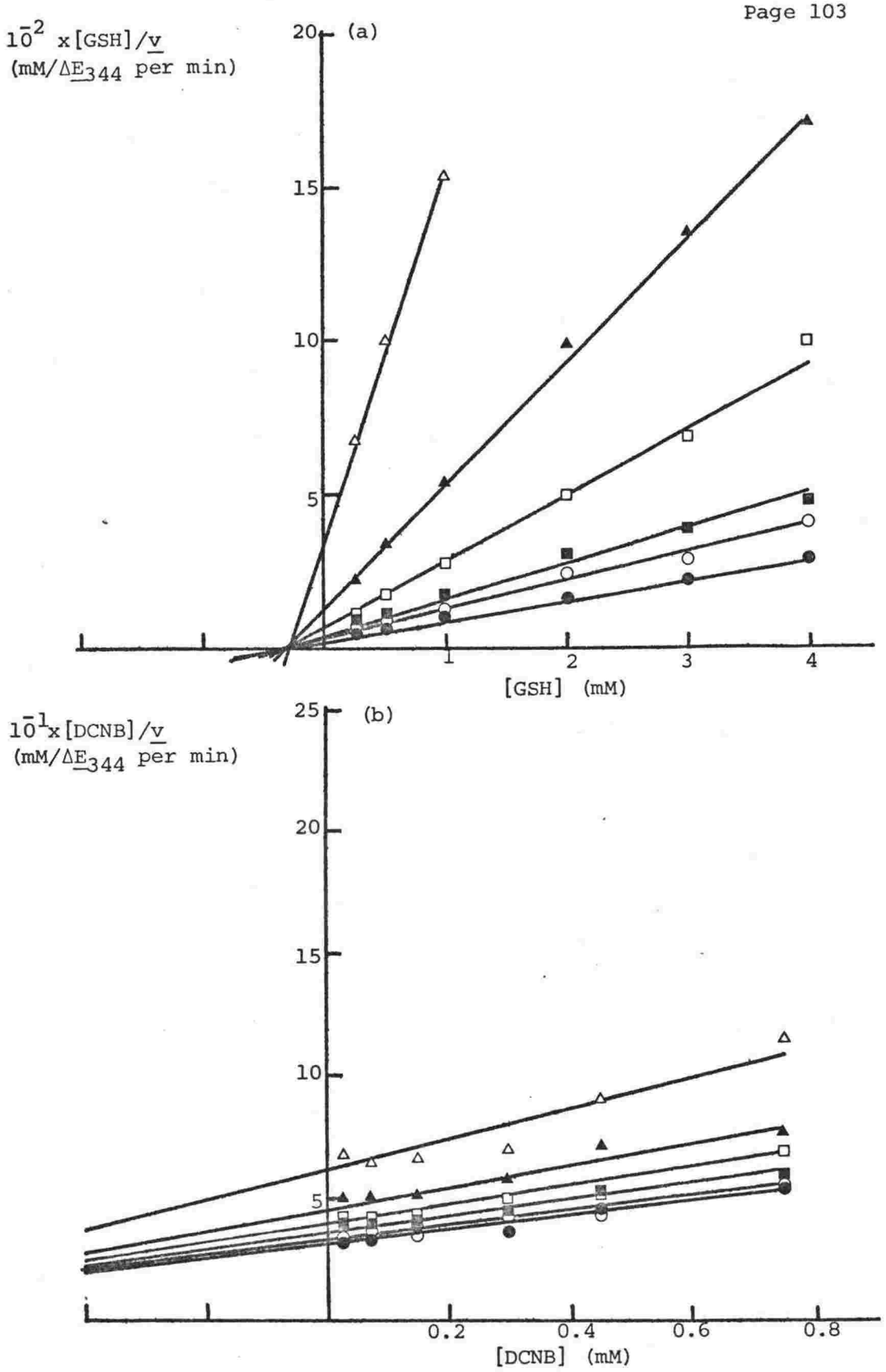


Fig. 49. [S]/v against [S] plots for pI 8.7 GSH S-aryltransferase at pH 8.7, 37°C.

In (a), DCNB concentrations were: ● , 0.75mM; ○ , 0.45mM; ■ , 0.30mM; □ , 0.15mM; ▲ , 0.075mM; △ , 0.025mM.

In (b), GSH concentrations were: ● , 4mM; ○ , 3mM; ■ , 2mM; □, 1mM; ▲ , 0.50mM; △ , 0.25mM.

Lines were fitted by eye using the coordinates of the intersection point calculated from the computed substrate dissociation constants. In (b), lines intersect at (-0.91, 0.60).

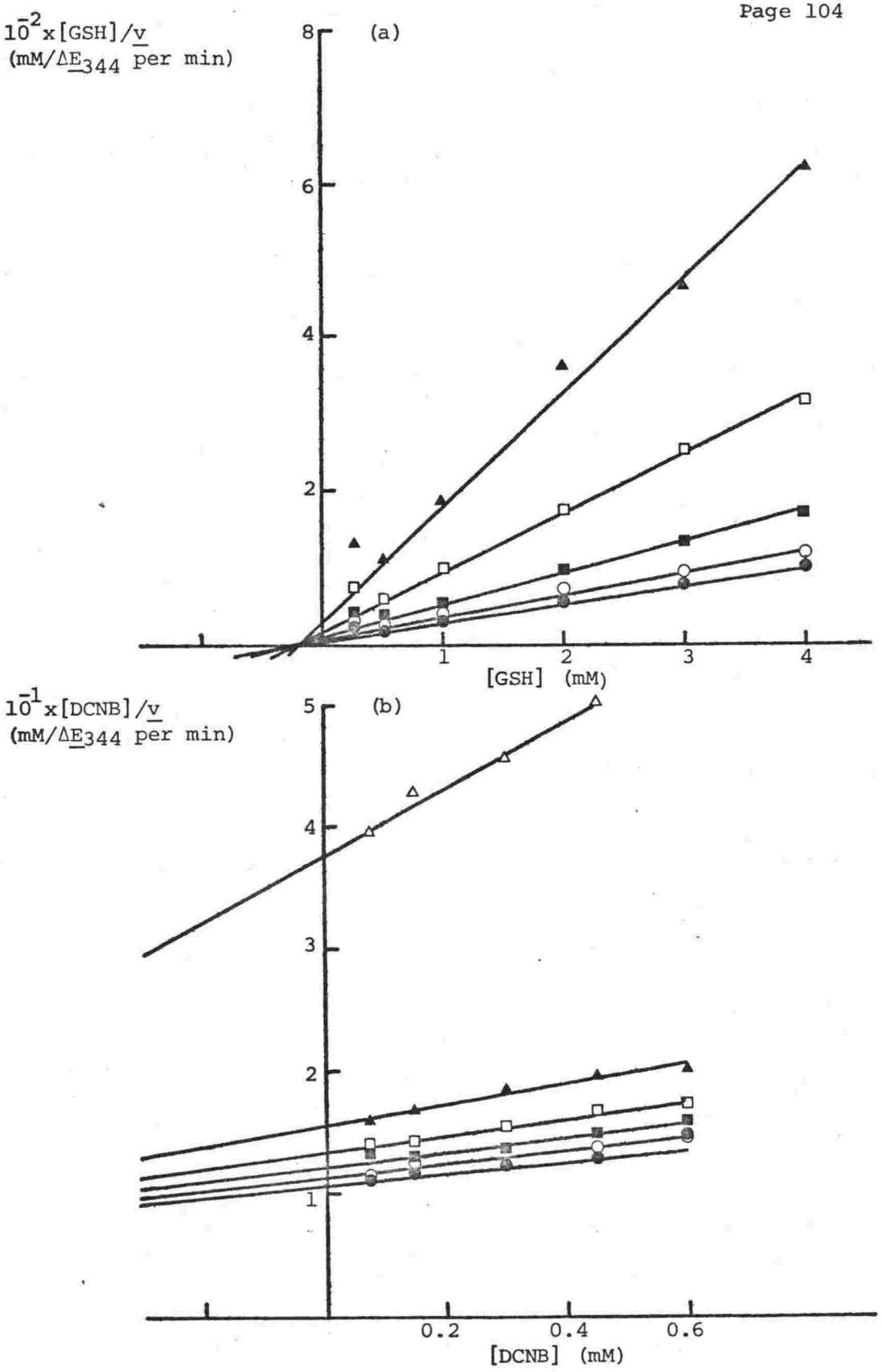


Fig. 50. [S]/v against [S] plots for pI 4.6 GSH S-aryltransferase at pH 8.65, 37°C.
In (a), DCNB concentrations were: ● , 0.60mM; ○ , 0.45mM; ■ , 0.30mM; □ , 0.15mM; ▲ , 0.075mM.
In (b), GSH concentrations were: ● , 4mM; ○ , 3mM; ■ , 2mM; □ , 1mM; ▲ , 0.50mM; △ , 0.25mM.
Lines were fitted by eye using the coordinates of the intersection point calculated from the computed substrate dissociation constants. In (b), lines intersect at (-1.2, 0.57).

K_a and K_b corresponding to K'_{GSH} and K'_{DCNB} respectively, and K_{ia} (and K_{ib}) corresponding to K_{GSH} (and K_{DCNB}) respectively.

Initial computing runs incorporated automatic weight revision which showed that the weights converged to a constant value closely proportional to v^2 - this is of interest since in many instances where rates are measured at constant enzyme concentration and the variances of v are essentially constant the weighting factors are equal to v^4, and v^2 weighting factors become appropriate when different enzyme levels are used to produce nearly identical velocities with equal standard errors and the data are adjusted to equal enzyme concentration for analysis (Cleland, 1967). Thus it seems that in this work, where the recorder sensitivity was adjusted to give similar reaction slopes (and apparent velocities), the second situation above is approximated and in the final computing runs v^2 weighting factors were used.

Reversal of the subscripts in the dissociation constants for each substrate [in (1)] gave the same answer for each parameter, indicating that the system is symmetrical and providing some justification for use of the model.

The computed kinetic constants at each temperature over the pH range studied are tabulated in Appendix III. The measured reaction velocities (v_o values) have been stored on punched computer cards for future reference.

Effect of pH

The variations of V_m and the substrate constants with H^+-concentration at each temperature are shown as log-log plots in Figs. 51-55 .

Dissociation constants of substrate-binding groups in the active site.

Application of Dixon's rules (Dixon & Webb, 1964; p.137) to the pK_{GSH} - pH plots in Figs. 51-55 suggested, as in the work of Clark et al (1967), that two groups in the active site with dissociation constants in

