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THE METABOLISM OF GAMMEXANE AND RELATED COMPOUNDS

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TO MY PARENTS

Abstract

1. A detailed kinetic study has been made of the glutathione S-aryl-transferases from the New Zealand grass grub (Costelytra zealandica) and from sheep liver. The insect enzyme behaves in accordance with a Michaelis-Menten model for two-substrate enzymes. It is inhibited by the sulphonphthaleins, phthaleins, fluoresceins and dicarboxylic acids competing with glutathione, while the sheep-liver enzyme is not susceptible to this type of inhibition.

From this, and other data obtained from a study of the variation of kinetics with pH, it is proposed that two basic groups (possibly lysine residues) are involved in the binding of glutathione to the insect enzyme, while only one such group appears in the sheep-liver enzyme. Binding of the aromatic substrate to the enzyme in both species may involve a histidine residue.

2. The accumulation of little significant radioactivity in diluant γ-pentachlorocyclohexene (γ-PCCH) during the in vitro metabolism of [^{14}C]γ-hexachlorocyclohexane (γ-HCH) suggests that the PCCH's are not formed as free intermediates during the metabolism of the HCH's. However, certain ambiguities introduced with the experimental techniques used preclude the complete exclusion of this possibility.

3. γ-HCH, γ-PCCH and δ-PCCH metabolized in vivo by M.domestica and C.zealandica and in vitro by preparations from both species, all produce as the principal metabolite a glutathione conjugate with chromatographic properties identical with those of authentic

S-(2,4-dichlorophenyl)glutathione. There is, however some doubt as to the identity of the S-substituent moiety.

4. The *in vitro* metabolism of γ-HCH and δ-PCCH is glutathione-dependent and is inhibited by various phthaleins and sulphonphthaleins. The *in vivo* metabolism of δ-PCCH in *C.zealandica* is profoundly affected by this type of compound, but its effects on the rate of metabolism *in vivo* of δ-HCH in *M.domestica* and *C.zealandica* are only marginal.

5. The enzyme concerned in the metabolism of δ-PCCH has been shown to differ from aryltransferase in *M.domestica* and *C.zealandica* by gel filtration techniques and by differences in activity in different enzyme preparations. The δ-PCCH-metabolising activity appears to be associated with a DDT dehydrochlorinase activity. In *M.domestica*, there appears to be, in addition, a second DDT dehydrochlorinase with only a low cross-specificity towards δ-PCCH.

Preface

The phenomenon of resistance towards pesticides is one of considerable interest to the biochemist and geneticist, and one of great economic significance in agriculture and public health. The use of ever increasing quantities of insecticides, to which we now appear to be irrevocably committed, is itself, however, a cause of growing concern in the field of public health and ecology. To minimise the risk of side effects from our heavy dependence on pesticides, and to increase their efficiency towards pests, we must design them, as well as synergists, to possess greater specificity of action. This requires a detailed study of the lethal effects and metabolic fate of existing pesticides, and of the nature of resistance towards them, in species both noxious and beneficient, that are likely to be affected by them.

In this thesis, the nature of resistance towards the commercial insecticide " lindane" ('Gammexane') is reviewed, and the experimental results presented, it is hoped, will contribute to the elucidation of this particular problem.

For the greater part of this work I have been supported by a University Grants Committee Postgraduate scholarship, and by a MacMillan Brown Agricultural Research Scholarship. I wish to express my thanks for this aid, which has enabled me to carry out the experimental work which is recorded in this thesis.

I wish to extend my thanks to all those who have made my years in the department a pleasure. To Professor J.N. Smith my debt of gratitude

is great. Without his patience, infectious enthusiasm and depth of experience, this work would have been arduous indeed. It is a privilege to have had him as a supervisor and teacher. To Associate Professor R. Truscoe and Dr. F. Darby my thanks for their invaluable help and encouragement; to the technical staff of both the chemistry and biochemistry departments; and last, but by no means least, to my fellow students who, in a variety of ways, have made this department an interesting and stimulating place of work.

CONTENTS

CHAPTER 1.

The Chemistry of the Hexachlorocyclohexanes and Related Compounds.

The toxic component of the commercial insecticide 'Gammexane' (lindane), is γ-1,2,3,4,5,6-hexachlorocyclohexane (γ-HCH) also commonly referred to as benzene hexachloride (BHC).

For cyclohexane derivatives, there are, in theory, 16 steric isomers, three of which exhibit stereoisomerism. If, however, ring inversion occurs (Fig.1.1a) the number of isolable isomers is halved; there will exist eight steric isomers, one of which possesses an enantiomorph.

That ring inversion occurs with HCH is suggested by the nature and number of isomers that it has been possible to isolate.

Data on the stereochemical characteristics of the possible isomers are listed in table I.1. (Orloff, 1954). Polar (p) bonds are those orientated parallel to the trigonal axis. The (a) column shows the least strained configuration, the (b) column the more strained, inverted form (see also Fig.I.1(b)).

Table I-1.

Isomer	a		b	
	No.meta,polar C-Cl bonds.	Configuration	Configuration	No.meta,polar C-Cl bonds.
β	0	eeeeee	pppppp	6
δ	0	peeeee	epppppp	4
α	0	ppeeee	eeppppp	2
θ	1	pepeee	epeppp	3
ϵ	0	peepee	eppepp	2
γ	1	pppeee	eeeppp (identical)	1
η	1	ppepee	eepepp	1
ι	3	pepepe	epepep (identical)	3

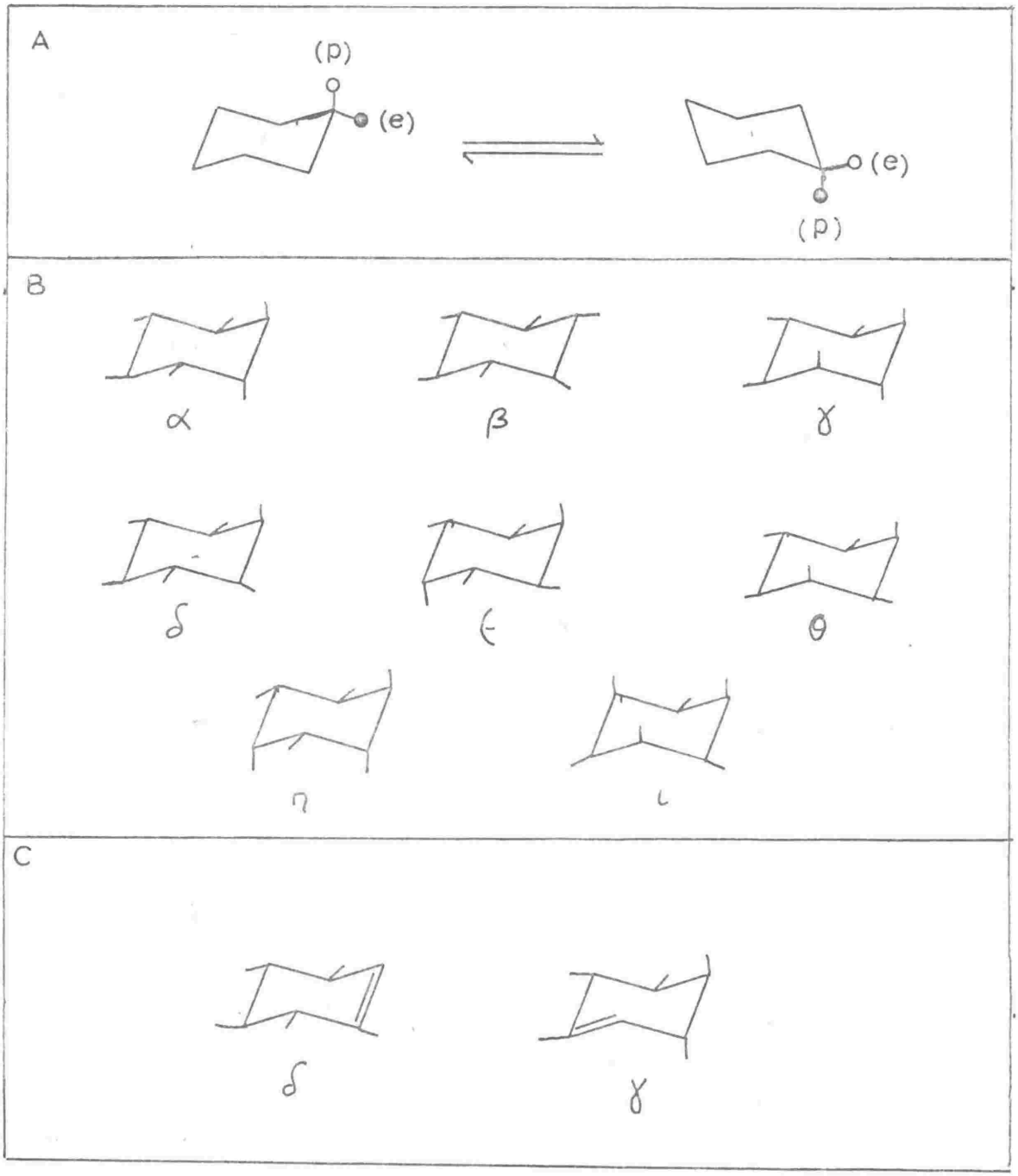


Fig.I.1:(a) Ring inversion in cyclohexane derivatives.

(b) Orientation of chlorine atoms in the isomers (least strained forms) of HCH.

(c) Orientation of chlorine atoms in δ- and γ-PCCH.

Interaction between chlorine atoms involved in meta, polar-orientated C-Cl bonds strains the configuration. The ϵ -isomer, possessing three such bonds, has not been isolated, and its existence seems improbable.

As yet, it has not been possible to relate the differing toxicities of these compounds to their configuration.

Two types of reaction undergone by these compounds are of significance to the history of metabolic studies on gammexane viz. dehydrochlorination and dechlorination.

Dehydrochlorination to the trichlorobenzenes (TCB) has been known for many years. Van der Linden (1912) quotes ratios of 1,2,4-TCB, 75.9%; 1,2,3-TCB, 17.6%; 1,3,5-TCB, 6.5% from the dehydrochlorination of γ -HCH.

Kinetic analysis shows that these reactions proceed via three successive E_2 eliminations of HCl (Crystol, 1947). In cases where elimination of a second molecule leads to the formation of a conjugated diene (e.g. the α -isomer), the first elimination is rate limiting. Where this is not so (β-, γ -, δ -isomers) the first and second steps compete for rate control. For the δ -isomer, the second step is limiting; for the γ-isomer, the first dominates the second by a small margin (Hughes, Ingold and Pasternak, 1953). From both of these isomers, the monodehydrochlorination products, γ -, and δ-pentachlorocyclohexene (PCCH) can be isolated, (Fig.I.1) (Pasternak, 1951; Hughes et al., 1953; Crystol, 1951; Orloff, 1954).

For the centrosymmetric β-isomer, trans elimination is impossible. Cis elimination occurs, possibly through a carbanion intermediate, at a rate 10^{-3} - 10^{-4} times that for the other HCH's.

Rate constants for the limiting steps for the dehydrochlorination of the α-, β-, γ-, and δ-isomers are set out in Table I.2 (Crystol, 1947).

Table I.2.

Isomer	K_1	K_2
α-	0.167-0.172	-
β-	3×10^{-6}	-
γ-	0.0435 - 0.0457	-
δ-	-	0.106 - 0.113

Differences in proportions of trichlorobenzenes obtained from alkaline dehydrochlorination of α-, β-, γ- and δ-HCH and of δ-PCCH, lead to the conclusion that, in each case, different intermediates must be involved. The occurrence of 1,2,3-TCB indicates that eliminations other than 1,2-eliminations are involved.

This reaction is the basis of the Armstrong method of assaying HCH and TCB in the presence of each other (Armstrong, Bradbury and Standen, 1951; Bradbury and Standen, 1954). The mixture is nitrated giving dinitrotrichlorobenzenes which, on alkaline hydrolysis, give the chlorodinitroresorcinols which are estimated at 410 mμ. At the same time, dehydrochlorination of the HCH occurs and the resulting TCB's are nitrated and the products hydrolysed as before. The original HCH concentration is then calculated from the increase in chlorodinitroresorcinols. The assay shows no interference from PCCH, benzene or chlorobenzene (Bradbury and Standen, 1957).

It is now apparent that dehydrochlorination is important in the metabolism of γ-HCH. Its exact significance is in dispute and will be discussed more fully later on.

The dechlorination of HCH, by removal of vicinyl chlorines by

a metal atom such as zinc, is the basis of the Schechter-Hornstein assay for HCH (Schechter & Hornstein, 1951) and for HCH & PCCH in the presence of each other (Sternburg & Kearns, 1956). HCH & PCCH are refluxed with a zinc/acetic acid/malonic acid mixture and thereby dechlorinated to benzene and chlorobenzene respectively. These are nitrated to m-dinitrobenzene and 1-chloro-2;4-dinitrobenzene. On treatment with KOH and methyl ethyl ketone, these form chromophores which can be assayed at different wavelengths. This method will obviously suffer from interference from benzene and chlorobenzene. Further, it has been shown (Clark, M.Sc. Thesis) that various possible metabolic intermediates of the HCH's will break down in the first part of the Schechter-Hornstein assay to give the chlorothiophenols. On nitration, these form compounds, including 1-chloro-2;4-dinitrobenzene, which will give a spurious result for PCCH in this assay. Again, the significance of this effect will become evident in the more detailed discussion of the metabolism of γ-HCH in Chapter 3.

CHAPTER 2.

The Role of Metabolism in Resistance to γ-HCH.

Resistance towards most insecticides is a complex phenomenon, being the compounded result of a variety of factors. For instance, factors cited as being of significance in resistance to DDT include behaviour, morphology, and rates of absorption and metabolism of the insecticide. Genetically, resistance to knockdown by DDT can be traced to a single gene pair, while resistance to the lethal effects is derived from a multiple gene inheritance.

Resistance towards γ-HCH in M.domestica appears to be dependent on a number of genes. This is reflected in the sometimes conflicting evidence concerning the extent to which metabolism of γ-HCH contributes to overall resistance. This evidence is reviewed by Openoorth (1956).

Openoorth (1954, 1955) reported a higher rate of conversion of α-, γ- & δ-HCH into water-soluble metabolites in two resistant strains of M.domestica than in a susceptible strain. This, he suggested, could be due either to an enhanced ability to detoxify HCH in the resistant strains, or to a decreased ability to metabolize the insecticide attributable directly to its toxic effects. As α-and δ-HCH have acute toxicities very much lower than that of the γ-isomer, the second alternative seems the less likely.

Bradbury & Standen (1960), working with four strains of M.domestica demonstrated a linear relationship between the rate of metabolism of γ-HCH and the log LD_{50}. This relationship does not, however, conclusively demonstrate that metabolism is critical in

determining the degree of resistance, and indeed the authors were of the opinion that metabolism contributed to the overall resistance only to a minor extent. Rates of absorption of the insecticide were stated to be of no significance.

Openoorth (1956), however, using the Schechter-Hornstein assay to follow the rate of disappearance of α-, γ-, and δ-HCH from live M.domestica, demonstrated linear relationships between 'breakdown capacity' for α-, γ-, and δ-HCH and the E.T.50 (time for 50% knockdown), and between the rate of absorption of γ-HCH and E.T.50. Rates of absorption decreased in the order $\gamma > \delta \gg \alpha$ and rates of metabolism in the order $\gamma > \alpha \gg \delta$.

The findings of Openoorth (1956) and of Bradbury and Standen (1960) may not be mutually exclusive, as, being based on different definitions of resistance, they are not strictly comparable. As quoted earlier, resistance to knockdown and to the lethal effects of DDT in M.domestica appear to be controlled by different factors. This may be so for γ-HCH, in which case there will be no simple relationship between L.D.50 and E.T.50.

Bridges and Cox (1959), proposed three types of resistance towards γ-HCH in M.domestica raised on a dieldrin-containing medium:-

1) Associated with an increased ability to metabolize γ-HCH.
2) A non-metabolic mechanism, linked with resistance to dieldrin.
3) Related to the fat content of the diet, but not to the fat content of the body of the fly.

M.domestica is atypical of most insects in that it exhibits, in the

resistant strain, a greater degree of resistance toward γ-HCH than towards isodrin and endrin. Busvine and Townsend (1963) investigating this anomalous cross-resistance spectrum found that the resistant strains of M.domestica metabolized γ-HCH at a greater rate than susceptible strains. Lucilia cuprina, with a more typical cross-resistance spectrum, showed no difference in rates of metabolism between resistant and susceptible strains. On these data, the authors proposed two mechanisms operating in M.domestica. The first is a non-specific resistance towards chlorinated hydrocarbon insecticides, possibly operating at the level of the nerve sheath. The second, responsible for the anomalous cross-resistance spectrum, is purely metabolic. These hypotheses parallel those of Bridges and Cox (1959).

Thus, the available data indicate the existence of at least four mechanisms by which a species may become resistant to γ-HCH. These are related respectively to absorption, to metabolism, to the diet, and to a generalized, non-metabolic resistance to chlorinated hydrocarbons. The last-named appears to be common to all species of resistant insects. The difficulty in assessing the importance of any one of the other mechanisms has lain in that these factors may not all be active in any one species. The findings of Busvine and Townsend (1963) show that in only a few species, (e.g. Musca domestica), is enhanced metabolism of γ-HCH likely to be a major factor in determining the degree of resistance. In such cases, studies leading to formulation of a Gammexane synergist would be profitable. In the others, blocking of what metabolism occurs would probably raise the toxicity of Gammexane only marginally.

CHAPTER 3.

The Metabolism of γ-HCH

In 1951, Davidow and Frawley, found that the acute toxicity of the isomers of HCH in rats decreased in the order $\gamma > \alpha > \delta > \beta$, while chronic effects, measured over a period of months, decreased in the order $\beta > \alpha > \gamma > \delta$. It was found that these compounds were preferentially stored in the adipose tissue. The α-, γ- and δ-isomers reached a steady level in from 4-6 weeks, while the tissue content of the β-isomer continued to rise after 12 weeks. On withdrawal of HCH from the diet, levels of α-, γ-, and δ-HCH fell to zero within 3 weeks, while the β-isomer was still detectable after 14 weeks. Obviously, some mechanism was eliminating the α-, γ-, and δ-isomers in preference to the β-isomer. Similar results were obtained with dogs.

Openoorth (1954) observed the conversion of γ-HCH and α- and δ-HCH (1955) into Schechter-Hornstein-negative metabolites in M.domestica. The rates of conversion were $\gamma > \alpha \gg \delta$ (1956).

The use of ^{14}C labelled α-, β-, γ-, and δ-HCH (Bradbury and Standen, 1956) and ^{36}Cl labelled α-, γ-, and δ-HCH (Bradbury and Standen, 1957) demonstrated for the first time the conversion of HCH isomers into water-soluble metabolites. Metabolism of the α- and γ-isomers resulted in the release of 4-5 g. equiv. of chloride ion per mole, and that of the δ-isomer by the release of 5-6 g. equiv. of chloride ion per mole. Rates of solubilization decreased in the order $\alpha > \gamma > \delta > \beta$. The release of non-integral amounts of chloride per molecule suggests the existence of a number of metabolites containing different amounts of chloride.

With reference to the relative rates of solubilization of the isomers, the results of Bradbury and Standen, and of Openoorth again appear to be incompatible. Again this could be due to a difference in techniques. The Schechter-Hornstein assay used by Openoorth (1954, 1955) is subject to interference by other compounds. It is possible that intermediates in the metabolism of -HCH could interfere with assays for the parent compound.

Bradbury and Standen (1957, 1960) subjected aqueous and 80% ethanol extracts of $[^{14}C]$ γ -HCH and $[^{14}C]$ α -HCH-dosed M.domestica to two dimensional chromatography. Eleven radioactive spots were found in each case, the patterns of distribution for each isomer being very similar. Electrophoretic behaviour suggested identity for 8 pairs of metabolites.

The production of identical metabolites from a pair of steric isomers suggests that the difference is removed by aromatization. This supported the idea that dehydrochlorination is an integral part of the metabolism of HCH.

Bradbury and Standen (1959) subjected water-soluble metabolites of $[^{14}C]\alpha$- and $[^{14}C]$ γ -HCH to alkaline hydrolysis, and succeeded in isolating and identifying by dilution analysis the six isomeric dichlorothiophenols. The proportions of each are shown below in table III.1.

Table III.1.

Isomer	% of water soluble metabolites	
	α	γ
2,4-	20,21	32
2,5-	19,21	16,14
2,6-	5	9
3,4-	22,19	5,4
3,5-	2	2
2,3-	2	1

The authors pointed out that hydrolysis to the thiophenol is a step in the standard assay for mercapturic acids (Parke and Williams, 1951), and suggested that substitution of one of the chlorines of the HCH by the sulphur of a thiol was involved. The identity of the thiol was tentatively proposed as being glutathione, which, as in the dehydrochlorination of DDT to DDE (Sternburg, Kearns and Moorefield, 1954), was found to be essential to the in vitro metabolism of γ-HCH.