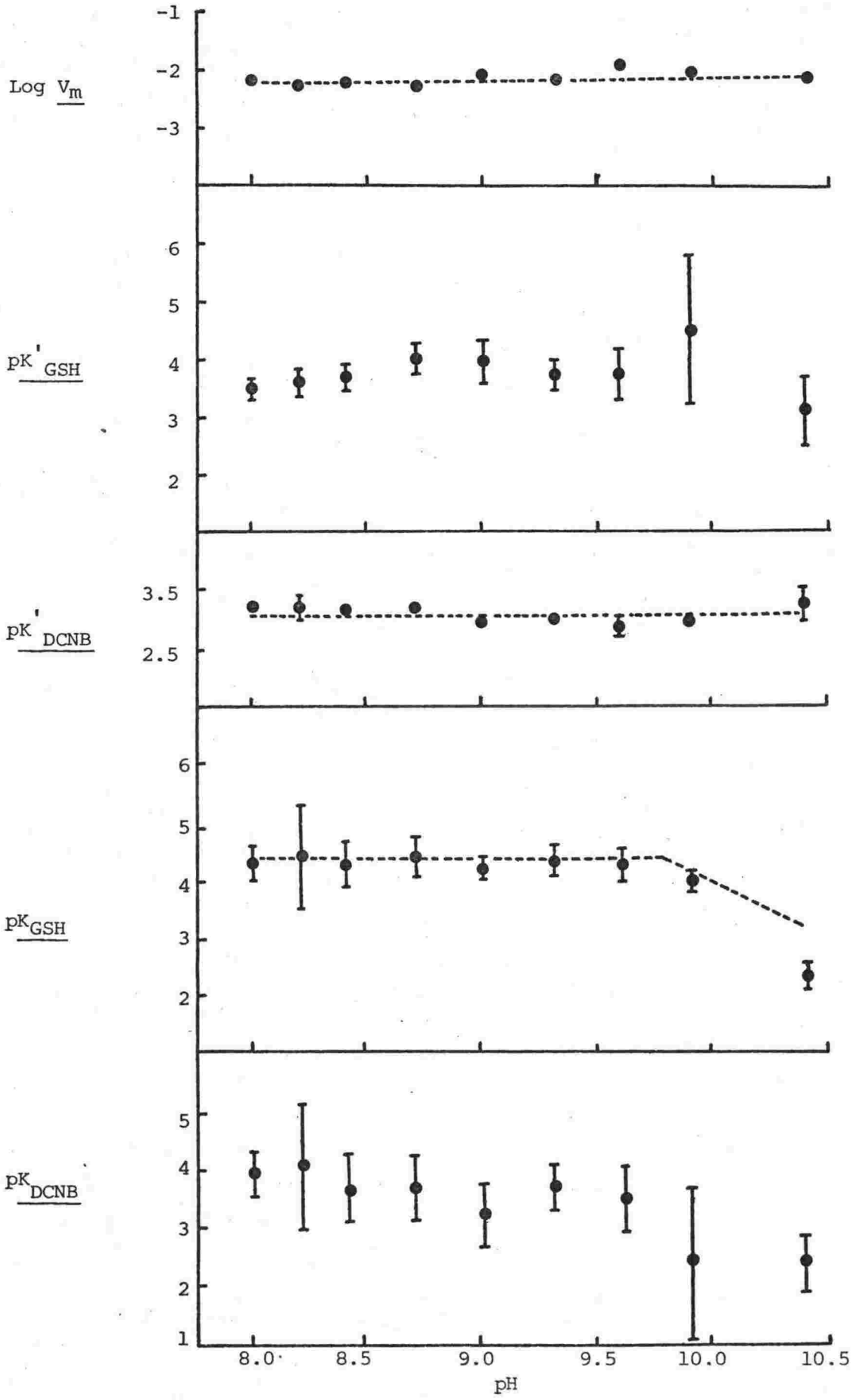
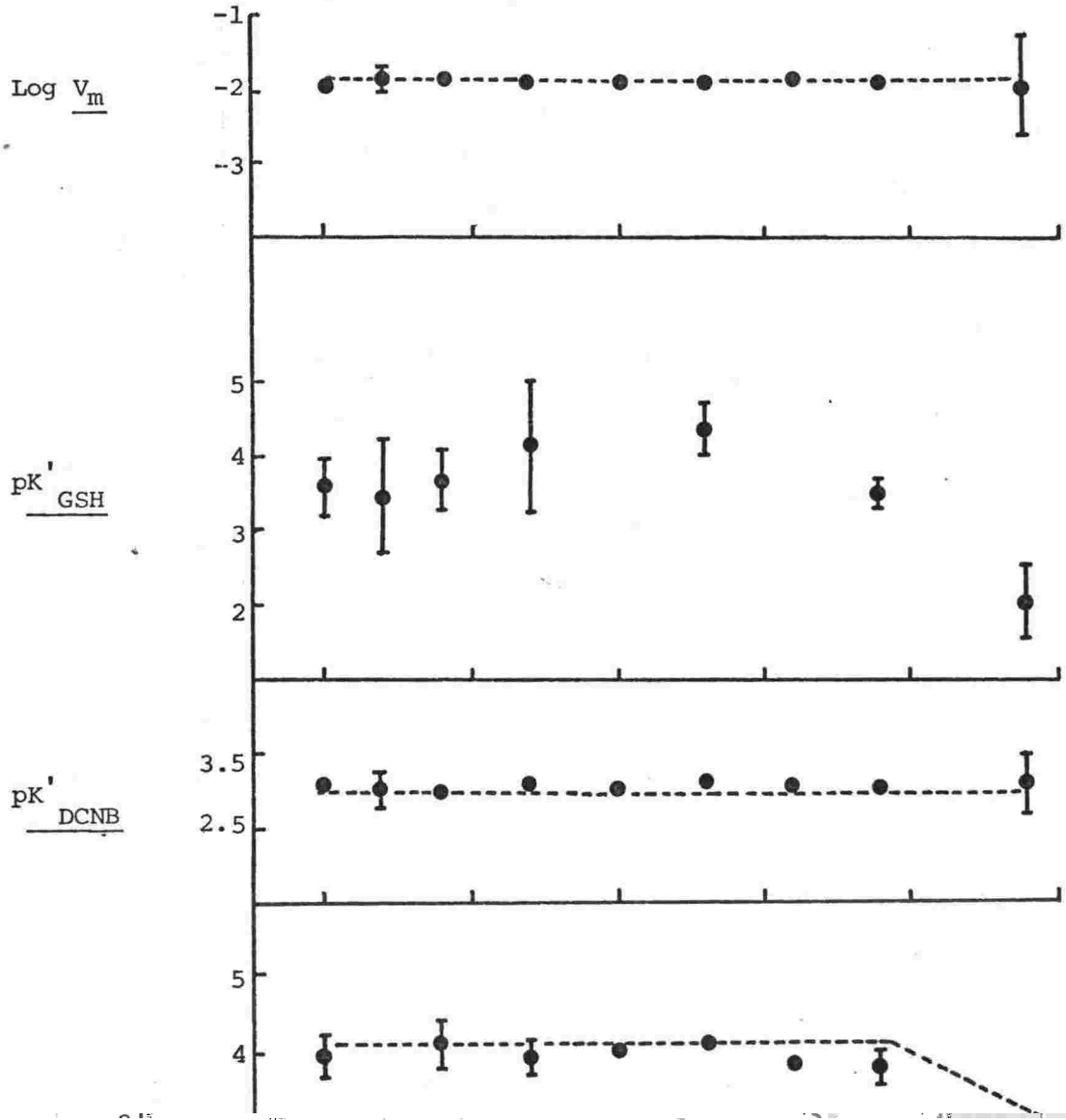


Fig. 51. Variation with pH of the kinetic constants of grass-grub pI 8.7 GSH S-aryltransferase at 15°C. Points are drawn showing their standard error. Fitted lines used the computed parameters.


Log V_m
-1
-2
-3
pK'_{GSH}
5
4
3
2
pK'_{DCNB}
3.5
2.5
5
4

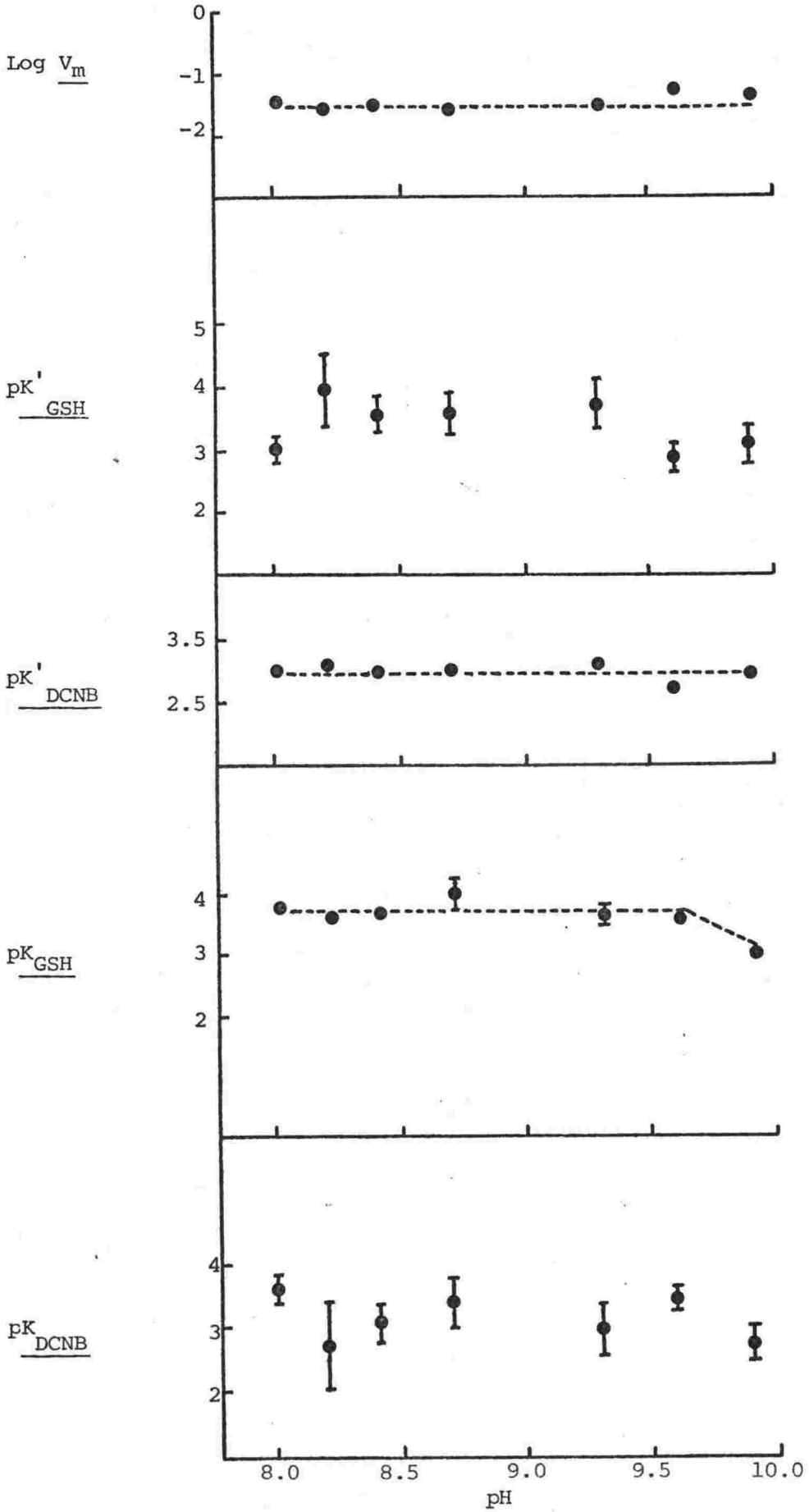


Fig. 53. Variation with pH of the kinetic constants of grass-grub pI 8.7 GSH S-aryltransferase at 30°C. Points are drawn showing their standard error. Fitted lines used the computed parameters.

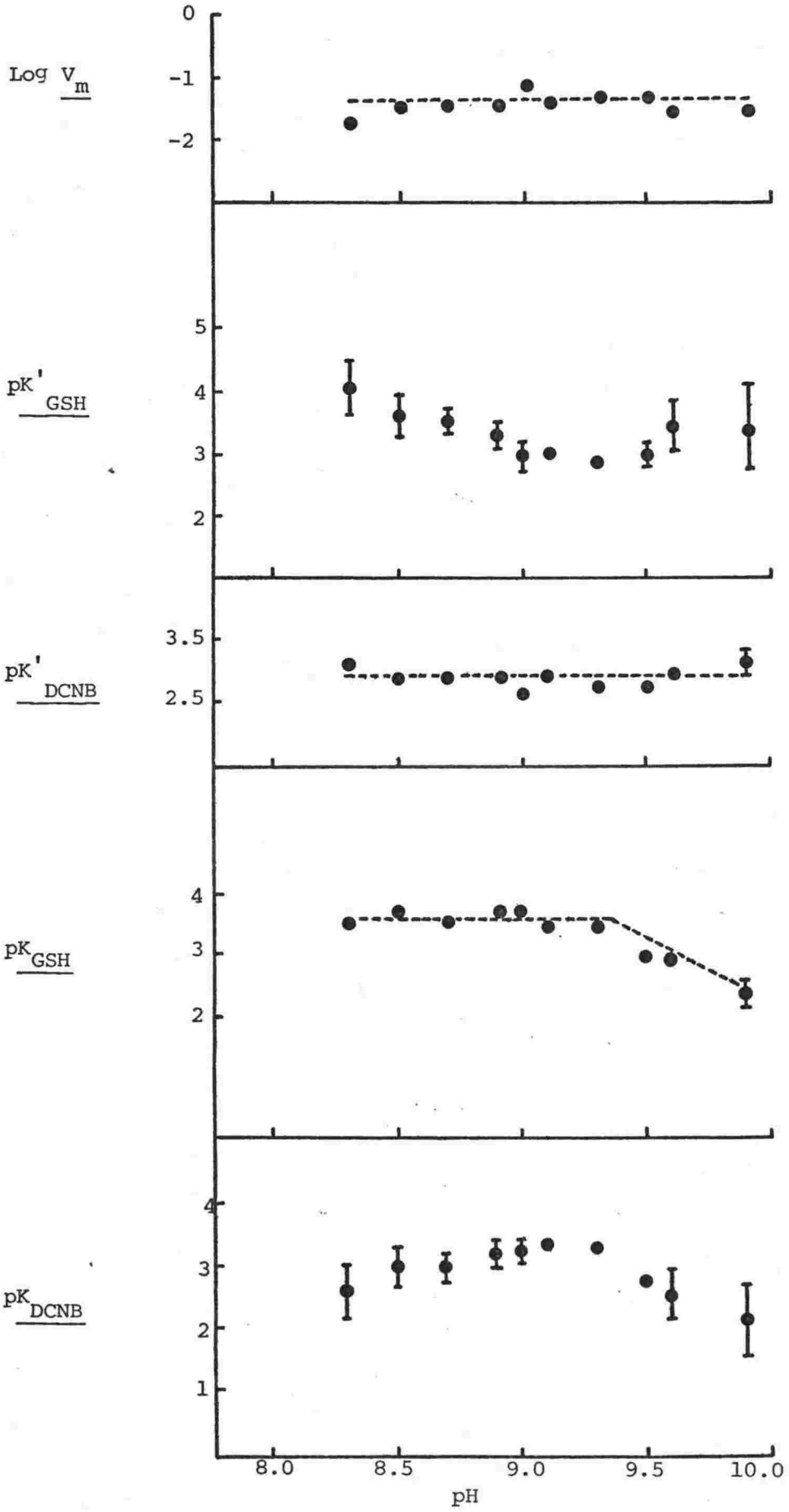


Fig. 54. Variation with pH of the kinetic constants of grass-grub pI 8.7 GSH S-aryltransferase at 37°C. Points are drawn showing their standard error. Fitted lines used the computed parameters.