This proposition was supported by the finding (Clark, Hitchcock and Smith, 1966) that the major metabolite of [^{14}C] γ-HCH in M.domestica was chromatographically and electrophoretically identical in behaviour to authentic S-(2,4-dichlorophenyl)glutathione. Sims and Grover (1965) found that γ-PCCH and α-tetrachlorocyclohexene formed ninhydrin-positive metabolites in vitro, a reaction for which GSH was essential. Gammexane itself did not appear to form such a conjugate. In live rats dosed with γ-HCH and γ-PCCH one urinary metabolite was found to be chromatographically and spectroscopically identical with 2,4-dichlorophenylmercapturic acid (Grover & Sims, 1965). The major metabolites were 2,3,5- and 2,4,5-trichlorophenol, present as sulphate, glucuronide, and free phenols. Grover and Sims suggested that these compounds could be responsible for some of the radioactive spots located chromatographically by Bradbury and Standen (1957, 1960) though these authors found less than 2% of metabolites soluble in organic solvents would extract into alkaline solution, and also that isotope dilution for 2,4,5-trichlorophenol in [^{14}C] α-HCH-dosed M.domestica gave negative results (Bradbury and Standen, 1958).

The findings of Cohen and Smith (1964) are of some interest here.

These authors found that, when incubated with locust faeces, S-(p-nitrophenyl)glutathione was degraded to the substituted cysteine, and mercapturic acid, p-nitrobenzoic acid, and an unidentified weakly-acidic component. Artifacts such as the last two mentioned above, could well contribute to the large number of metabolites found by Bradbury and Standen (1957, 1960).

There seems to be a marked species difference here. In vertebrates, the major products of metabolism appear to be the trichlorophenols and derivatives thereof. Jondorf, Parke and Williams (1955) showed that 1,2,4-trichlorobenzene was metabolized to 2,3,5- and 2,4,5-trichlorophenol, plus a small amount (0.3%) of the trichlorophenylmercapturic acid. It was suggested that oxidation to the 1,2-dihydroxy-1,2-dihydro-3,4,6- trichlorobenzene, and dehydration to the phenols was involved. Though Woodward, Davidow and Lehmann (1948) found that α-HCH administered to rabbits gave only a trace of TCB, and none of the phenols, the evidence of Grover and Sims (1965) strongly suggests that in rats, at least, the major pathway in HCH metabolism involves initial dehydrochlorination to the TCB, with subsequent oxidation to the phenol.

The mechanism of conjugation of GSH with γ-HCH, which appears to be a major pathway in insects, and at least a supporting one in vertebrates, is at present obscure, there being some controversy as to the role of γ-PCCH in the degradation of its parent compound.

Sternburg and Kearns (1956), subjected whole resistant M.domestica dosed with γ-HCH to the Schechter-Hornstein process, and showed that at one hour after dosing up to 40% of the absorbed dose had been converted to a form which gave 1-chloro-2,4-dinitrobenzene (CDNB). The authors

interpreted this to mean that the primary metabolite was γ-PCCH, which was later converted into Schechter-Hornstein-negative metabolites. However, Bradbury and Standen (1958) demonstrated, by means of an isotope-dilution technique, that at no time did the amount of γ-PCCH accumulated exceed 3% of the [^{14}C] γ-HCH administered to M.domestica. This result was confirmed by Bridges (1959) for both γ- and δ-isomers. It was found that at least three compounds had been formed which would give dinitrochlorobenzene on being subjected to the Schechter-Hornstein process.

1) PCCH
2) An acid-labile fraction which yields chlorodinitrobenzene on treatment by the Schechter-Hornstein process from which zinc has been omitted.
3) A water-soluble metabolite which gives CDNB on treatment by the full Schechter-Hornstein process.

Traces of free chlorobenzene were also detected.

Further, Bridges and Cox (1959) showed that injection of flies with γ-PCCH prior to treatment with [^{14}C] γ-HCH does not lead to a build-up of radioactive γ-PCCH.

These experiments do not show conclusively that γ-PCCH is not an intermediate in the metabolism of γ-HCH. It will be seen later that the PCCH's are metabolized much faster than are the parent compounds. If they were intermediates in the metabolism of the HCH's, they would not be expected to accumulate. The seemingly conclusive experiment of Bridges and Cox (1959) does not take into account the relative rates of

penetration, and the solubility in the tissues of γ-HCH and γ-PCCH. If the total concentration of the γ-PCCH in the environment of the enzyme responsible for its further degradation is very much lower than the K_m, then there would be no accumulation of labelled γ-PCCH. However, these experiments do show, quite conclusively, that only some of the Schechter-Hornstein-positive metabolites observed by Sternburg and Kearns (1956) can have been -PCCH. It could be argued that compounds such as S-(pentachlorocyclohexyl)glutathione, or S-(trichlorocyclohexadienyl)glutathione, might be expected to give CDNB in the Schechter-Hornstein reaction. It has been found (Clark, Hitchcock and Smith, 1966) that the S -(dichlorophenyl)glutathiones do not give rise to CDNB.

In spite of the doubt cast on the conclusions drawn by Sternburg and Kearns (1956), most recent publications have been based on an explicit assumption of their correctness. In fact, though γ-HCH and γ-PCCH give rise to the same metabolic end-products, no causal relationship has been demonstrated between the two sets of data. Neither has it been demonstrated that γ-HCH does not itself have one of its chlorines substituted by the sulphydryl of GSH.

A similar state of confusion exists as to the nature of the enzyme catalysing the conjugation of glutathione with γ-PCCH and (if direct conjugation does, in fact, occur) with γ-HCH. Grover and Sims (1965) claim that the glutathione S-aryltransferase of rats, which conjugates 2,4-dichloronitrobenzene with GSH, is the same as that coupling γ-PCCH to GSH, on the grounds that the ratio of the activities of their

preparation toward the two substrates did not change through several purification steps. They also report, however, that even at 0.5 mM. the γ-PCCH does not inhibit the activity of the preparation towards the dichloronitrobenzene.

Ishida and Dahm (1965a) have isolated a Sephadex fraction of a Musca domestica preparation which catalyses the degradation of 1,2,3,4-tetrachloro-1,2,3,4-tetrahydronaphthalene, δ-PCCH, γ-PCCH, α-HCH, γ-HCH and δ-HCH.

The substrates are given in decreasing order of reactivity towards the preparation. GSH is a necessary cofactor, but it has not been determined whether, in fact, GSH conjugates are formed. A change in the activity of the preparation towards the different substrates on ageing suggests that there are a number of enzymes. Variations in the ratio of the activities of preparations made at different stages of the life cycle support this idea (Ishida and Dahm, 1965b). In the same work it was observed that the preparation lost different amounts of activity towards α-HCH and γ-HCH when passed through two different ion-exchange columns. The authors concluded that there were at least three enzymes in the preparation with differing specificities for these two substrates. It does seem equally valid, however, to postulate only two enzymes, with complete specificity, which are denatured to differing extents on the two columns. Partial loss activity is not a good criterion for postulating the existence of a new enzymic species. However, the other evidence for a multiplicity of enzymes seems quite strong, and this automatically weakens the concept of any one of them being the aryltransferase.

It would now be appropriate to review the known glutathione transferases, insofar as it seems that the enzymes involved in the metabolism of the HCH's and PCCH's must be of this group.

CHAPTER 4.

The Glutathione Transferases

The detoxication of foreign compounds as the alkylated N-acetylcysteines has been known for many years (Baumann and Preusse, 1879; Jaffé, 1879). For some time, it has been postulated that GSH was the source of the cysteine moiety of these mercapturic acids. The administration of many foreign compounds excreted as mercapturic acids was observed to be followed, in mammals, by a depression of the body levels of GSH, particularly in the liver (Nakashima, 1934; Yamamoto, 1940; Binet and Wellers, 1951; Barnes, James and Wood, 1959). Bray, Franklin and James (1959a) found that S-(p-chlorobenzyl)GSH was formed in rat liver from p-chlorobenzyl chloride. This was converted to the cysteine derivative by liver slices and homogenates, and by glutathionase.

Booth, Boyland and Sims (1961) demonstrated the complete process in rats. N-Acetyl-S-(2-chloro-4-nitrophenyl)-L-cysteine was found to be formed from 3,4-dichloronitrobenzene in three steps, each of which could be demonstrated *in vitro* (see Fig. IV.1).

The first step catalysed the conjugation of glutathione and 3,4-dichloronitrobenzene to give S-(2-chloro-4-nitrophenyl)glutathione.

The second step, catalysed by a kidney homogenate, involved the removal of the glycyl and glutamyl residues to give the substituted cysteine. Two enzymes may be involved.

The third step, catalysed by liver homogenate, acetylated the cysteine, giving the mercapturic acid. This does not appear to occur in guinea pigs (Bray and James, 1959).

The enzyme catalysing the first step, which has since been

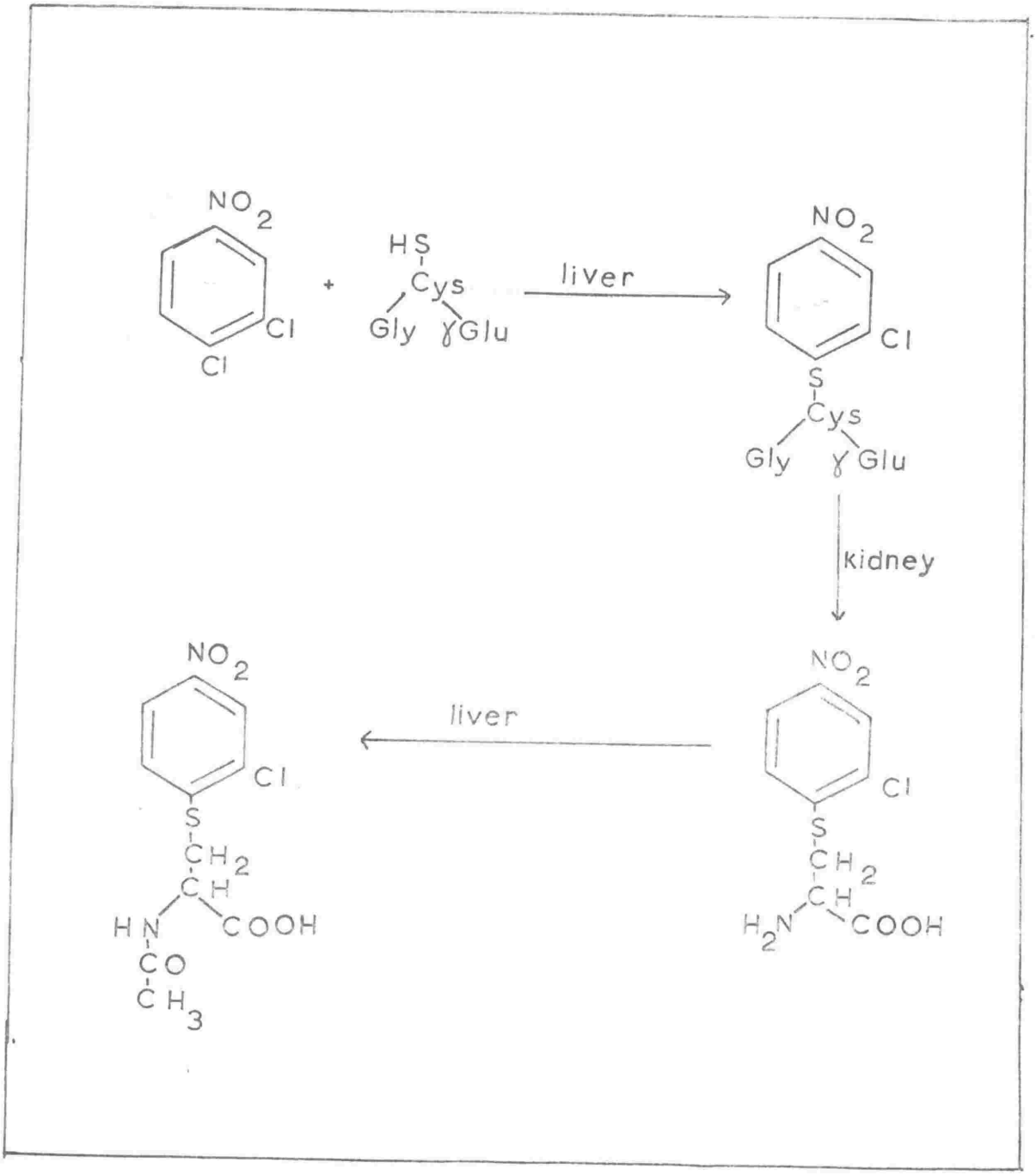


Fig.IV.1: Production of mercapturic acid from 3,4 dichloro-nitrobenzene as in rats.

named glutathione S-aryltransferase, is of the type with which we are principally concerned. Three other distinct enzymes have since been discovered. These will be discussed in more detail below.

1) The aryltransferase:-

The vertebrate enzyme, the first to be studied, has been found to be specific for glutathione, but with a wide range of aromatic substrates. These include 3,4-dichloronitrobenzene (Booth, Boyland and Sims, 1961), various polychloronitrobenzenes (where the nitro group is replaced by some S-derivative) (Bray, Hybs, James and Thorpe, 1953; Betts, James and Thorpe, 1955; Betts, Bray, James and Thorpe, 1957); 4-nitropyridine-N-oxide and 4-nitroquinoline-N-oxide (Al Kassab, Boyland and Williams, 1963), phenoltetrabromphthaleindisulphonic acid (B.S.P.) (Grover and Sims, 1964), and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (Boyland and Williams, 1964; Booth, Boyland and Williams, 1961).

The enzyme has a broad pH optimum (pH 7.5-9.0) (Booth, Boyland and Williams, 1961) and is sensitive to organic solvents (Boyland and Williams, 1965), but is stable to dialysis.

The invertebrate enzyme is specific for GSH and conjugates a similar range of aromatic substrates (Cohen, Turbert and Smith, 1964). The enzyme appears to be more stable than that of vertebrates towards organic solvents, and is stable towards dialysis. The optimum pH of fly and grass-grub enzymes with 3,4-dichloronitrobenzene or 2,4-dinitrochlorobenzene as substrate is 8.3 (Clark, Darby and Smith, 1967).

The molecular weight of the locust enzyme, as determined on Sephadex G-100, is about 65,000.

substrates possess an aromatic nature. This casts some doubt on the claim of Grover and Sims (1965) that the aryltransferase conjugates the PCCH's. It also seems possible on this basis that 1,2,3,4-tetrachloro-1,2,3,4-tetrahydronaphthalene (Ishida and Dahm, 1965a) is metabolized by the aryltransferase, by analogy with 1,2-epoxy-1,2,3,4-tetrahydronaphthalene, while the HCH and PCCH isomers are metabolized by some different enzyme.

2) Glutathione S-epoxytransferase:-

This enzyme catalyses the conjugation of a variety of aliphatic and aromatic epoxides with GSH (Boyland and Williams, 1965). In vivo, the epoxides are produced from compounds which do not themselves conjugate with GSH, by a microsomal oxidising system requiring molecular oxygen and reduced NADP. Such compounds include naphthalene, 1,2-dihydronaphthalene (Boyland and Sims, 1961), phenanthrene (Boyland and Sims, 1962; Sims, 1962), benzanthracene (Boyland and Sims, 1964), and possibly chlorobenzene (Gessner and Smith, 1960). Many of the GSH conjugates in this group, and their derivatives, are acid-labile. For example, N-acetyl-S-(2-hydroxy-1,2-dihydro-1-naphthyl)-L-cysteine yields on acidification the S-(1-naphthyl)mercapturic acid, with minor amounts of naphthalene and of 1- and 2-naphthol. It is tempting to speculate on the possibility of such a premercapturic acid being the acid-labile precursor of chlorobenzene derived from γ-HCH-dosed M.domestica (Bridges, 1959).

This enzyme has only been demonstrated so far in the supernatant of kidney and liver homogenates from rat and ferret (Boyland and Williams, 1965).

The enzyme is specific towards GSH, and is stable towards

dialysis. Exposure to pH values below 5.0 destroys its activity. The optimum pH with 2,3-epoxypropylphenyl ether as substrate is pH 6.5.

3) Glutathione S-alkyltransferase:-

This enzyme catalyses in vertebrates the conjugation of a number of alkyl halides with GSH (Bray and James, 1958; Grenby and Young, 1960; James, 1961; Boyland and Williams, 1965; and Johnson, 1963, 1966). In vitro known substrates include ethyl bromide and chloride, methyl chloride, and iodoacetic acid. Higher homologues do not appear to react in vitro, though Bray and James (1958) claim that alkyl halides of up to eight carbon atoms in chain length are conjugated in vivo.

The enzyme is unstable towards acetone, but is stable towards ethanol at concentrations of up to 25%. It is denatured by dialysis against water, but not against 2% sodium chloride solution. The optimum pH for the rat and sheep liver enzyme is between pH 8.0 and 8.5.

4) Glutathione S-alkenyltransferase:-

Boyland and Chasseaud (1966) observed a fourth type of activity in which GSH is added to an activated double bond system. A general formula for active substrates is R-CH = CH-R' where R = H or COOEt, R' = NO_2, CHO, or COOEt. The enzyme is unaffected by dialysis and has an optimum pH of 8.0.

It is tempting to think that this enzyme might be responsible for conjugating the PCCH's, giving an S-(pentachlorocyclohexyl)glutathione. The electron-withdrawing effect of neighbouring chlorines might be sufficient to activate the double bond to the extent required by this enzyme.

A fourth enzyme that might fall into this category is DDT dehydrochlorinase (Sternburg, Kearns and Moorefield, 1954; Lipke and Kearns, 1959a,b). This has an absolute requirement for GSH. Although there is no evidence for it, it seems possible that the reaction might proceed via the formation of a transitory conjugate, followed by elimination of GSH. There seems to be no other plausible explanation for the rigid requirement for GSH, though the fact that glutamylcysteine inhibits, and cysteinylglycine activates, this reaction complicates this simple picture.

Sternburg and Kearns (1956) claim that this enzyme does not dehydrochlorinate γ-HCH. Ishida and Dahm (1965a) however, claim that their γ-HCH-solubilizing preparation will dehydrochlorinate DDT, and that the substrates are mutually inhibitory. It is quite possible that different enzymes are involved.

Ishida and Dahm's housefly preparation contains an enzyme, or enzymes metabolizing the HCH's and PCCH's, which cannot, at present, be positively identified with any of those discussed previously. The optimum pH towards all isomers is about pH 8. The molecular weight of the fraction is about 55,000 as determined on Sephadex G-100.

CHAPTER 5

The Kinetics of Glutathione S-Aryltransferase

As will have been seen in the introduction, the enzymes involved in the conjugation reaction during the metabolism of the HCH's and PCCH's are, if not identical with the aryltransferase, probably very similar to it, having similar specificities, pH optima, elution characteristics, and sensitivity to inhibition by the sulphonphthaleins. In view of this, the aryltransferase, being the most readily available, and the most easily studied of the glutathione transferases, is taken here as a model. From it, we can pick out features which may provide a means of comparing properties of the different enzymes, or which may support the idea of only one enzyme being involved. Also, in view of the similar behaviour of the enzymes towards the sulphonphthalein inhibitors, it may be possible, by studying the nature of the inhibition in the aryltransferase model, to design an inhibitor that would act as an effective synergist for γ-HCH.

A.

Materials & Methods

Melting points are uncorrected.

Substrates:-

1-Chloro-2,4-dinitrobenzene (CDNB) was prepared by the action of a 50/50 (v/v) mixture of fuming nitric acid and fuming sulphuric acid on chlorobenzene. The product, recrystallized from ethanol, had m.p. 50.1°.

(B.D.H.) 1-nitro-2,4-dichlorobenzene (DCNB), recrystallized from ethanol, m.p. 43°.

(Chemical Procurement Laboratories, U.S.A.) 2,4,6-Trinitrobenzene-sulphonic acid, m.p. 83° and

2,4-Dinitrobenzenesulphonic acid, recrystallized as the barium salt, m.p. of the free acid 107°.

2,4-Dinitrobenzenesulphonamide was prepared by the action of PCl_5 on the sulphonic acid, followed by reaction with ammonia solution (sp. gr. 0.91) of the resulting chloride. The product was recrystallized from ethanol, m.p. 154°.

Picryl chloride was prepared by the action of $POCl_3$ on pyridine picrate. The product was washed with water and recrystallized from ethanol, m.p. 82°.

Reduced glutathione (Sigma) m.p. 190° (decomp.).

L-cysteine hydrochloride (Sigma) m.p. 174° (decomp.).