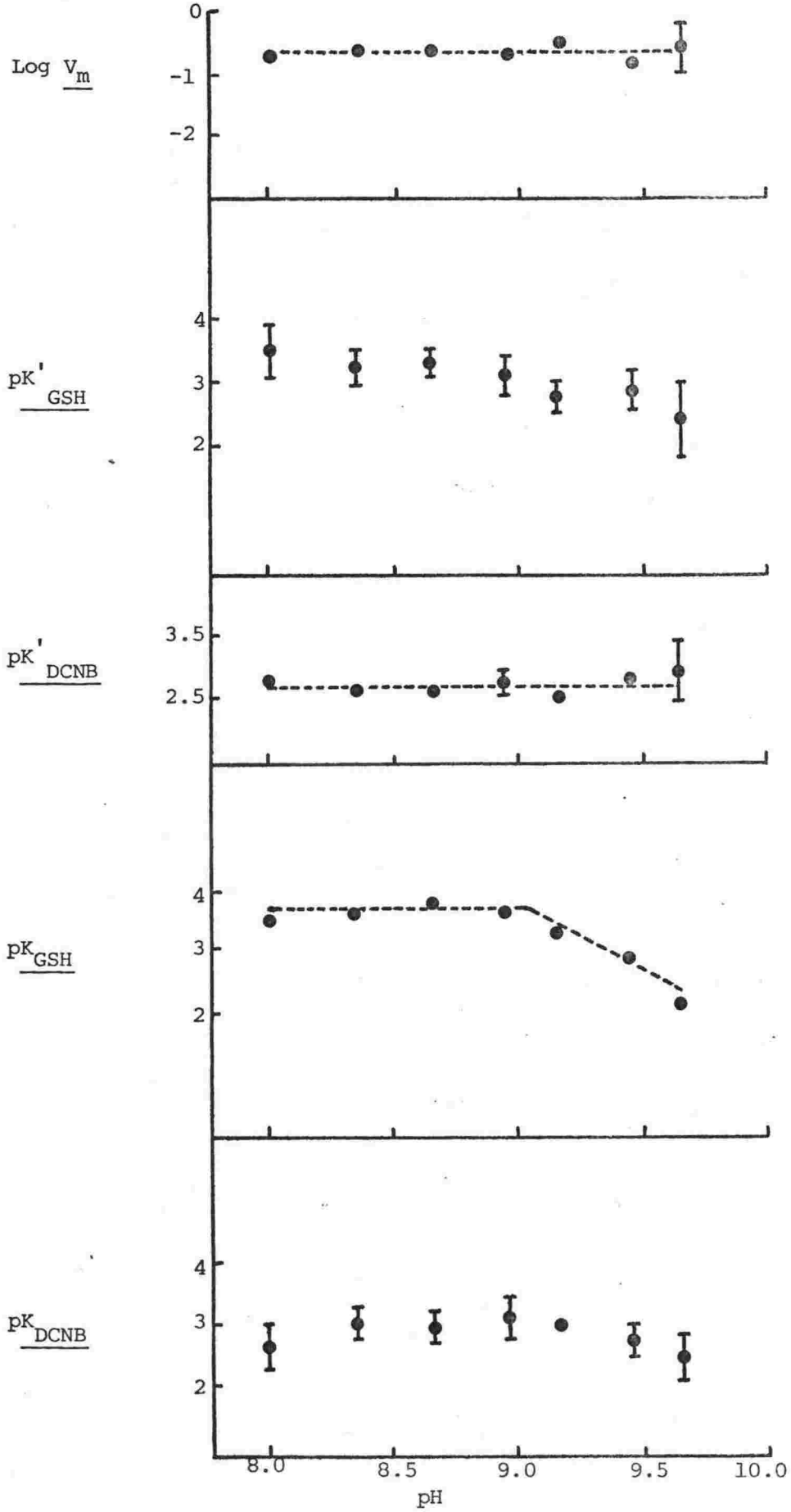


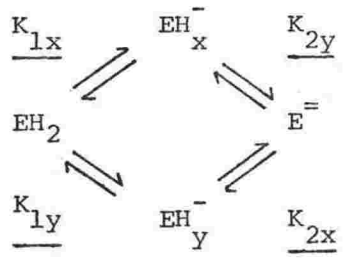
Fig. 55. Variation with pH of the kinetic constants of pI 4.6 GSH S-aryltransferase at 37°C. Points are drawn showing their standard error. Fitted lines used the computed parameters.

the range pH 9-10 are involved in binding of the substrate glutathione.

There are two situations to consider for calculation of the dissociation constants, assuming that loss of two protons occurs.

(a) non-simultaneous proton release -

The ionization pathway for the general case, for which the active site can be treated as an unsymmetrical dibasic acid, is given in Scheme II:



Scheme II Ionization pathways considering the active site as an unsymmetrical dibasic acid

and the pH-function, f_{eh_2}, describing variation of EH_2-concentration with H^+-concentration ($[EH_2] = [E]_t / f_{eh_2}$) is

$$f_{eh_2} = 1 + \underline{K_{1x}}/H + \underline{K_{1y}}/H + \underline{K_{1x}}\underline{K_{2y}}/H^2 \qquad (2)$$

For the mathematical calculation of the dissociation constants (2) reduces to

$$f_{eh_2} = 1 + \underline{K_1}/H + \underline{K_1}\,\underline{K_2}/H^2 \qquad (3)$$

which is of the same form as the equation obtained for the single ionization pathway

$$EH_2 \underset{+H^+}{\overset{\underline{K_a}}{\rightleftharpoons}} EH^- \underset{+H^+}{\overset{\underline{K_b}}{\rightleftharpoons}} E^= \qquad (4)$$

viz. $f_{eh_2} = 1 + \underline{K_a}/H + \underline{K_a}\,\underline{K_b}/H^2 \qquad (4a)$

The regression coefficients $\underline{K_1}$ and $\underline{K_2}$ in (3) are thus molecular dissociation constants (Dixon & Webb, 1964; p.124), and are equal to the true (group) dissociation constants only when the $\underline{pK}$ values are widely separated, i.e. when either of the $EH_2 \longrightarrow E^{=}$ pathways in Scheme II is dominant.

The variation of $\underline{K_{GSH}}$ with H^+-concentration is therefore described by

$$\underline{K_{GSH}} = \underline{\tilde{K}_{GSH}} \ (1 + \underline{K_1}/H + \underline{K_1}\ \underline{K_2} / H^2) \qquad (5)$$

where $\underline{\tilde{K}_{GSH}}$ is the pH-independent value of $\underline{K_{GSH}}$,

H is the H^+-concentration, and

$\underline{K_1}$ and $\underline{K_2}$ are as described above.

(b) concerted proton release -

the relevant equilibrium is

$$EH_2 \overset{\underline{K}}{\rightleftharpoons} E^{=} + 2H^+ \qquad (6)$$

and the variation of $\underline{K_{GSH}}$ with H^+- concentration is described by

$$\underline{K_{GSH}} = \underline{\tilde{K}_{GSH}} \ (1 + \underline{K}/H^2) \qquad (7)$$

The $\underline{K_{GSH}}$ values (Appendix III) were fitted to (5) and (7) using the weighted linear regression procedure of Wilkinson (1961). Calculations were done on a Hewlett - Packard 9810A programmable calculator and a Hewlett - Packard 2100A computer running in Time-Share BASIC.

Equation (5) gave negative values for $\underline{K_1}$ and $\underline{K_2}$ with large coefficients of variation, which precluded calculation of a heat of

ionization for the group(s) assuming non-simultaneous proton release.

The best fit was obtained using equation (7). This implies that $K_1 << K_2$ in (3) and, hence $f_{eh_2} \equiv 1+(K_1 K_2)/H^2$. This is mechanistically difficult to reconcile but does provide a value for the dissociation constant which can be used for calculation of the heat of ionization. Results are shown in Table 28. The absolute value of pK_2 obtained from (5) is included for comparison.

Table 28. Dissociation constants for GSH S-aryltransferase calculated assuming concerted proton release

temp. (°C)	$p\tilde{K}_{GSH}$ ±S.E.	pK ±S.E. [from (7)]	pK_e ±S.E.*	$\lvert pK_2 \rvert$ [from (5)]
15	4.34±0.13	19.54±0.23	9.77±0.11	9.78±0.11
22	4.05±0.05	19.87±0.22	9.93±0.11	9.58±0.60
30	3.75±0.08	19.19±0.12	9.59±0.06	9.47±0.13
37	3.59±0.06	18.72±0.14	9.36±0.07	8.94±0.79
37**	3.70±0.02	18.05±0.07	9.03±0.03	8.99±0.13

*pK_e is assumed equal to $0.5pK$

**pI 4.6 enzyme; all other values are for pI 8.7 enzyme.

An attempt was made to refine the dissociation constants (Wilkinson, 1961) by re-fitting the K_{GSH} data to the equation

$$p\left(\frac{K_{GSH}}{K^\circ_{GSH}}\right) = p\left(\frac{\tilde{K}_{GSH}}{\tilde{K}^\circ_{GSH}}\right) + p\left(\frac{K}{K^\circ}\right) \cdot \frac{K^\circ}{H^2+K^\circ} \qquad (8)$$

which was derived by application of the linear part of a Taylor expansion

to the logarithmic form of (7). The K°_{GSH} values were calculated from (7) using the previously obtained values of $\tilde{K}^{\circ}_{GSH}$ and K° (from $p\tilde{K}_{GSH}$ and pK, respectively, in Table 28). The weights were revised (Wilkinson, 1961) for the logarithmic fit. The residual mean squares reached a constant value after 3 - 5 cycles of iteration, but no significant improvements in the coefficients of variation of the dissociation constants were obtained.

Heat of Ionization of substrate-binding groups in the active site of pI 8.7 GSH S-aryltransferase

Determination of the pK_{GSH} – pH curves at several temperatures enables measurement of change of pK of the ionizing group(s) involved in enzyme -GSH binding from the displacement of the bends (Dixon & Webb, 1964; p.165). The heat of ionization of the corresponding group then can be obtained from the equation (Dixon & Webb, 1964; p.166)

$$\Delta H_i = -2.303\, R T^2 \frac{dpK}{dT} \qquad (9)$$

or

$$\Delta H_i = 2.303 R \frac{dpK}{d(1/T)} \qquad (10)$$

where R = 1.986 cal/mol per °K

The heat of ionization of the relevant groups in the active site was obtained from a weighted linear regression of pK_e against $(1/T)$ using the pK_e values in Table 28. For comparison, the calculations were repeated using $|pK_2|$. Results are plotted in Fig. 56. The heat of ionization is given in Table 29.