Inhibitors:-

Phenol red (Ph.R.) (Phenolsulphonphthalein)

Bromphenol blue (B.P.B.) (3,3', 5,5'-tetrabromophenolsulphonphthalein)

Bromthymol blue (B.T.B.) (3,3'-dibromothymolsulphonphthalein)

Bromcresol purple (B.C.P.) (3,3'-dibromocresolsulphonphthalein)

Bromcresol green (B.C.G.) (3,3', 5,5'-tetrabromcresolsulphonphthalein)

Chlorophenol red (C.P.R.) (3,3'-dichlorophenolsulphonphthalein)

BSP. (Phenoltetrabromphthaleindisulphonic acid, disodium salt)

3,3', 5,5' Tetrabromophenolphthalein (TBPP).

3,3', 5,5' Tetrabromophenolphthalein ethyl ester (TBPOEt)

Eosin-2,4,5,7-tetrabromofluorescein

2,5-dichlorofluorescein

4,5-di-iodo-1,8-dimethylfluorescein

Rose bengal-4,5,6,7-tetrachloro-2',4',5',7'-tetrabromofluorescein.

The above were obtained as commercial indicators and were not subjected to further purification.

Dicarboxylic acids:-

Succinic, glutaric, pimelic, adipic, suberic, sebacic, traumatic, tetradecanedioic, and thapsic acids were kindly donated by the Food Chemistry Division, D.S.I.R.

The following were commercial samples:-

Benzene m-disulphonic acid

Flavianic acid (2,4-dinitro-1-naphthol-7-sulphonic acid), m.p. 150°.

2,3-Dimercaptopropan-1-ol (B.A.L.)

Buffers:-

pH 8-10	$Na_4P_2O_7/HCl$	0.1M in pyrophosphate
pH 6-8	Na_2HPO_4/KH_2PO_4	0.1M in phosphate
pH 4-6	$NaOOC.CH_3COOH$	0.1M in Na^+.

Unless otherwise specified, all experiments were carried out at pH 8.3, and at 37°C.

Enzyme Preparations:-

Acetone powders of flies (Musca domestica) and grass grubs (Costelytra zealandica) were prepared. Homogenates (prepared in an M.S.E. overhead blender) of adult flies, or of 3rd instars of C.zealandica, in an equal weight of water were spun at 10,000g for 30 mins. at 0°. The supernatant was poured into 20 volumes of acetone at -15°, the protein was filtered off on the pump, and dried at 4° at 0.5 mm. Hg.

The grass-grub powder was used in experiments concerning the

conjugation of both CDNB and DCNB. Where CDNB was used, a stock enzyme solution containing 2 mg. of acetone powder per ml. of buffer was used, and for DCNB, 20 mg. per ml.

The fly powder was used only with CDNB, and the stock solution contained 20 mg. per ml.

Sheep liver homogenate:-

A sheep liver was bought from a butcher on the day following slaughter (Freshly slaughtered sheep did not have significantly higher levels of enzyme). Portions were homogenized with an equal weight of buffer, pH 8.3, and cellular debris removed at 1500g for 10 minutes at 0°. The pH was adjusted to 5, protein was sedimented at 10,000g for 30 min. at 0°, and the pH of the supernatant taken back to 8.3. The clear red solution was diluted twentyfold for use as a stock solution, for experiments using CDNB as substrate.

If the pH precipitation were not performed, readings taken below pH 6 would be obscured by protein precipitation. This was not significant when the insect acetone powders were used.

The assay:-

Depending upon the type of experiment being performed, different procedures were required. The method was basically that of Booth et al. (1961).

1) Determination of $K_{m_{GSH}}$ and $K_{i_{GSH}}$:-

A two-cuvette system was used. The blank contained, in a final volume of 3 ml., 0.05 ml. of enzyme stock solution, and CDNB or DCNB to final concentrations of 0.1 mM or 0.75 mM, respectively, added in 0.05 ml.

of ethanol. The reaction system contained GSH to the required concentration (added in from 0.01 to 0.1 ml. of buffer at the required pH), inhibitor (if used) to the required concentration (added in buffer, from 0.01 to 0.2 ml.), 0.05 ml. of stock enzyme solution, and CDNB or DCNB to final concentrations of 0.1 mM or 0.75 mM; final volume 3 ml. Reagents were added in the order given.

Where CDNB was the substrate, reaction velocities were measured directly on a recording spectrophotometer for the first minute of the reaction time by measuring the increase in optical density at 344 mμ. due to the formation of S-(2,4-dinitrophenyl)glutathione. Corrections were made for the spontaneous reaction, which is significant above pH 6.5.

Where DCNB was the substrate, optical densities at 344 mμ. were measured against the blank before addition of the enzyme, and after incubation for 5 minutes. A small correction had to be made for the enzyme in the initial reading. No correction was required for spontaneous reaction which is insignificant below about pH 9.5.

2) Determination of K_m and K_i with respect to DCNB and CDNB.

A similar procedure to the above was used. The blank contained only 0.05 ml. of enzyme solution in 3 ml. total volume. In the reagent tubes, GSH, to 2 mM, was added in 0.05 ml. of buffer, and 0.05 ml. of enzyme solution, and CDNB or DCNB were added in from 0.25 to 2.5 ml. of buffer. If used, inhibitors were added to the required concentration in from 0.01 ml. to 0.1 ml. of buffer.

All components, except enzyme and GSH solutions, were held at 37°C. The enzyme and GSH solutions were kept on ice.

Comments on the assay:

1) As might be expected from chemical characteristics, CDNB reacted faster, enzymically, than did DCNB, and also gave a significant spontaneous reaction (see Fig.V.1). For this reason, a recording spectrophotometer must be used when CDNB is the substrate. Under all conditions examined, DCNB gave a linear reaction curve for at least 5 minutes, enabling the more sensitive "Uvispek" to be used.

2) As a matter of routine, GSH was always added to the system before the enzyme, and before the substrate. With some of the GSH transferases (Boyland and Sims, 1965), failure to observe this precaution results in denaturation. In fact, with this enzyme, adding it after the substrate appeared to make little difference to its activity.

3) In studying K_m and K_i for CDNB and DCNB, these substrates were added in aqueous solutions. It is possible that, if they were added in ethanol, the different ethanol concentrations might have affected the reaction to different extents.

4) GSH solutions were kept on ice to minimise loss through oxidation. This does not, however, appear to be critical. Even at 37°C, the GSH content of a 0.3 M solution in pyrophosphate buffer at pH 8.3 fell by only 10% in 90 minutes (GSH was measured by the rate of spontaneous reaction with CDNB). Making up this solution with EDTA to 6 mM did not decrease the loss.

5) An attempt was made to use this assay as an assay for glutathione. The enzyme appears to be quite specific for GSH, and, as the conjugates

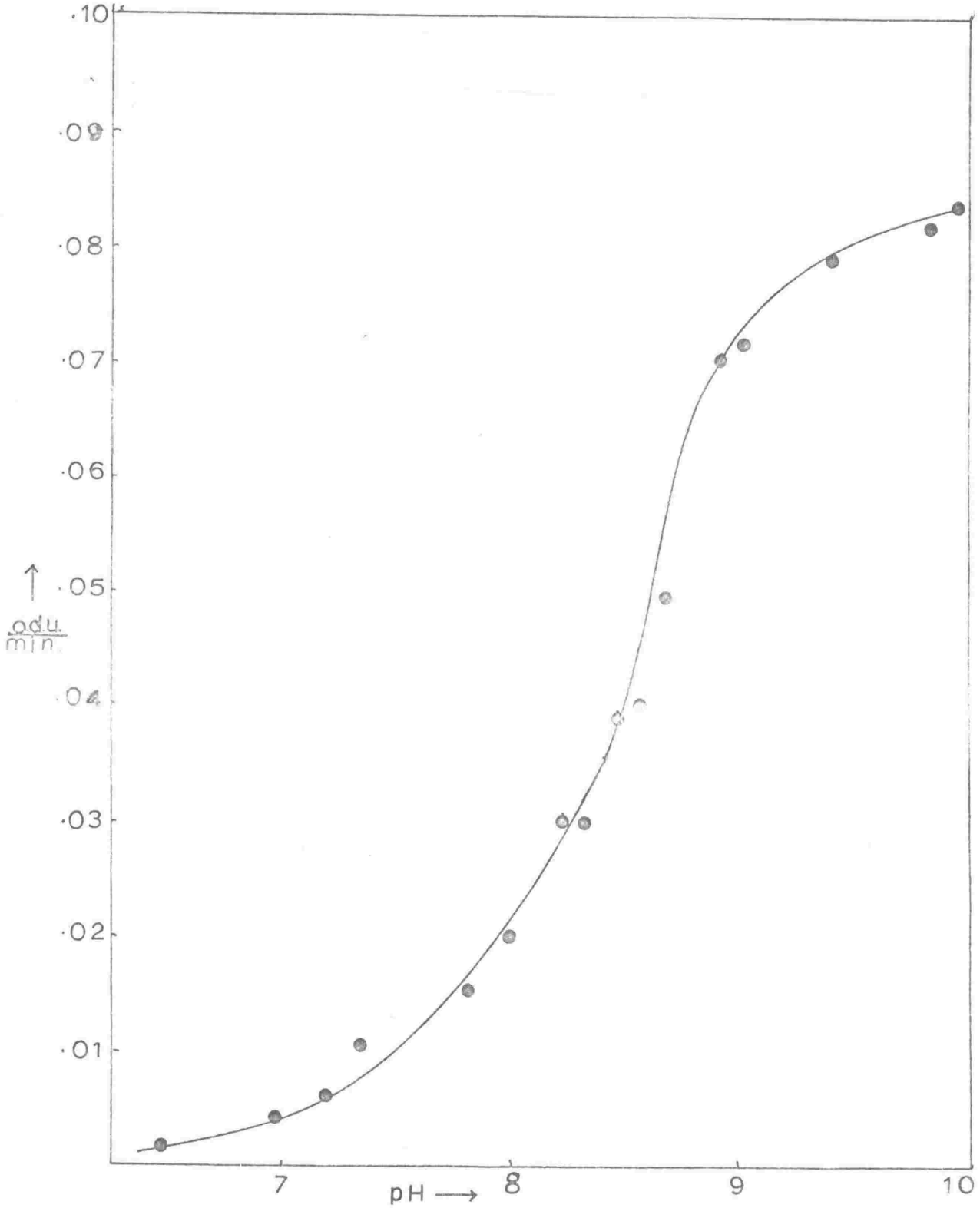


Fig.V.1: Variation with pH of the rate of spontaneous reaction of 2,4-dinitrochlorobenzene with GSH.

with CDNB and DCNB both have maximum molecular absorptions of about 10,000, it should, in theory, be possible to detect GSH down to about 3 μg/ml. CDNB is unsuitable - its high spontaneous rate of reaction with thiols removes the specificity from the reaction. DCNB appears, at the moment, to be unsuitable. With present enzyme preparations, the reaction rate is so low that protein precipitation obscures the results before the reaction is complete.