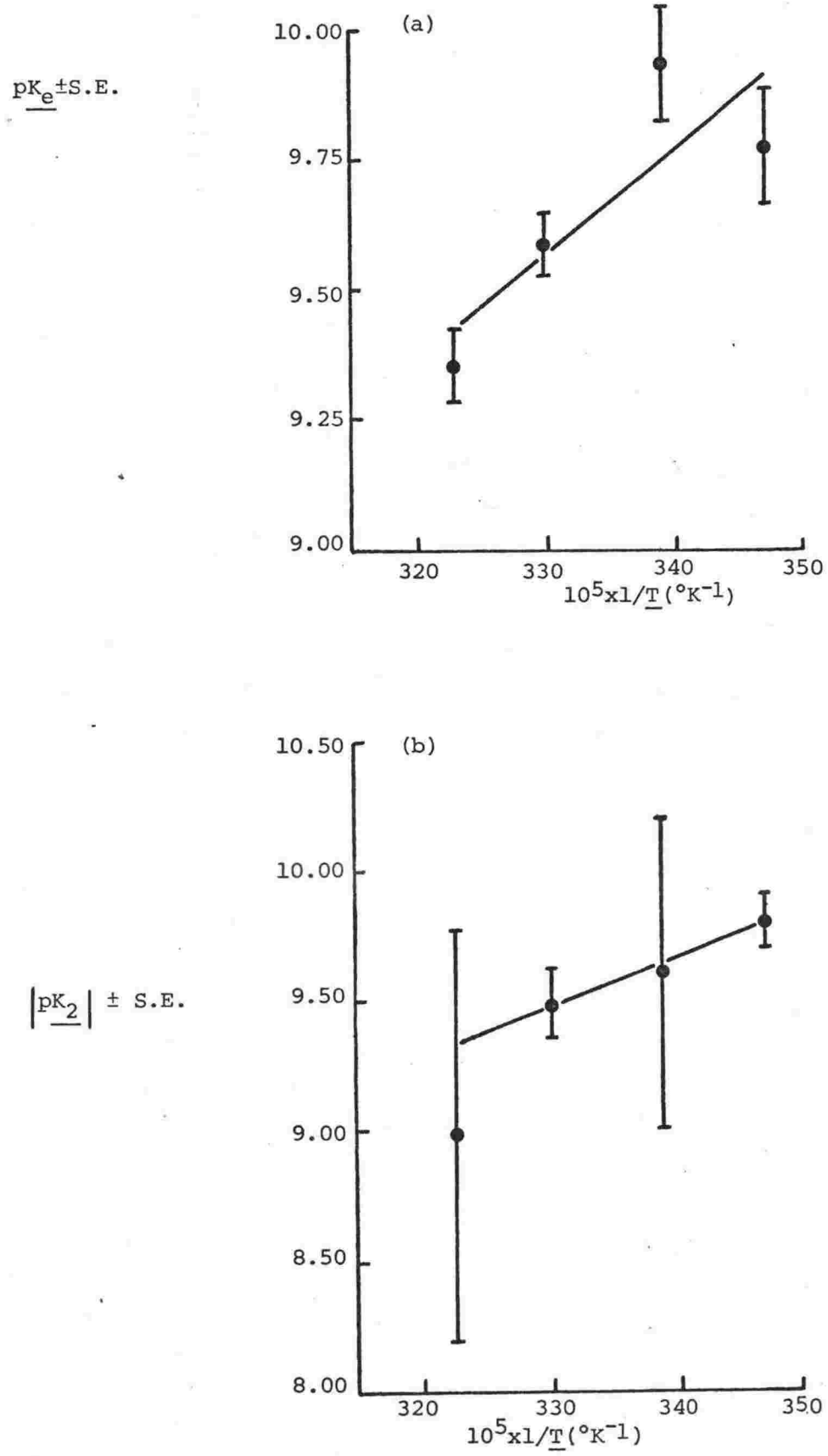


Fig. 56. Effect of temperature on the dissociation constant of group(s) in the active site of grass-grub pI 8.7 GSH S-aryltransferase.

Table 29. Heat of ionization of substrate-binding groups in the active site of pI 8.7 GSH S-aryltransferase

$\Delta H_i \pm$ S.E. (kcal/mole)	$\Delta H_i \pm$ S.E. (kJ/mole)	
9.44±3.63	39.5±15.2	(using the pK_e values)
8.73±1.54	36.6± 6.5	(using the $\lvert pK_2 \rvert$ values)

Enthalpy of Activation

It was apparent from the plots of log V_m against pH (Figs.51-55) that V_m was essentially constant with pH and accordingly a mean log V_m ($\overline{\log V_m}$) and its standard error were calculated at each temperature (Table 30).

Table 30. Mean log V_m values for pI 8.7 GSH S-aryltransferase

temp. (°C)	$-\overline{\log V_m} \pm$ S.E.M.
15	2.173±0.103
22	1.874±0.030
30	1.570±0.091
37	1.413±0.068

The enthalpy of activation (ΔH^*) was obtained from a weighted linear regression of $\overline{\log V_m}$ against ($1/T$) (Dixon & Webb, 1964; p.152)

$$\frac{d\overline{\log V_m}}{d(1/T)} = -\frac{\Delta H^* + RT}{2.303R} \qquad (11)$$

The apparent activation energy is given by

$$E = \Delta H^* + RT \qquad (12)$$

and thus

$$\Delta H^* = E - RT \qquad (13)$$

Results are given in Table 31.

Table 31. Activation energies for pI 8.7 GSH S-aryltransferase

Quantity	Value ± S.E. (kcal/mole)	Value ± S.E. (kJ/mole)
E	13.64±0.97	57.1±4.1
ΔH^* (37°C)	13.02±0.93	54.3±3.9

Thermodynamics of substrate binding to pI 8.7 GSH S-aryltransferase

The calculated data (see Figs.51-55) showed that $\overline{K'_{DCNB}}$ was essentially constant with pH, and a mean $\overline{pK'_{DCNB}}$ ($p\overline{K'_{DCNB}}$) and its standard error were calculated at each temperature (Table 32).

Table 32. Mean $\overline{pK'_{DCNB}}$ values for pI 8.7 GSH S-aryltransferase

Temp. (°C)	$\overline{pK'_{DCNB}}$ ± S.E.M.	$10^3\overline{K'_{DCNB}}$ (M)
15	3.121±0.130	0.757
22	3.102±0.051	0.790
30	2.948±0.095	1.128
37	2.949±0.082	1.127

The enthalpy of binding of DCNB to the enzyme -GSH complex was calculated from the equation (derived from IV.180; Dixon & Webb, 1964; p.151)

$$\Delta H^\circ = -2.303\,R\,\frac{d\overline{pK'_{DCNB}}}{d(1/T)} \qquad (14)$$

by a weighted linear regression of $\overline{pK'_{DCNB}}$ against $(1/T)$.

The standard free energy change for the combination was calculated from (Dixon & Webb, 1964; IV.179)

$$\Delta G^\circ = -2.303RT\,\overline{pK'_{DCNB}} \qquad (15)$$

and the entropy change of the process was calculated from (Dixon & Webb, 1964; IV.181)

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \qquad (16)$$

Similar calculations were made for binding of GSH to the enzyme using the pH-independent values $p\tilde{K}_{GSH}$ given in Table 28. Results are given in Table 33.

Table 33. Thermodynamic parameters for pI 8.7 GSH S-aryltransferase

parameter	value ± S.E. (kcal/mole)	value ± S.E. (kJ/mole)
ΔH° (DCNB binding)	-4.21±1.21	-17.62±5.06
ΔG° (DCNB binding, 37°C)	-4.18±0.12	-17.50±0.50
ΔS° (DCNB binding, 37°C)	0±4*	0±18**
ΔH° (GSH binding)	-13.41±0.93	-56.13±3.87
ΔG° (GSH binding, 37°C)	-5.25±0.03	-21.96±0.12
ΔS° (GSH binding, 37°C)	−26±3*	−110±13**

* cal/mol per °K

**J/mol per °K

DISCUSSION

It was intended at the outset that this thesis would continue the work of Clark (1967) and extend the kinetic studies of the active site of grass grub GSH S-aryltransferase. A large supply of grubs was obtained, and stored deep-frozen, for purification of the enzyme on a scale larger than previously attempted. Early results showing unanticipated instability, and the discovery of enzyme multiplicity, necessitated some divergence from the initial aims. Thus, discussion of the results can be divided into three rather broad sections: the instability of GSH S-aryltransferase in crude homogenates and attempts to prevent the loss of activity; the detection and partial purification of multiple forms of the enzyme; and studies of the purified enzymes.

INSTABILITY OF GSH S-ARYLTRANSFERASE

The observed instability of grass grub GSH S-aryltransferase is at variance with the results of earlier workers. Clark et al (1967) found that activities of aqueous solutions remained constant at 5°C for at least three weeks, whereas Balabaskaran (1972) found a 10% loss in 15h at 5°C. The results presented here show a loss of up to 60% in 15h at neutral pH and 5°C when deep frozen grubs were used, and that the instability increased markedly with pH - the half-life for loss of 50% of the initial activity decreased from greater than 28h at pH 6 to 1.3 - 1.6h at pH 9 with either CDNB or DCNB as second substrate. The non-linearity of the log (activity) against time plots suggests that some factor other than inherent instability of the enzyme causes the loss of activity; in contrast, the similar plots for enzyme solutions from live grubs were linear. The earlier workers appear to have used only live grubs as their enzyme source, and the finding in this work that enzyme solutions from live grubs were

considerably more stable implicated the freezing procedure in the instability. Work initially, therefore, concentrated on overcoming and explaining the nature of the instability because further bulk supplies of the grubs were not available.

Proteolytic Inactivation

It is well known (Dixon & Webb, 1964; p.32) that freezing and thawing of tissues may disrupt subcellular particles, and that loss of enzyme activity in crude tissue extracts is due in part to proteolysis by lysosomal enzymes and other peptidases (Wilkinson, 1970). The little information available on insect lysosomes (Lockshin, 1969) suggests that they behave similarly to vertebrate lysosomes and that they may be involved in metamorphosis. Measurement of grass grub acid phosphatase activity as a lysosomal marker established that such activity was not sedimented during the normal procedures for lysosomal preparation and, further, the addition of Triton X-100 to all fractions gave only a slight increase of this activity. This, together with the abrasive action of the soil particles ingested by the grubs, suggests that the lysosomes were ruptured in the homogenizing procedure. However, proteolysis by lysosomal enzymes cannot explain the increased lability of GSH S-aryltransferase at high pH as they act optimally at approximately pH 5.

The frozen grubs gradually darkened upon storage at -20°C, indicating that phenolic oxidations similar to those that occur in the tanning of insect cuticular protein may be occurring in the frozen state. It is often assumed that the freezing of tissues is sufficient to prevent deterioration. However, a liquid phase will exist at temperatures down to the eutectic point and this may not be reached for tissues until well below the temperatures attained by normal domestic freezers - a point substantiated by the observation (Sussman & Chin, 1966) of a liquid water phase in frozen cod tissue at about - 70°C.

Direct inactivation by tyrosinase-catalysed oxidation of tyrosine residues has been demonstrated for some enzymes (Sizer, 1950), and *o*-quinones produced from low molecular weight phenols may react with lysine or cysteine residues either intra- or intermolecularly. The addition of tyrosinase inhibitors to the homogenizing solutions or the 13 000*g* supernatant had no effect on the loss of GSH *S*-aryltransferase activity. Incorporation of sodium dithionite and several thiols to reduce or act as scavengers for quinones likewise had no effect, with the exceptions of cysteine and, particularly, GSH which went some way towards stabilizing the activity. The addition of soluble or insoluble polyvinylpyrrolidone to the extraction medium or the supernatants failed to improve the enzyme stability, although PVP has been used successfully to decrease inhibition by polyphenols during enzyme isolation from plants (e.g. Hulme *et al*, 1964; Walker & Hulme, 1965). Assuming a similarity between grass grub phenolases and those from other sources, these results eliminate such oxidation as a factor in the loss of GSH *S*-aryltransferase activity.