It is however, possible that with a substrate of intermediate activity, perhaps p-chloronitrobenzene, and with an enzyme preparation of higher purity, this system might provide a simple assay for GSH.

B.

Uninhibited Kinetics

(i) Of the potential substrates tested, only CDNB and DCNB participated in the enzyme-catalysed reaction. Picryl chloride and trinitrobenzenesulphonic acid react spontaneously very rapidly at pH as low as 6. The 2,4-dinitrobenzenesulphonic acid and sulphonamide do not react spontaneously below about pH 10. None of the compounds appears to inhibit the enzymic conjugation of CDNB with GSH.

L-Cysteine reacts spontaneously with CDNB but does not participate in an enzymic conjugation. When present with GSH in the enzyme system, it appears to have a slight activating effect (5 - 10%). This may be due to reduction of any oxidized glutathione in the system.

(ii) K_m with respect to GSH with DCNB and CDNB as substrates, and K_m for these substrates, were determined for the grass grub enzyme. $K_{m_{GSH}}$ with CDNB as substrate and $K_{m_{CDNB}}$ were determined for the fly and the sheep-liver enzymes.

The four substrate constants for the grass grub enzyme were determined by multiple Burke-Lineweaver plots, using the method of Florini and Vestling (1957) (Fig.V.2), with CDNB as substrate.

Results:

Grass Grub:-

K_m GSH (0.75 mM DCNB)	1.58×10^{-3}M $\pm$ 10%
K_m DNCB (2 mM GSH)	5.0×10^{-4}M $\pm$ 10%
V_m DCNB (2 mM GSH)	0.03 o.d.u./min/mg. acetone powder
K_m GSH (0.1 mM CDNB)	1.6×10^{-3}M $\pm$ 10%
K_m CDNB (2 mM GSH)	1.6×10^{-4}M $\pm$ 10%

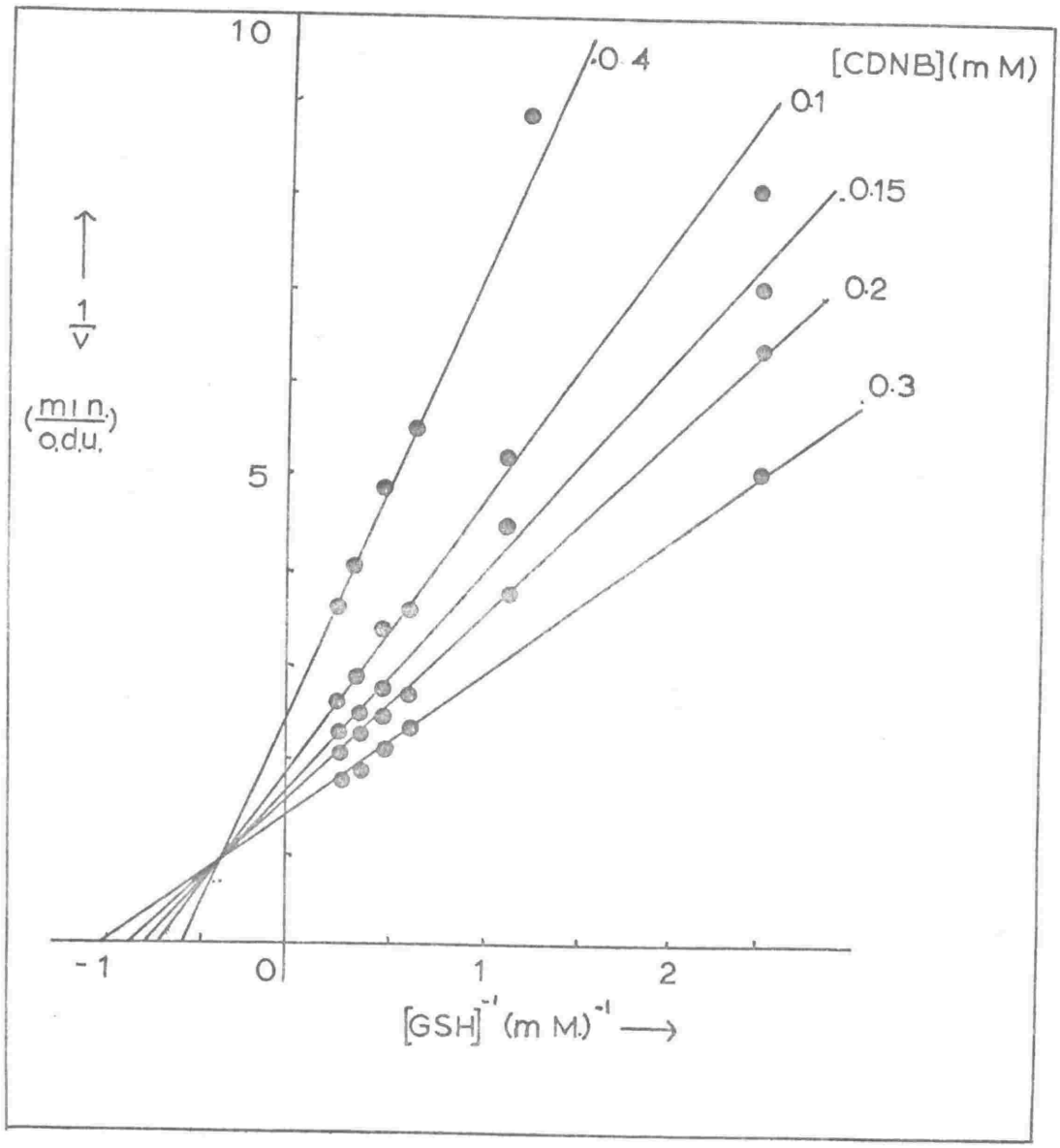


Fig. V.2: Multiple Burke-Lineweaver plot for C. zealandica glutathione S-aryltransferase.

$V_{m_{CDNB}}$ (2 mM GSH) 3.3 o.d.u./min/mg. acetone powder.

Substrate constants:-

$K_g = 1.7 \times 10^{-3}$ M 5% $K'g = 1.5 \times 10^{-3}$M ± 5%

$K_d = 8.7 \times 10^{-5}$M 5% $K'd = 8.0 \times 10^{-5}$M ± 5%

$$\frac{K'g}{K_g} = \frac{K'd}{K_d} = 0.91 \pm 10\%$$

V_m = 10 o.d.u./min/mg. acetone powder ± 10%

Sheep Liver:-

$K_{m_{GSH}}$ (0.1 mM CDNB) 7.6×10^{-4} ± 10%

$K_{m_{CDNB}}$ (2 mM GSH) 2.0×10^{-4} ± 10%

M.domestica:-

$K_{m_{GSH}}$ (0.1 mM CDNB) 1.2×10^{-3} ± 10%

$K_{m_{CDNB}}$ (2 mM GSH) 8.0×10^{-5} ± 10%

(iii) Discussion:-

The linearity of the reciprocal plots, and the constancy of the ratio between the paired substrate constants for the Costelytra enzyme indicates that, for this enzyme, Michaelis-Menten kinetics apply and that substrates combine in random order. It is assumed that the same situation applies to the other enzymes dealt with here, but this needs to be verified.

The ratio of the substrate constants is, within the limits of experimental error, close to unity. Thus, it can be seen that the effect of one substrate upon the affinity of the enzyme for the other is low, if not negligible. This is borne out by the close similarity of the $K_{m_{GSH}}$ obtained with the two different substrates.

It will be noticed that the value of $V_{m_{CDNB}}$ (velocity at

saturating substrate concentration, obtained by extrapolation, at 2 mM GSH) is greater than $V_{m_{DCNB}}$ by a factor of about 10^2. From this, one concludes that the rate constant (assuming irreversibility) for the reaction of the former substrate when on the enzyme, must be 100 times that of the latter. A more accurate comparison could be made if the true V_m were determined for DCNB.

On the available data, the M.domestica enzyme seems to have similar kinetic properties to that of C.zealandica. The sheep liver enzyme differs considerably from both.

C.

Inhibited Kinetics:- (see Appendix A.)

(i) Of the potential inhibitors examined, the following gave no significant inhibition at concentrations of up to 1 mM: benzene m-disulphonic acid, oxidised glutathione, B.A.L., and succinic, glutaric, adipic, pimelic, and suberic acids.

(ii) With the insect enzymes, the phthalein-type dyes and the rest of the dicarboxylic acids inhibited competitively with respect to GSH, and non-competitively with respect to the aromatic substrate. The characteristic plots obtained are shown for bromcresol green (Fig. V.3). The values of the K_i obtained with the insect enzymes are listed below.

Grass grub enzyme:-

Inhibitor	pK_a	$Ki_{GSH} \times 10^6$ (0.75 mM DCNB)	$K_{i_{DCNB}} \times 10^6$ (2 mM GSH)	$Ki_{GSH} \times 10^5$ (.1 mM CDNB)	$K_{i_{CDNB}} \times 10^6$ (2 mM GSH)
B.P.B.	4.23	0.45	0.76		

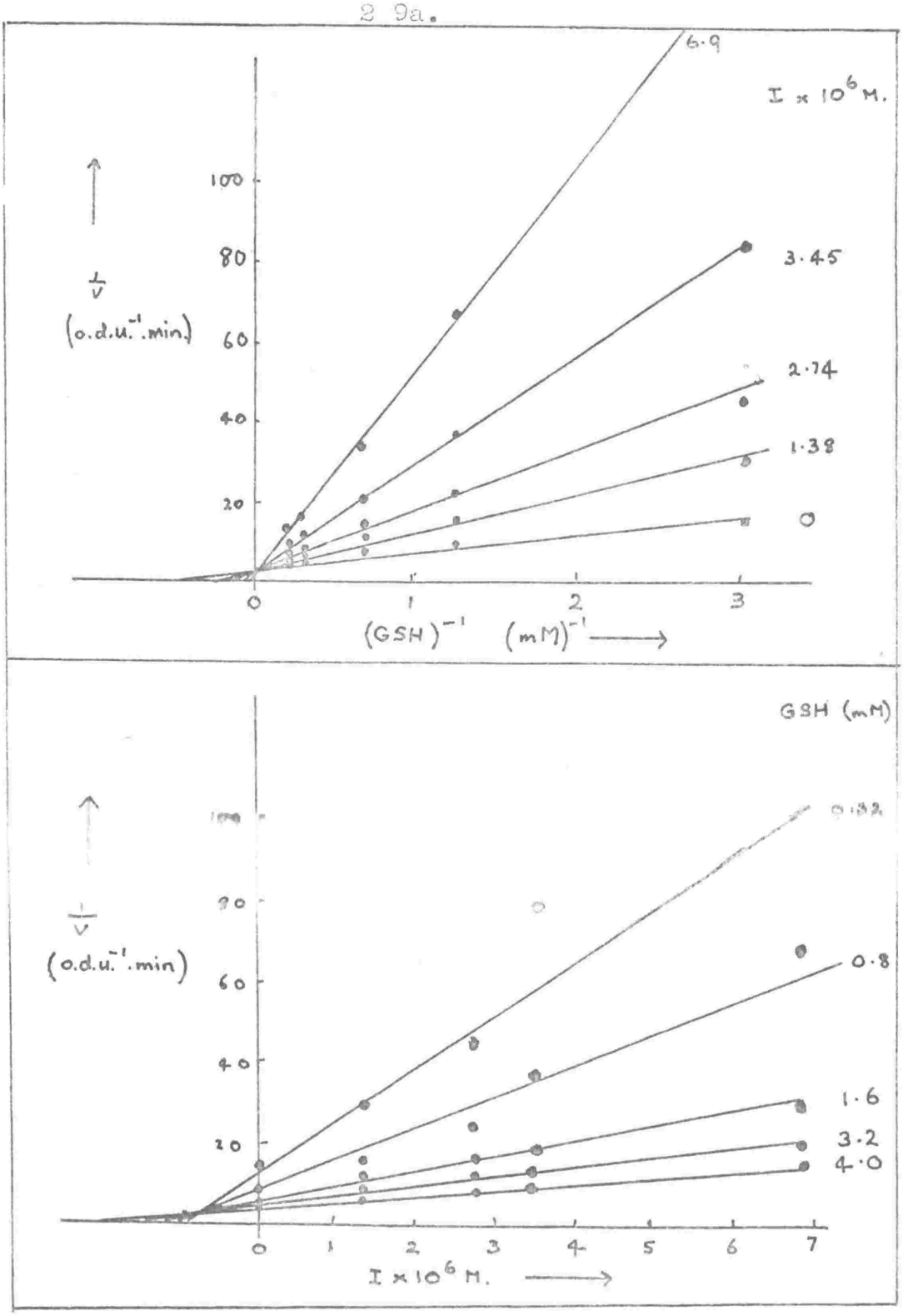


Fig.V.3(i): Inhibition of Costelytra Glutathione S-Aryl-transferase by bromocresol Green. (Competitive towards GSH).

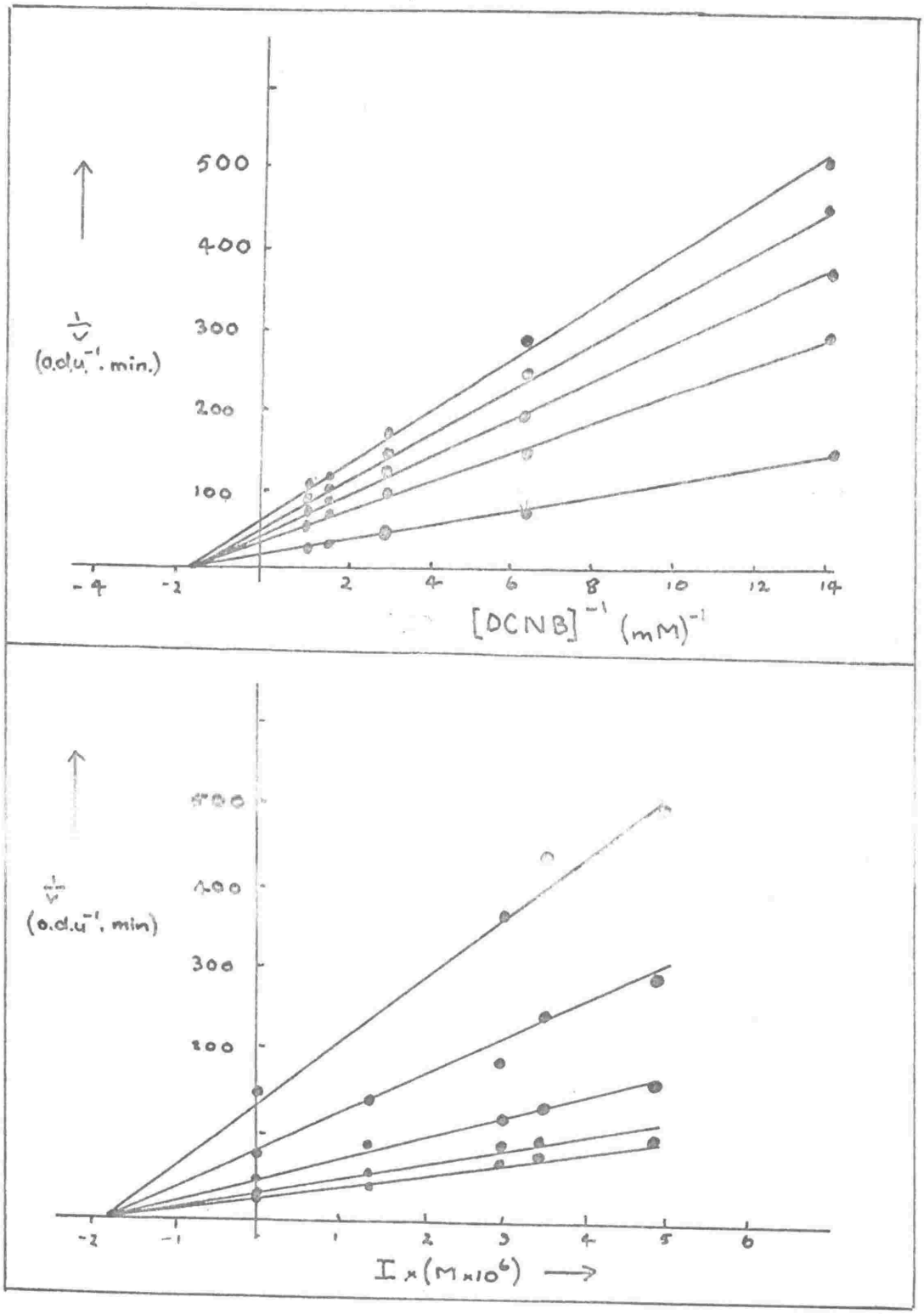


Fig.V.3(ii): Inhibition of Costelytra Glutathione S-Aryltransferase by Bromocresol Green. (Non-competitive towards DCNB.)

Grass grub enzyme (continued):-

Inhibitor	pKa	K_i GSH x 10^6 (0.75 mM DCNB)	K_i DCNB x 10^6 (2 mM GSH)	K_i GSH x 10^6 (.1 mM CDNB)	K_i CDNB x 10^6 (2 mM GSH)
B.C.G.	4.92	0.82	1.1	0.4	2.2
C.P.R.	5.95	1.10	1.25		
B.C.P.	6.33	1.67	-		
B.T.B.	7.34	11.6	17.20		
Phenol Red	8.08	19.9	-		
BSP	8.66	1.25	8.9	8.9	
TBPP	-			6	
TBPOEt.	-			7	
Eosin		3.53			
Rose Bengal		5.5			
Di-iodofluorescein		7.0			
Dichlorofluorescein		91.5			
Flavianic acid				7.0	17.5
Azelaic acid				770	
Sebacic acid				55	
Traumatic acid				55	
Tetradecanedioic acid				100	
Thapsic acid				75	
Fly enzyme:-					
Bromphenol Blue				5.5	
Bromcresol Green				9.3	
Bromcresol purple				15.8	
Phenol Red				48	

The sheep-liver enzyme was inhibited by none of the phthalein-type compounds with the exception of BSP, which gave 16% inhibition at 2×10^{-4} M. Grover and Sims (1964) reported that BSP inhibited the vertebrate enzyme by competition with the aromatic substrate: it does, in fact, form a conjugate itself, and this is the basis of the BSP test for liver function. Flavianic acid gave 16% inhibition at 4×10^{-4} M. The dicarboxylic acids did not inhibit the sheep enzyme.

(iii) The variation of percentage inhibition of the grass-grub enzyme with pH by bromcresol green, B.S.P., chlorophenol red, and phenol red is shown in Fig.V.4. It will be seen that in each case, the inhibition is independent of the state of ionization of the inhibitor. Each curve rises gently to a maximum at about pH 9, and then falls off rapidly.

(iv) Discussion:-

Binkely (1961) has shown that in mammalian renal glutathionase, BSP and bromcresol green compete with acceptor dipeptides - possibly for cationic sites on the enzyme. Sayre and Roberts (1958) show that in dog glutaminase, BSP and bromcresol green compete with the cofactor $PO_4^{\equiv}$ - again an ionic mechanism was invoked.

It was at first assumed that a similar situation existed in the case of the aryltransferase. On paper, it is easy to show that the glutathione molecule can be superimposed on the phthalein-type dyes so that its carboxyls coincide with the two phenolic groups (or the sulphonic acid groups in BSP), or with one phenolic group and the acid residue involved in the lactone ring (see Fig.V.5). Cohen (1963) observed that phenolphthaleindisulphonate did not inhibit,