The instability shows some resemblance to problems encountered by Krieger and Wilkinson (1970) during preparation of microsomal mixed function oxidases from larvae of the southern armyworm (*Prodenia eridania*) and other lepidopterous larvae. They isolated an endogenous inhibitor from the gut contents of *Prodenia* and showed it to be a soluble protease, molecular weight about 26 000, with no clearly defined pH optimum - the steady increase of inhibitor-activity and the low degree of inhibition of this by DFP paralleled the findings with grass grub extracts. In this work, removal of the hind gut contents from live grass grubs before homogenization had a stabilizing effect on GSH *S*-aryltransferase, although the activity in crude extracts from live grubs was relatively stable.

Removal of the gut contents lowered the total activity obtained and hence the selective removal of a labile member of a family of enzymes cannot be excluded, although it seems improbable. Krieger & Wilkinson (1970) showed that the Prodenia inhibitor was not ingested - this, also, cannot be excluded for the grass grub inhibitor.

Substrate Protection

Of a wide range of compounds tested to stabilise the enzyme, only the substrate GSH had any marked effect. The use of enzyme-saturating concentrations of GSH in the assays of aged, inactivated, samples failed to restore activity thus indicating irreversible inhibition. The effect of GSH appeared to be primarily to decrease the rate of loss of activity rather than to prevent loss, although gradual oxidation to GSSG could account for the continued enzyme decrease. The lack of protection by other thiols (apart from the slight effect of cysteine) and reducing agents implies that the effect of GSH is due to its specific capacity as a substrate, rather than to its reducing potential or the reactivity of the thiol. It could, therefore, be shielding the active site or maintaining the enzymes in a conformation resistant to proteolysis. Such effects may be restricted to one of the enzymes.

Stabilization by Partial Purification

Acetone powder preparation or preliminary maceration in acetone prior to extraction with water did not stabilize the GSH S-aryltransferase activity.

The enzyme stability was greatly improved by ammonium sulphate fractionation provided that an initial fraction was collected at the critical concentration of 2.3 - 2.4M-$(NH_4)_2SO_4$ - above this concentration fractions containing enzyme were stable. The pH of fractionation had little effect on the pattern of enzyme precipitation or on the stability of the fractions. The activity precipitating before the critical $(NH_4)_2SO_4$ concentration was extremely unstable and its inactivation showed similar

pH- dependence to the crude supernatant. Addition of an unstable fraction to one of the stabilized fractions restored the instability, thus confirming the presence of an inhibitor and suggesting that it may be a high molecular weight polyelectrolyte, although many simple organic compounds can be salted out at high ionic strength (Dixon & Webb, 1961). The $(NH_4)_2SO_4$- stabilized fractions were not affected by pH variation and thus resembled the crude 13 000g supernatant from live grubs. Further stabilization was achieved by isoelectric focusing or DEAE-cellulose ion exchange chromatography.

MULTIPLICITY AND PURIFICATION OF GSH S-ARYLTRANSFERASE

Isoelectric focusing of dialysed $(NH_4)_2SO_4$ fraction separated two major GSH S-aryltransferases with isoelectric points of 4.6 and 8.7 and suggested the presence of several others. The smaller peaks occurred as shoulders on the large main peaks and may have arisen by re-mixing during elution from the focusing column. The two major enzymes were present in approximately equal amounts (of activity), were stable to dialysis and were stable when stored at pH values from 5 to 9.5. The peaks coincided when assays were done with CDNB and DCNB. The ratio of activity in the two peaks after isoelectric focusing of the $(NH_4)_2SO_4$ fraction probably does not reflect the ratio *in vivo* because the two enzymes could reasonably be expected to fractionate differently (Dixon & Webb, 1961). The fractionation was performed almost at the isoelectric point of the pI 4.6 enzyme which, therefore, is probably the more insoluble of the two and should precipitate at lower ionic strength.

It was predicted from the isoelectric points that DEAE- cellulose chromatography at a pH between the two values should result in adsorption of the pI 4.6 enzyme but not the pI 8.7 enzyme. This was confirmed and isoelectric focusing of the ion exchange peaks gave peaks with pI values identical to those obtained without prior ion exchange. The *I* = 0.01M ion

exchange peak focused as a single peak at pH 8.7 with 90% recovery. The $\underline{I}$ = 0.20M peak focused in two peaks, 68% at pH 4.8 and 26% at pH 8.7 - the activity at pH 8.7 was greater than that expected from tailing of the first peak on ion exchange, suggesting conversion of the pI 4.6 to the pI 8.7 enzyme. The third peak ($\underline{I}$ = 0.11M) on DEAE-cellulose focused into peaks at pH 5.8 (48%) and pH 8.7 (31%), with smaller peaks at pH 6.7 and 7.55 and shoulders at pH 4.75, 4.90 and 8.20; the pI 8.7 peak and pI 4.7 shoulder probably arose from tailing or emergence of these enzymes on ion exchange. The enzymes therefore elute from ion exchange according to their pI values, in agreement with the ion exchange mechanism for the retention process (Morris & Morris, 1964; p.326).

The presence of both major GSH $\underline{S}$-aryltransferases in the $(NH_4)_2SO_4$-fraction from live grass grubs was verified by ion exchange and isoelectric focusing. The smaller peaks, including that at pH 5.8, observed with frozen grubs were not found. This confirms that the two major enzymes, at least, are present naturally and do not arise by modification in the unstable crude extracts. However, the activity ratio for the two was about 2.5:1 (pI 8.7 : pI 4.6) compared with 1:1 for frozen grubs. The finding, when refocusing the pI 4.6 enzyme, of some pI 8.7 enzyme is further evidence for conversion of the acidic to the basic enzyme. This could arise from initial binding of the pI 8.7 enzyme with a strongly acidic molecule with subsequent dissociation of the complex during ion exchange or isoelectric focusing. The formation of multiple enzyme peaks in this way has been reported (Wilkinson, 1970). Binding of the pI 4.6 enzyme to a strongly basic molecule is also possible, but evidence was not found for conversion of the pI 8.7- to the pI 4.6- enzyme.

Isoelectric focusing of the total activity from one live grub showed the presence of both major enzymes. This tends to deny the existence of genetic variants homogenous for one or other of the major enzymes. The

activity ratio for the two enzymes was the same as in isoelectric focusing of the $(NH_4)_2SO_4$- fraction from live grubs, thus demonstrating that changes do not occur during salt fractionation.

Only GSH S-aryltransferase activity that precipitated in the stabilized $(NH_4)_2SO_4$- fraction was studied further. It is possible, therefore, that the activity in the unstable fractions represents additional variants of the enzyme that are present in amounts too small to be seen in the focusing of a single grub extract.

Multiplicity or an Experimental Artifact?

The presence of multiple protein zones in ion exchange chromatography or isoelectric focusing need not indicate inherent heterogeneity. Artifical zoning in ion exchange may occur for several reasons (see Boman, 1963; Morris & Morris, 1964; Cann, 1966). Starting buffer development in particular, is very sensitive to changes of pH and ionic strength, and it is generally accepted with gradient elution that re-chromatography under conditions expected to improve resolution is necessary to demonstrate homogeneity of a peak. Variation of the conditions for DEAE- cellulose chromatography gave identical elution patterns for GSH S-aryltransferase. The peaks were not re-chromatographed but were checked by isoelectric focusing and the agreement of results, whether or not ion exchange was used, is considered to be good evidence against artifical zoning. This eliminates the formation of artifacts by complexing with Ampholine® - such complexes have been detected in polyacrylamide gel isoelectric focusing (Frater, 1970) but have not been reported in density gradient column focusing.

The strong anion exchanger TEAE- cellulose gave results identical to DEAE- cellulose even though tighter binding of the adsorbed enzyme, shown by elution at higher ionic strength, was expected. However, a study

(Benerito et al, 1965) of the conditions necessary for conversion of DEAE-cellulose to the quaternary salt concluded that BIO-RAD TEAE-cellulose was more likely to be the weakly basic DEAE- with only slight conversion to TEAE-, i.e. BIO-RAD were probably selling an improperly labelled product at the time our lot was purchased.

Molecular weight measurements of the pI 4.6 and pI 8.7 enzymes from isoelectric focusing showed that both were identical, within a range of about 3 000. Peaks of activity were not detected at integral multiples of 20 000 up to a molecular weight of 80 000, in agreement with the finding of Balabaskaran (1972). However, activity measurements alone cannot demonstrate the formation of inactive species by association or dissociation. The experimental error in the molecular weight measurements precludes the drawing of any conclusion about either enzyme arising from proteolysis of the other.

It was suggested (Sizer, 1950) as a follow up to the studies of enzyme inactivation by tyrosinase that other non-proteolytic enzymes may attack native enzymes to give multiple forms. Microheterogeneity due to deamidation of asparagine or glutamine residues has been found with cytochrome c (Flatmark, 1966) and rabbit muscle aldolase (Midelfort & Mehler, 1972). Successive deamidations gave enzymes with successively lower isoelectric points. Flatmark (1966) also found that the minimum rate of deamidation at 37°C occurred at pH 5; rapid deamidation occurred at high $(NH_4)_2SO_4$ concentrations. McKerrow & Robinson (1971) found the deamidation of asparagine in synthetic peptides to be particularly favoured in phosphate buffer, at extreme pH or at high ionic strength. The difference of 4 pH units between the pI values of the two major grass grub GSH S-aryltransferases is considerably greater than that found in these deamidation studies and it is unlikely that the pI 4.6 enzyme is derived

from the pI 8.7 enzyme in this way. However, unequivocal proof of the nature of the heterogeneity depends on isolation of the enzymes as stable, homogeneous species.

Purification

The presence of two enzymes with widely separated pI values suggested a simple separation from each other and from the bulk protein by a combination of anion- and cation- exchange chromatography. However, the behaviour on the cation exchange columns was not reproducible, differed from that predicted from DEAE- cellulose ion exchange and isoelectric focusing, and the approach was discontinued. The strong inhibition of GSH S-aryltransferase by dicarboxylic acids (Clark, 1967; Balabaskaran, 1972) may be a factor in the lower recoveries and it may be necessary to use GSH or a competitive inhibitor of the GSH- binding site to elute the enzyme(s) from cation exchange columns.

The majority of the GSH S-aryltransferase activity precipitated within a range of 0.4M-$(NH_4)_2SO_4$. The purification achieved was only 7-fold and the main advantage of the step was that it gave a relatively stable enzyme. There was some loss of activity during dialysis of the $(NH_4)_2SO_4$- fraction but the concomitant protein precipitation resulted in overall purification. A second $(NH_4)_2SO_4$- fractionation gave a further 2-fold purification and was marginally more effective when carried out at pH 8 instead of at the same pH.

DEAE-cellulose chromatography did not increase the specific activity of the enzyme and the step was omitted from the large scale purification.

Isoelectric focusing gave the best purification and was particularly effective for the pI 8.7 enzyme when a wide pH range focusing was followed by re-focusing over a narrow range. The method was much less effective for the pI 4.6 enzyme because the majority of the protein was isoelectric

in the same region. The large scale focusing was not greatly affected by the protein precipitation that occurred at the acid end of the range - the precipitated material migrated into the anode solution and was eluted before aryltransferase.

Substrate-Specific Elution

Attempts at specific elution of adsorbed pI 4.6 GSH S-aryltransferase from DEAE-cellulose were unsuccessful. One of the requirements to effect specific elution (Yoshida, 1970) is that a change of charge occurs on binding of the substrate, or analogue, to the enzyme. At pH 6.3 the predominant form of GSH has both carboxyls and the amino group ionized (Waley, 1966), and one interpretation of the results is that GSH binds to the enzyme through the ammonium and one carboxylate group so that no change of charge occurs. If binding occurs in this way a similar failure could be expected using cation exchange columns. Because adsorption to ion exchange supports is probably dependent on localized groups of charge, elution is not mandatory with alteration of the charge balance and may depend on the relationship between the sites of adsorption and the active site. A conformational change upon substrate binding has also been suggested as a reason for substrate specific elution (Yoshida, 1970) - however, this could also lead to tighter binding. There may have been some adsorption of GSH to the column which would have prevented enzyme-substrate interaction, but this seems difficult to overcome with an amphoteric substrate. Elution with GSH-competitive inhibitors may be a better approach.

Affinity Chromatography

The requirements for effective inhibition of GSH S-aryltransferase are well defined (Clark, 1967; Balabaskaran & Smith, 1970; Balabaskaran, 1972), and it was anticipated that covalent attachment

of fluorescein derivatives to agarose through the phthalic acid ring would give a good chance for successful preparation of an affinity adsorbant.

The coupling reaction proceeded satisfactorily when dimethyl-formamide was used as solvent to overcome the insolubility of the inhibitors. It was not possible to measure the degree of substitution of the gels because of the lengthy and varied washing procedure necessary to remove tightly adsorbed inhibitor. The molar ratios of reagents were the same as used by Cuatrecasas (1970) in the preparation of oestradiol-Sepharose and coupling of a similar magnitude (0.5μmoles/ml gel) was probably achieved.

The conditions chosen for batch testing of the Eosin - Sepharoses resulted in loss from the supernatant of 40 - 50% of the GSH S-aryl-transferase activity and 10 - 15% of the protein; the Fluorescein - Sepharose was about half as effective. The adsorption was not dependent on ionic strength, buffer ions or length of the hydrocarbon chain between inhibitor and matrix. Adsorption of only 10U/ml gel is too inefficient for large scale purification at enzyme concentrations of 50 - 100 U/ml. Only 20% of the adsorbed enzyme could be eluted by 2mM-GSH.

Passage of GSH S-aryltransferase solutions through columns of the coupled inhibitors resulted in almost complete disappearance of the enzyme when small volumes were applied. With 5'-CF-AEA-Sepharose, 28% of the activity appeared in the breakthrough volume when 48mg acetone powder (in 5ml) was applied; the appearance of a dark band at the top of the column indicated non-specific protein adsorption.

Variations of pH, ionic strength, addition of ethanol or GSH were ineffective in releasing the enzyme from the 5'-CE-AHA-Sepharose column; 7% of the activity was released by 4mM-GSH in 0.1M-$Na_4P_2O_7$ buffer, but subsequent passage of 120mM-GSH had no effect.

The low recovery from the columns may be due to very high affinity of the coupled inhibitor for the enzyme, but then the batch method should have shown greater effect. Use of solutions of inhibitors with K_i values of about 10^{-6}M may be necessary to elute tightly bound enzyme. A more likely explanation for the low recoveries is that the extremely hydrophobic surface presented by the inhibitor resulted in denaturation of the enzyme. A recent study of the application of hydrophobic bonding to protein purification (Porath et al, 1973) showed that adsorption increases strongly with increasing ionic strength, is enhanced further by decrease of pH, and that high salt concentrations often promote such strong adsorption that a protein may be desorbed only by severe reduction of ionic strength and/or lowering the polarity of the medium and/or elevation of pH.

Comparative Studies of GSH S-transferases

The pattern of precipitation of activity by $(NH_4)_2SO_4$ fractionation of the 13 000g supernatant was similar for GSH S-aryl-, S-alkyl- and S-alkene-transferases. However, the stabilities to dialysis differed markedly - aryltransferase activity decreased 20% whereas alkene- and alkyl-transferase activity increased 20% and 70% respectively. The specific activity with each substrate increased as a result of protein precipitation during the dialysis.

Alkenetransferase activity chromatographed on DEAE-cellulose similarly to aryltransferase except that activity was not detected in the I = 0.20M peak. Alkyltransferase activity was too low to be detected after ion-exchange chromatography.

The isoelectric focusing results were more conclusive although the relatively large amounts of aryltransferase tended to swamp the smaller peaks. The pI 4.6 enzyme had activity in approximately the same

proportions with all three substrates; the pI 5.0 - 5.45 peak had considerably more alkene-/alkyl-transferase activity than aryltransferase activity and the pI 8.7 peak had predominantly aryltransferase activity. Re-focusing of the activity between pH 4.4 - 5.5 over the range pH 4 - 6 gave similar results but with a slight shift in position of each peak. The differences in substrate specificity shown after isoelectric focusing provide further evidence against the two major enzymes arising from artificial zoning during isoelectric focusing or ion exchange chromatography.

STUDIES OF PURIFIED GSH S-ARYLTRANSFERASES

The optimum pH for activity of the focused enzymes was pH 8.6, a difference of +0.3 pH units from the values previous workers (Clark, 1967; Balabaskaran, 1972) found for (presumably) the mixture of enzymes.

The substrate specificity of the enzymes with three 1-halo-2,4-dinitrobenzenes was studied in a search for a substrate more suitable than CDNB for kinetic studies. Both enzyme-catalysed reactions and the non-enzymic reaction had greatest velocity with the bromo derivative. The ratio (enzymic velocity/non-enzymic velocity) was greatest with CDNB, implying greatest sensitivity with this substrate. The decrease in enzymic rate with IDNB may reflect a decrease in affinity of the enzyme for the molecule due to increased size of the halogen or steric hindrance of the approach of GSH. Little work has been done on interaction with the aromatic substrate site of grass grub GSH S-aryltransferase - measurement of substrate dissociation constants would be valuable and may indicate a better approach to affinity chromatographic purification.

Enthalpy of Activation

Gibson (1953) has derived an expression for a one-substrate enzyme relating the apparent activation energy (obtained from a plot of log v against $1/T$) and the true activation energy (obtained from a plot of log V_m against $1/T$). The relationship for a two-substrate enzyme has not been presented in the literature and is derived in Appendix II. It can be seen that, in plots of log v against $1/T$, only when the substrate constants are invariant with temperature does the slope give the true activation energy of the reaction.

The apparent activation energy, E, for grass grub GSH S-aryltransferase at pH 8.3 was similar for the pI 4.6 and pI 8.7 enzymes. The values with CDNB were only slightly less than with DCNB, in agreement with the observation (Dixon and Webb, 1964; p.158) that the activation energy seems to be more characteristic of the enzyme than of the substrate. The activation energy for the non-enzymic reaction of DCNB and GSH was about twice that for the enzymic reaction; E for the non-enzymic reaction of DCNB and GSH was not determined because of extremely low reaction rates.

The true activation energy (ΔH^*) for the pI 8.7 enzyme and DCNB, calculated from the Arrhenius plot of $\overline{\log V_m}$, was very similar to E. The ΔH values for GSH binding to the enzyme and DCNB binding to the enzyme-GSH complex were negative showing that the binding is exothermic. Thus the enzyme appears to function in the usual manner by lowering the energy barrier over which the complex must pass. Formation of the enzyme-GSH complex was accompanied by a marked loss of entropy; the significance of this is not clear.

Effect of pH and temperature on the substrate dissociation constants

The results show that V_m and K'_{DCNB} are essentially invariant with pH for the pI 8.7 enzyme over the temperature range studied and for the

pI 4.6 enzyme at 37°C. pK'_{GSH} and pK_{DCNB} did not follow a pattern compatible with theory and interpretation of their variation has not been attempted.

The pK_{GSH} - pH plots showed changes of slope of approximately -2 and, accordingly, the data were fitted using pH functions for an integral slope change of -2. The assumption of an integral slope is necessary because a non-integral value suggests observation of an additive pK due to titration of several different residues within the active site and interpretation would then be impossible. The value for pK_e (37°C) of 9.36 agrees well with the value (9.2) obtained by Clark et al (1967) from their pK_m - pH plot. The decrease of (positive) slope in the pK_{GSH} - pH plots indicates (Dixon & Webb, 1964; p.137) that the ionizations correspond to groups in the free enzyme or substrate, and, because the pH-dependence of pK_i (Clark et al, 1967) showed that the ionizations are not of the substrate, it can be concluded that the groups are in the active site. The ionizations can thus be assigned to Tyr-OH, Cys-SH, Lys ε-NH_2 or free α-NH_2 groups using the pK values given by Dixon & Webb (1964; p.144). However, for several reasons (Mahler & Cordes, 1966; p.292) considerable uncertainty exists in assignment on this basis alone:- the observed pK may not reflect a true group dissociation; the behaviour may reflect titration of several residues; and the pK values may differ considerably from the values for the groups free in solution (the hyper-reactivity of amino acid residues in enzyme active sites is well known, e.g. Shaw, 1970). In addition, this work, and that of Clark et al (1967), was conducted at high ionic strength which may have displaced the pK values from those at low ionic strength. Ideally, the pK values should be determined at a range of ionic strength and extrapolated to zero to give a more

accurate comparison with literature values.

The absence of an inflexion in the log V_m - pH plots shows that binding of GSH to the enzyme suppresses the ionizations or displaces them to pH values outside the range studied. This differs from the results of Clark *et al* (1967) which showed that ionization still occurred in the enzyme-GSH complex, although it was not clear whether one or two groups then ionized.

Comparison of the heat of ionization with the ΔH_i values given by Dixon & Webb (1964; p.144), together with consideration of the pK value, suggests that the groups are α- or ε-NH_2, although a phenolic -OH cannot be excluded due to the large coefficient of variation in the experimental ΔH_i . The ΔH_i is similar to one value for Cys-SH (Marini *et al*, 1971) but this group can probably be excluded by virtue of the higher pK of the enzyme group. It seems likely, therefore, that enzyme-GSH binding occurs between the two carboxylate groups of GSH and two positively charged (Lys ε-NH_2) groups in the active site.

Further insight into the nature of the enzyme-GSH binding may be obtained by measurement of the affinity of GSH S-aryltransferase for GSH derivatives in which ionization of either of the carboxylic acid groups or of the amino group has been prevented, and by measurement of the inhibition constants of some derivatives with the thiol group reacted or removed.

The accuracy of the kinetic constants was less than was hoped to be achieved and, with the high coefficient of variation of ΔH_i for the enzyme groups, does not completely resolve the assignment of the active site binding groups. At first sight the KINTRAC appears ideally suited to kinetic studies of this nature but several disadvantages became apparent during the course of the experiments. To make full use of

the instrument the seven positions in the cell basket should be used, but the time required to add two substrates and enzyme to each cell limits the usable enzyme concentration and the choice of aromatic substrate. Thus, CDNB could not be used because by the time the reaction was started in the seventh cell it would have become non-linear in the first. Use of DCNB allowed full use of the instrument but the longer observation time required due to the slower rates meant that there was probably little advantage over single analyses using CDNB, where the enzymic rates were greater but the non-enzymic rate was more significant. Accuracy could be improved by several repetitions of the rate measurements at each substrate concentration but this would take about 8h for a complete run at one pH and requires a particularly stable enzyme preparation. A more active enzyme preparation would still allow use of DCNB and maximum use of the KINTRAC. However, a better approach for future kinetic studies would be the use of a rapid reaction technique incorporating either continuous or stopped flow measurements. The continuous flow technique would appear to be satisfactory for observation of the initial stages of the reaction with CDNB before it became non-linear. The ultimate aim of future kinetic studies should be measurement of the individual rate constants of each stage of the reaction.

APPENDIX I

Ionic Strength of tetra-Sodium Pyrophosphate Solutions

Routine GSH S-aryltransferase assays in these and other studies (Clark, 1967; Balabaskaran, 1972) have used as buffer 0.10M-$Na_4P_2O_7$ adjusted to pH 8.30 with HCl. Calculations using the Henderson-Hasselbach equation and the equilibrium,

$$HP_2O_7^{3-} \rightleftharpoons P_2O_7^{4-} + H^+$$

(for which pK_a = 8.95 at 25°C; Dawson et al, 1969; p.481) show that such a solution has I = 0.755M at 25°C. Accordingly, buffers for the kinetic experiments were prepared with this ionic strength and the variation with pH of the $Na_4P_2O_7$ concentration required to give this value is shown in Fig. 57. The variation of ionic strength of 0.100M - $Na_4P_2O_7$ / HCl between pH 7.95 - 9.95 is also illustrated.

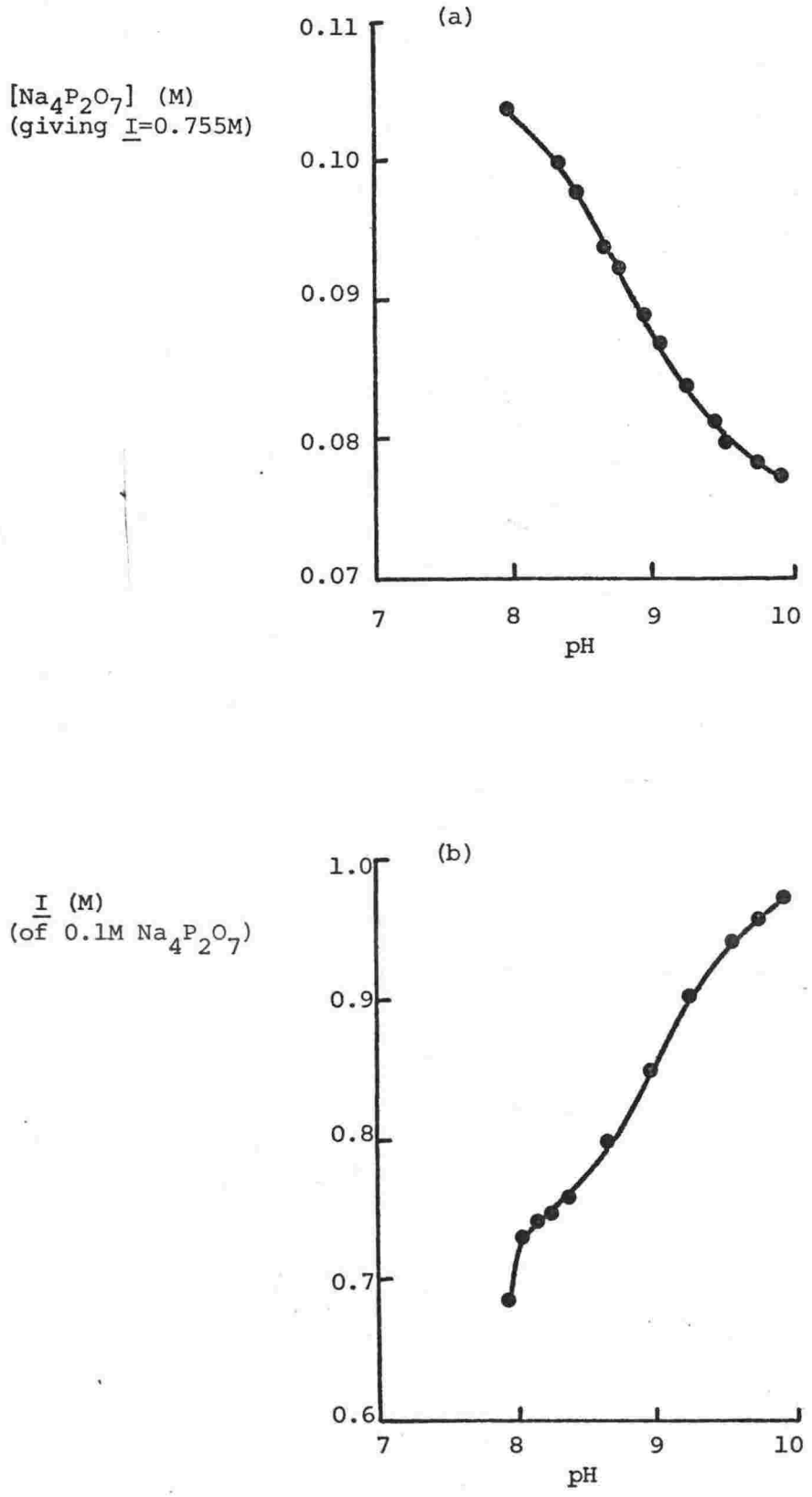


Fig. 57. (a) Variation with pH of the $Na_4P_2O_7$ concentration required to give an ionic strength of 0.755M at 25°C when adjusted with HCl.
(b) Variation with pH of the ionic strength of 0.10M-$Na_4P_2O_7$ buffer when adjusted with HCl.

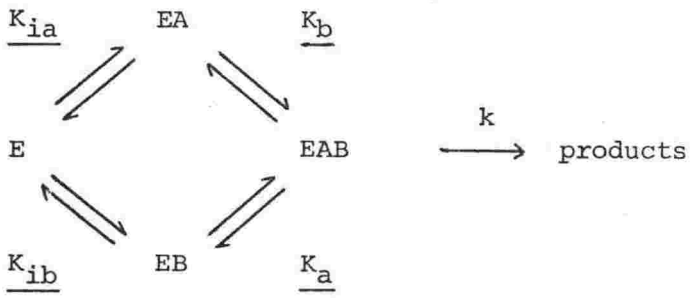
APPENDIX II

Activation energies of Two-substrate Enzyme Reactions

Gibson (1953) has derived a relationship between the true and apparent activation energies for one-substrate enzymic reactions. The relationship for a two-substrate enzyme is derived below.

The Random Bi Bi mechanism (Cleland, 1963) is considered:

i.e.



and $$\underline{v} = \frac{keab}{ab + bK_a + aK_b + K_{ia}K_b} \qquad (1)$$

If the rate limiting step of the reaction is the rate of breakdown of the EAB complex, then from the Arrhenius equation

$$2.303\frac{\partial \log k}{\partial(1/\underline{T})} = \frac{-\Delta H^*}{R} \qquad (2)$$

where ΔH^* is the true activation energy of the reaction and R is 1.986 cal/mol per °K.

If the EAB concentration is kept constant while the temperature is varied, $\partial \log \underline{v}/\partial(1/\underline{T})$ equals $\partial \log \underline{k}/\partial(1/\underline{T})$. Experimentally, a constant EAB concentration is achieved when the enzyme is saturated with both substrates. Thus the true activation energy is obtained from a plot of log $\underline{V_m}$ against $1/\underline{T}$.

At non-saturating substrate concentrations

$$\frac{\partial \log \underline{v}}{\partial (1/\underline{T})} = \frac{\partial}{\partial (1/\underline{T})} \left[\log \left(\frac{keab}{ab+b\underline{K_a}+a\underline{K_b}+\underline{K_{ia}}\,\underline{K_b}} \right) \right]$$

$$= \frac{\partial \log k}{\partial (1/\underline{T})} - \frac{\partial}{\partial (1/\underline{T})} \left[\log \left(ab+b\underline{K_a}+a\underline{K_b}+\underline{K_{ia}}\,\underline{K_b} \right) \right]$$

$$= \frac{\partial \log k}{\partial (1/\underline{T})} - \left[\frac{a\underline{K_b}\frac{\partial \log \underline{K_b}}{\partial (1/\underline{T})} + b\underline{K_a}\frac{\partial \log \underline{K_a}}{\partial (1/\underline{T})} + \underline{K_{ia}}\underline{K_b}\left(\frac{\partial \log \underline{K_{ia}}}{\partial (1/\underline{T})} + \frac{\partial \log \underline{K_b}}{\partial (1/\underline{T})} \right)}{ab + b\underline{K_a} + a\underline{K_b} + \underline{K_{ia}}\,\underline{K_b}} \right] \quad (3)$$

If E is the apparent activation energy obtained from a plot of log $\underline{v}$ against $1/\underline{T}$, then

$$\frac{\partial \log \underline{v}}{\partial (1/\underline{T})} = -\frac{E}{2.303R} \quad (4)$$

and

$$E = \Delta H^* + 2.303R \left[\frac{a\underline{K_b}\frac{\partial \log \underline{K_b}}{\partial (1/\underline{T})} + b\underline{K_a}\frac{\partial \log \underline{K_a}}{\partial (1/\underline{T})} + \underline{K_{ia}}\,\underline{K_b}\left(\frac{\partial \log \underline{K_{ia}}}{\partial (1/\underline{T})} + \frac{\partial \log \underline{K_b}}{\partial (1/\underline{T})} \right)}{ab + b\underline{K_a} + a\underline{K_b} + \underline{K_{ia}}\,\underline{K_b}} \right] \quad (5)$$

When there is no effect of one substrate upon combination of the other with the enzyme, (5) reduces to

$$E = \Delta H^* + 2.303R \left[\frac{\underline{K_a}}{a + \underline{K_a}} \frac{\partial \log \underline{K_a}}{\partial (1/\underline{T})} + \frac{\underline{K_b}}{b + \underline{K_b}} \frac{\partial \log \underline{K_b}}{\partial (1/\underline{T})} \right] \quad (6)$$

which is of the same form as the equation for a one-substrate enzyme (Gibson, 1953) with the addition of a term for the second substrate.

Thus, in plots of log $\underline{v}$ against $1/\underline{T}$ the true activation energy is obtained only when the substrate constants are invariant with temperature.

APPENDIX III

COMPUTED KINETIC DATA

To facilitate presentation of the data, Cleland's (1963) nomenclature (as described in the Results section) has been used. K_{ia} ($\equiv K_{GSH}$) values that appeared anomalous were omitted from the calculations of the enzyme dissociation constants; such values are indicated below.

pI 8.7 Enzyme; Temperature = 15°C

(1) V_m

pH	V_m (ΔE_{344}/min)	S.E. (V_m)	$10^{-3}w(V_m)$	-log V_m	S.E. (log V_m)
8.00	.0064	.0008	1141	2.194	.054
8.20	.0054	.0010	946	2.268	.080
8.40	.0066	.0009	1179	2.181	.059
8.70	.0054	.0004	4979	2.268	.032
9.00	.0090	.0014	508	2.046	.068
9.30	.0089	.0013	605	2.051	.063
9.60	.0119	.0031	106	1.925	.113
9.90	.0094	.0014	531	2.027	.065
10.40	.0075	.0029	119	2.123	.168

(2) K_{ia} ($\equiv K_{GSH}$)

pH	10^3K_{ia} (M)	S.E. (10^3K_{ia}) (M)	$w(10^3K_{ia})$	pK_{ia}	S.E. (pK_{ia})
8.00	.058	.046	469	4.237	.344
8.20	.033	.075	177	4.482	.987
8.40	.053	.047	447	4.276	.385
8.70	.037	.035	798	4.432	.411
9.00	.068	.033	918	4.168	.211
9.30	.047	.029	1123	4.328	.268
9.60	.058	.039	646	4.237	.292
9.90	.116	.032	962	3.936	.120
10.40	4.849	1.759	0.33	2.314	.158

(3) K_{ib} ($\equiv K_{DCNB}$)

pH	$10^3 K_{ib}$ (M)	S.E.($10^3 K_{ib}$) (M)	w($10^3 K_{ib}$)	pK_{ib}	S.E.(pK_{ib})
8.00	.124	.124	64.4	3.907	.434
8.20	.074	.198	25.5	4.131	1.162
8.40	.185	.235	18.2	3.733	.522
8.70	.205	.291	11.8	3.688	.616
9.00	.575	.696	2.1	3.240	.526
9.30	.124	.210	22.6	3.670	.426
9.60	.410	.568	3.1	3.387	.602
9.90	3.9	12.1	0.01	2.409	1.347
10.40	3.64	4.04	0.06	2.439	.482

(4) K_a ($\equiv K'_{GSH}$)

pH	$10^3 K_a$ (M)	S.E.($10^3 K_a$) (M)	w($10^3 K_a$)	pK_a	S.E.(pK_a)
8.00	.335	.125	63.5	3.475	.162
8.20	.258	.165	36.6	3.588	.278
8.40	.195	.109	84.2	3.710	.243
8.70	.092	.056	322.8	4.036	.264
9.00	.117	.110	82.5	3.932	.408
9.30	.207	.112	79.8	3.684	.235
9.60	.196	.199	25.1	3.708	.441
9.90	.030	.092	116.7	4.523	1.332
10.40	.717	.958	1.1	3.144	.580

(5) K_b ($\equiv K'_{DCNB}$)

pH	$10^3 K_b$ (M)	S.E.($10^3 K_b$) (M)	w($10^3 K_b$)	pK_b	S.E.(pK_b)
8.00	.721	.136	53.9	3.142	.082
8.20	.587	.174	33.1	3.231	.129
8.40	.685	.141	50.1	3.164	.089
8.70	.513	.070	202.5	3.290	.059
9.00	.987	.196	25.9	3.006	.086
9.30	.947	.174	32.9	3.024	.080
9.60	1.38	.428	5.5	2.860	.135
9.90	1.02	.188	28.0	2.991	.080
10.40	.537	.343	8.5	3.270	.277

pI 8.7 Enzyme; Temperature = 22°C

(1) V_m

pH	V_m (ΔE_{344}/min)	S.E.(V_m)	$10^{-3}w(V_m)$	$-\log V_m$	S.E.($\log V_m$)
8.00	.0113	.0019	266.3	1.947	.073
8.20	.0133	.0057	30.4	1.876	.186
8.40	.0147	.0025	161.9	1.833	.074
8.70	.0132	.0017	338.6	1.879	.056
9.00	.0142	.0012	646.4	1.848	.037
9.30	.0125	.0009	351.0	1.903	.031
9.60	.0144	.0022	207.4	1.842	.066
9.90	.0153	.0026	152.2	1.815	.074
10.40	.0123	.0201	2.5	1.912	.710

(2) K_{ia} ($\equiv K_{GSH}$)

pH	$10^3 K_{ia}$ (M)	S.E.($10^3 K_{ia}$) (M)	$w(10^3 K_{ia})$	pK_{ia}	S.E.(pK_{ia})
8.00	.126	.097	107	3.900	.344
8.20*	.027	.166	36	4.569	2.67
8.40	.085	.068	218	4.071	.347
8.70	.128	.058	297	3.893	.197
9.00	.099	.022	1961	4.004	.097
9.30	.077	.020	2569	4.114	.113
9.60	.154	.045	494	3.812	.127
9.90	.159	.051	388	3.799	.139
10.40	8.135	9.205	0.01	2.090	.491

*omitted from calculations of $p\tilde{K}_{ia}$ and pK_e

(3) K_{ib} ($\equiv K_{DCNB}$)

pH	$10^3 K_{ib}$ (M)	S.E.($10^3 K_{ib}$) (M)	$w(10^3 K_{ib})$	pK_{ib}	S.E.(pK_{ib})
8.00	.401	.585	2.92	3.397	.634
8.20	.095	.703	2.02	4.022	3.214
8.40	.428	.678	2.17	3.369	.688
8.70	1.61	3.94	0.06	2.793	1.063
9.30	1.27	1.51	0.44	2.896	.516
9.90	.265	.132	57	3.577	.216
10.40	.678	.533	3.52	3.169	.341

(4) K_a ($\equiv K'_{GSH}$)

pH	$10^3 K_a$ (M)	S.E.($10^3 K_a$) (M)	w($10^3 K_a$)	pK_a	S.E.(pK_a)
8.00	.240	.223	20.0	3.620	.404
8.20	.291	.557	3.2	3.536	.831
8.40	.209	.211	22.5	3.680	.438
8.70	.065	.143	48.8	4.187	.955
9.30	.042	.043	529	4.377	.445
9.90	.499	.196	27.2	3.302	.171
10.40	8.59	10.14	0.01	2.066	.513

(5) K_b ($\equiv K'_{DCNB}$)

pH	$10^3 K_b$ (M)	S.E.($10^3 K_b$) (M)	w($10^3 K_b$)	pK_b	S.E.(pK_b)
8.00	.767	.189	28.0	3.115	.107
8.20	1.01	.584	2.9	2.996	.251
8.40	1.05	.237	17.8	2.979	.098
8.70	.814	.141	50.1	3.089	.075
9.00	.849	.098	104	3.071	.050
9.30	.694	.066	228	3.159	.041
9.60	.835	.169	35.1	3.078	.088
9.90	.832	.183	29.8	3.080	.096
10.40	.716	.656	2.3	3.145	.398

pI 8.7 Enzyme; Temperature = 30°C

(1) V_m

pH	V_m (ΔE_{344}/min)	S.E. (V_m)	$10^{-3}w(V_m)$	$-\log V_m$	S.E.($\log V_m$)
8.00	.028	.005	37.92	1.551	.079
8.20	.023	.004	71.81	1.631	.069
8.40	.027	.004	56.33	1.575	.069
8.70	.024	.003	113.20	1.627	.053
9.30	.025	.004	80.01	1.609	.062
9.60	.049	.012	7.24	1.310	.104
9.90	.040	.009	11.41	1.396	.102

(2) K_{ia} ($\equiv K_{GSH}$)

pH	$10^3 K_{ia}$ (M)	S.E.($10^3 K_{ia}$) (M)	$w(10^3 K_{ia})$	pK_{ia}	S.E.(pK_{ia})
8.00	.177	.058	293	3.752	.142
8.20	.237	.072	195	3.625	.132
8.40	.196	.052	367	3.708	.115
8.70*	.099	.044	516	4.004	.193
9.30	.251	.068	218	3.600	.118
9.60	.267	.054	341	3.573	.088
9.90	1.06	.143	49	2.975	.059

*omitted from calculations of $\tilde{pK}_{ia}$ and pK_e

(3) K_{ib} ($\equiv K_{DCNB}$)

pH	$10^3 K_{ib}$ (M)	S.E.($10^3 K_{ib}$) (M)	w($10^3 K_{ib}$)	pK_{ib}	S.E. (pK_{ib})
8.00	.238	.119	71	3.623	.217
8.20	1.80	2.96	0.11	2.745	.714
8.40	.741	.549	3.3	3.130	.322
8.70	.367	.314	10	3.435	.372
9.30	1.07	1.03	0.94	2.971	.418
9.60	.354	.124	65	3.451	.152
9.90	1.80	.948	1.11	2.745	.229

(4) K_a ($\equiv K'_{GSH}$)

pH	$10^3 K_a$ (M)	S.E.($10^3 K_a$) (M)	w($10^3 K_a$)	pK_a	S.E.(pK_a)
8.00	.940	.367	7.4	3.027	.170
8.20	.125	.194	26.6	3.903	.674
8.40	.326	.218	21.0	3.487	.290
8.70	.277	.164	37.3	3.588	.257
9.30	.212	.188	28.4	3.674	.385
9.60	1.54	.630	2.5	2.812	.178
9.90	.757	.491	4.1	3.121	.282

(5) K_b ($\equiv K'_{DCNB}$)

pH	$10^3 K_b$ (M)	S.E.($10^3 K_b$) (M)	w($10^3 K_b$)	pK_b	S.E.(pK_b)
8.00	1.27	.280	12.7	2.896	.096
8.20	.948	.195	26.3	3.023	.089
8.40	1.23	.238	17.6	2.910	.084
8.70	1.02	.163	37.9	2.991	.069
9.30	.901	.168	35.3	3.045	.081
9.60	2.05	.558	3.2	2.688	.118
9.90	1.29	.365	7.5	2.889	.123

pI 8.7 Enzyme; Temperature = 37°C

(1) V_m

pH	V_m (ΔE_{344}/min)	S.E.(V_m)	$10^{-3}w(V_m)$	$-\log V_m$	S.E.($\log V_m$)
8.30	.023	.002	175.00	1.640	.046
8.50	.040	.009	13.13	1.398	.095
8.70	.038	.004	51.82	1.420	.050
8.90	.038	.006	24.84	1.420	.072
9.00	.080	.024	1.75	1.097	.130
9.10	.039	.002	341.40	1.409	.019
9.30	.056	.006	26.18	1.252	.048
9.50	.055	.009	12.24	1.260	.071
9.60	.034	.006	28.34	1.473	.076
9.90	.033	.009	11.90	1.483	.121

(2) K_{ia} ($\equiv K_{GSH}$)

pH	$10^3 K_{ia}$ (M)	S.E.($10^3 K_{ia}$) (M)	$w(10^3 K_{ia})$	pK_{ia}	S.E.(pK_{ia})
8.30	.319	.055	321	3.496	.075
8.50	.211	.039	653	3.676	.080
8.70	.273	.030	1105	3.564	.048
8.90	.221	.030	1077	3.656	.059
9.00	.228	.030	1137	3.642	.057
9.10	.384	.011	7906	3.416	.012
9.30	.341	.018	2978	3.467	.023
9.50	.935	.052	373	3.029	.024
9.60	1.076	.180	31	2.968	.073
9.90	3.873	.935	1	2.412	.105

(3) K_{ib} ($\equiv K_{DCNB}$)

pH	$10^3 K_{ib}$ (M)	S.E.($10^3 K_{ib}$) (M)	w($10^3 K_{ib}$)	pK_{ib}	S.E.(pK_{ib})
8.30	2.64	2.72	.14	2.578	.447
8.50	.969	.703	2.02	3.014	.315
8.70	.914	.298	11.30	3.039	.142
8.90	.576	.210	22.70	3.240	.158
9.00	.527	.166	36.20	3.278	.137
9.10	.436	.026	1422	3.361	.026
9.30	.457	.051	389	3.340	.048
9.50	1.58	.356	7.80	2.801	.098
9.60	2.77	2.31	.20	2.558	.362
9.90	6.74	10.1	.01	2.171	.651

(4) K_a ($\equiv K'_{GSH}$)

pH	$10^3 K_a$ (M)	S.E.($10^3 K_a$) (M)	w($10^3 K_a$)	pK_a	S.E.(pK_a)
8.30	.085	.084	139	4.071	.429
8.50	.263	.205	23.7	3.580	.339
8.70	.341	.118	71.3	3.476	.150
8.90	.462	.188	28.2	3.335	.177
9.00	1.06	.518	3.7	2.975	.212
9.10	.943	.076	173	3.025	.035
9.30	1.25	.217	21.2	2.903	.075
9.50	.921	.310	10.4	3.036	.146
9.60	.345	.306	10.7	3.462	.385
9.90	.361	.596	2.8	3.442	.717

(5) K_b ($\equiv K'_{DCNB}$)

pH	$10^3 K_b$ (M)	S.E.($10^3 K_b$) (M)	w($10^3 K_b$)	pK_b	S.E.(pK_b)
8.30	.704	.106	88.7	3.152	.065
8.50	1.21	.303	10.9	3.917	.109
8.70	1.14	.157	40.5	2.943	.060
8.90	1.20	.229	19.1	2.921	.083
9.00	2.45	.789	1.6	2.611	.140
9.10	1.07	.055	327	2.971	.022
9.30	1.67	.205	23.8	2.777	.053
9.50	1.56	.287	12.1	2.807	.080
9.60	.887	.219	20.9	3.052	.107
9.90	.628	.275	13.2	3.202	.190

pI 4.6 Enzyme; Temperature = 37°C

(1) V_m

pH	V_m (ΔE_{344}/min)	S.E.(V_m)	w(V_m)	-logV_m	S.E. (logV_m)
8.00	.171	.030	1 110	.767	.076
8.35	.224	.043	548	.650	.083
8.65	.225	.033	901	.648	.064
8.95	.200	.056	316	.699	.122
9.15	.281	.064	247	.551	.099
9.45	.175	.043	549	.757	.107
9.65	.291	.252	16	.536	.376

(2) K_{ia} ($\equiv K_{GSH}$)

pH	$10^3 K_{ia}$ (M)	S.E.($10^3 K_{ia}$) (M)	w($10^3 K_{ia}$)	pK_{ia}	S.E.(pK_{ia})
8.00	.387	.050	401	3.412	.056
8.35	.252	.037	740	3.599	.064
8.65	.222	.027	1330	3.654	.053
8.95	.290	.063	252	3.538	.094
9.15	.545	.047	450	3.264	.037
9.45	1.61	.177	32	2.793	.048
9.65	6.98	2.17	0.2	2.156	.135

(3) K_{ib} ($\equiv K_{DCNB}$)

pH	$10^3 K_{ib}$ (M)	S.E.($10^3 K_{ib}$) (M)	w($10^3 K_{ib}$)	pK_{ib}	S.E.(pK_{ib})
8.00	2.32	1.83	0.3	2.635	.343
8.35	1.07	.54	3.5	2.971	.219
8.65	1.18	.55	3.3	2.928	.202
8.95	0.86	.54	3.4	3.066	.273
9.15	1.01	.25	15.7	2.996	.108
9.45	2.00	.80	1.5	2.699	.174
9.65	2.96	2.08	0.2	2.529	.305

(4) $\underline{K_a}$ ($\equiv \underline{K'_{GSH}}$)

pH	$10^3\underline{K_a}$ (M)	S.E.($10^3\underline{K_a}$) (M)	w($10^3\underline{K_a}$)	$\underline{pK_a}$	S.E.($\underline{pK_a}$)
8.00	.304	.256	15.2	3.517	.366
8.35	.570	.315	10.1	3.244	.240
8.65	.462	.228	19.2	3.335	.214
8.95	.730	.515	3.8	3.137	.306
9.15	1.59	.63	2.5	2.799	.172
9.45	1.14	.64	2.4	2.943	.244
9.65	2.86	4.10	0.1	2.544	.623

(5) $\underline{K_b}$ ($\equiv \underline{K'_{DCNB}}$)

pH	$10^3\underline{K_b}$ (M)	S.E.($10^3\underline{K_b}$) (M)	w($10^3\underline{K_b}$)	$\underline{pK_b}$	S.E.($\underline{pK_b}$)
8.00	1.83	.37	7.21	2.738	.088
8.35	2.43	.52	3.73	2.614	.093
8.65	2.46	.41	6.03	2.609	.072
8.95	2.17	.69	2.08	2.664	.138
9.15	2.95	.73	1.86	2.530	.107
9.45	1.42	.42	5.75	2.848	.128
9.65	1.22	1.31	0.58	2.914	.466

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