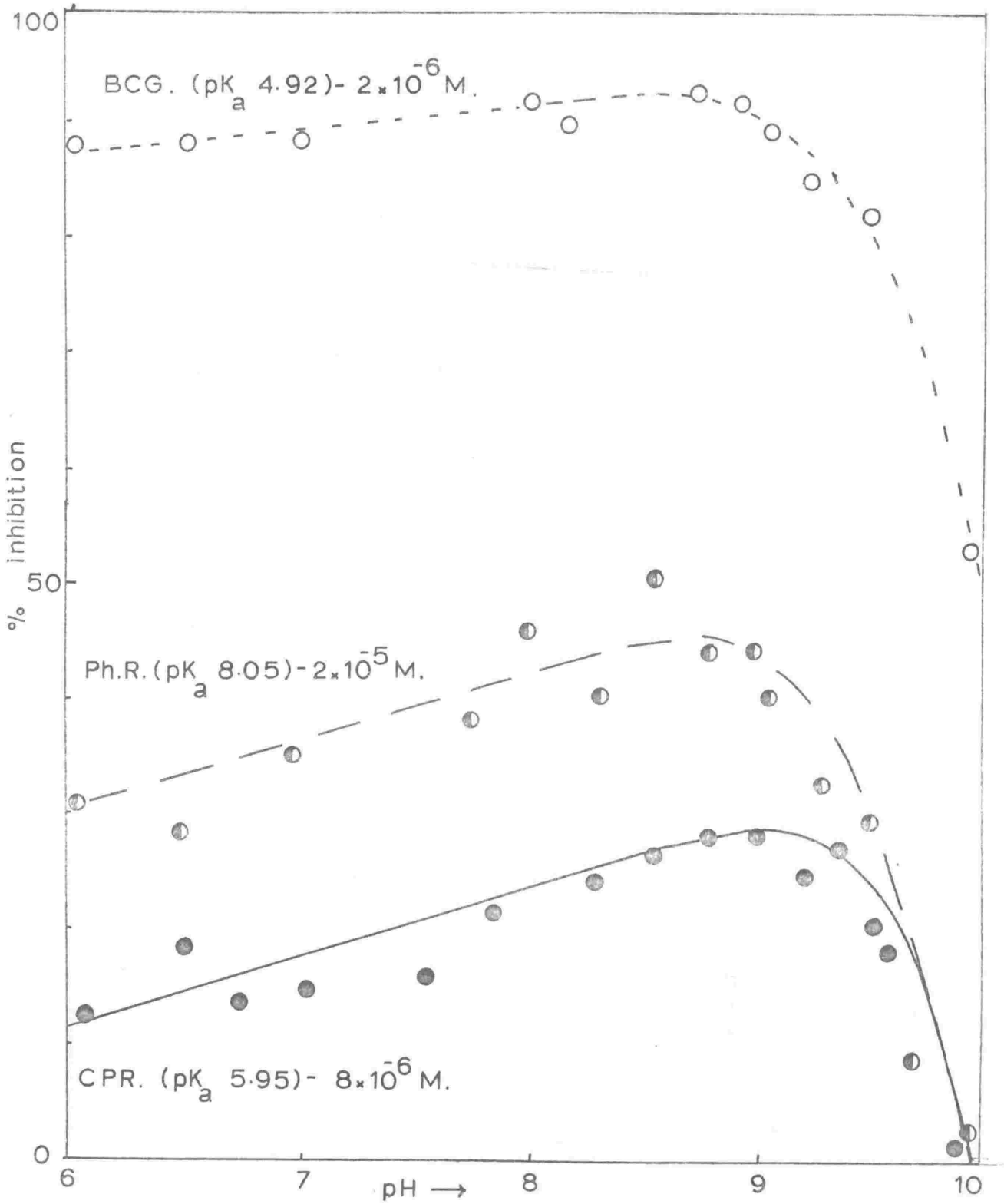
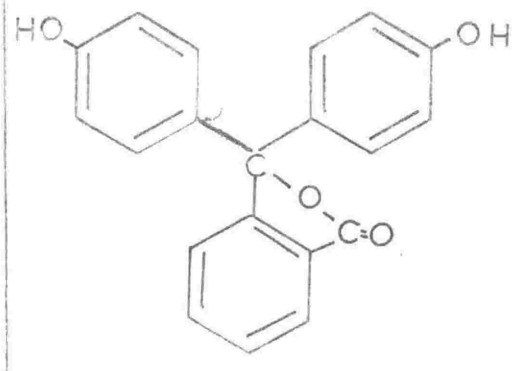
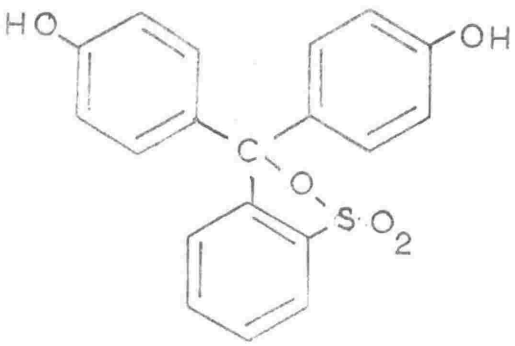


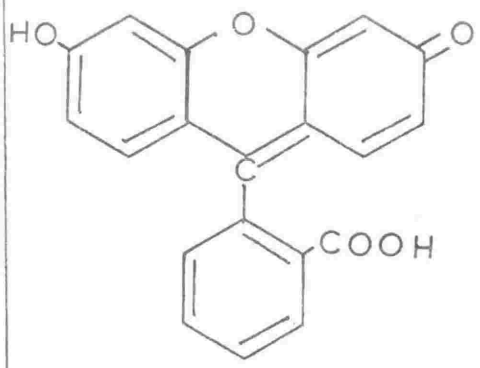
Fig.V.4: Variation with pH of percentage inhibition of C.zealandica aryltransferase by bromcresol green, chlorophenol red and phenol red.



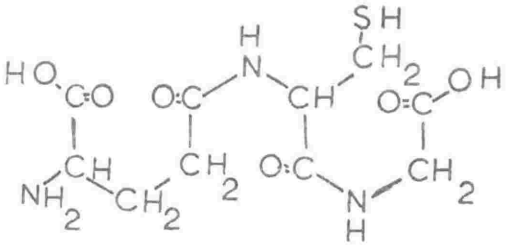
Phenolphthalein



Phenolsulphonphthalein



Fluorescein



Glutathione

Fig.V.5: Possible structural relationship between glutathione and the phthalein type dyes.

thus precluding the participation of the sulphonate groups in BSP, and at the same time suggesting that the inhibitory powers of BSP were due to the effect of its bromines on the carboxyl group. The participation of at least one phenolic residue was suggested by the fact that the rhodamines (Cohen, 1963) did not inhibit and that halogenation of the phenolic rings will increase the inhibitory power of the compound. Finally, the idea of competition between a pair of acid functions and the carboxyls of GSH is supported by the fact that the long-chain dicarboxylic acids are quite strong competitors. From Fig. V.6, it will be seen that as the inter-carboxyl chain length approaches and exceeds that for GSH, (C_{11}) the K_i reaches a steady minimum. Data obtained in the next series of experiments also support the idea that two cationic groups are involved in the binding of GSH to the enzyme.

However, as far as inhibition by the phthalein type compounds goes, a simple ionic model is insufficient. For instance, the relationship between pK_a and K_i for the sulphonphthaleins (Fig. V.7) shows the opposite trend to that expected for a purely ionic model. If it were simply a matter of electrostatic attraction between cationic and anionic groups, one would expect that the lower the pK_a of the anionic group, the less affinity that group should have for a proton or a protonated residue. Hence the lower the pK_a, the higher the K_i. In fact, the reverse situation holds.

Secondly, that tetrabromphenolphthalein and its ethyl ester have nearly identical K_i's shows that, if the lactone-forming acid residue is involved, it is not as an ionizing entity.

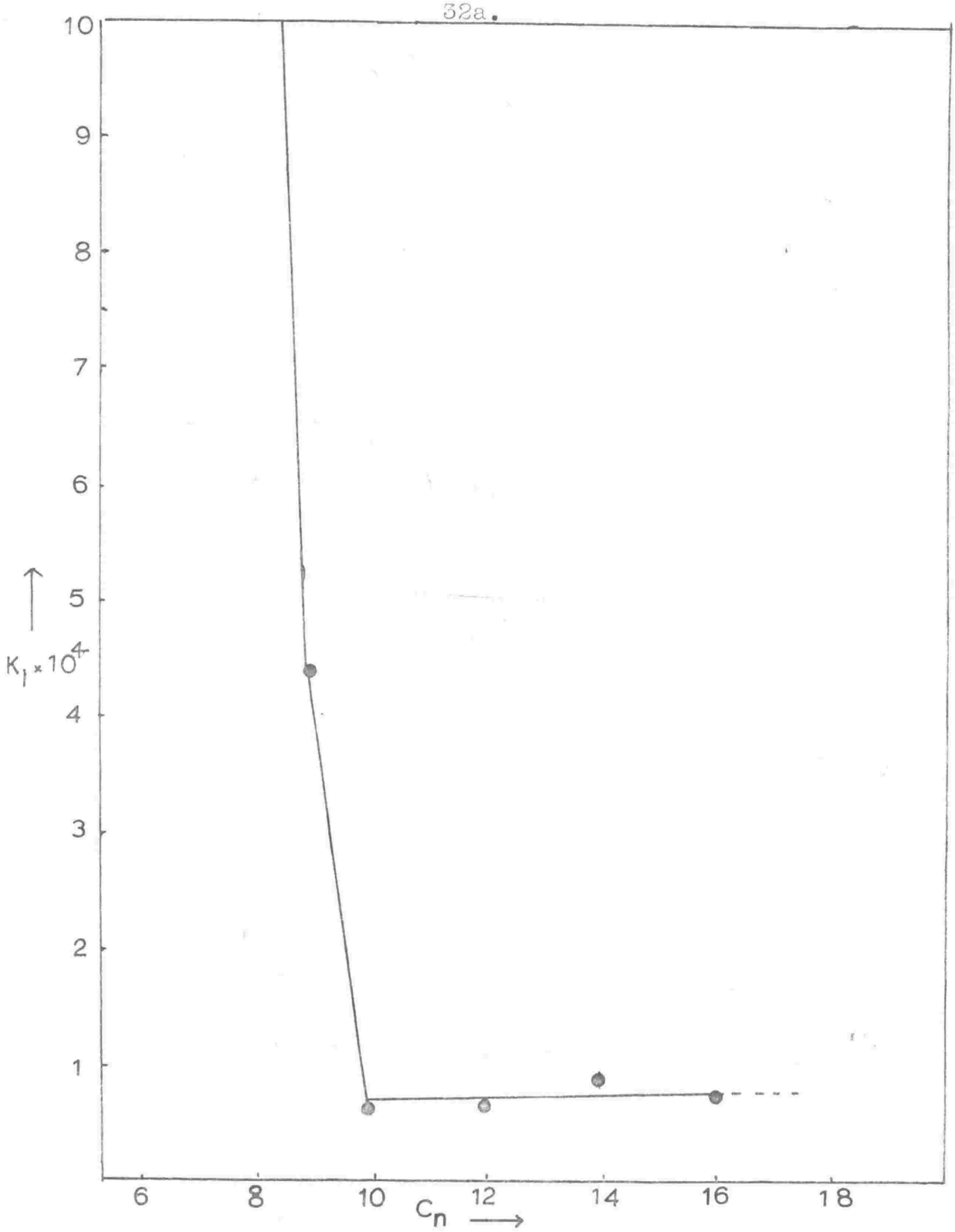


Fig.V.6: Relationship between chain-length and Ki for the dicarboxylic acids.

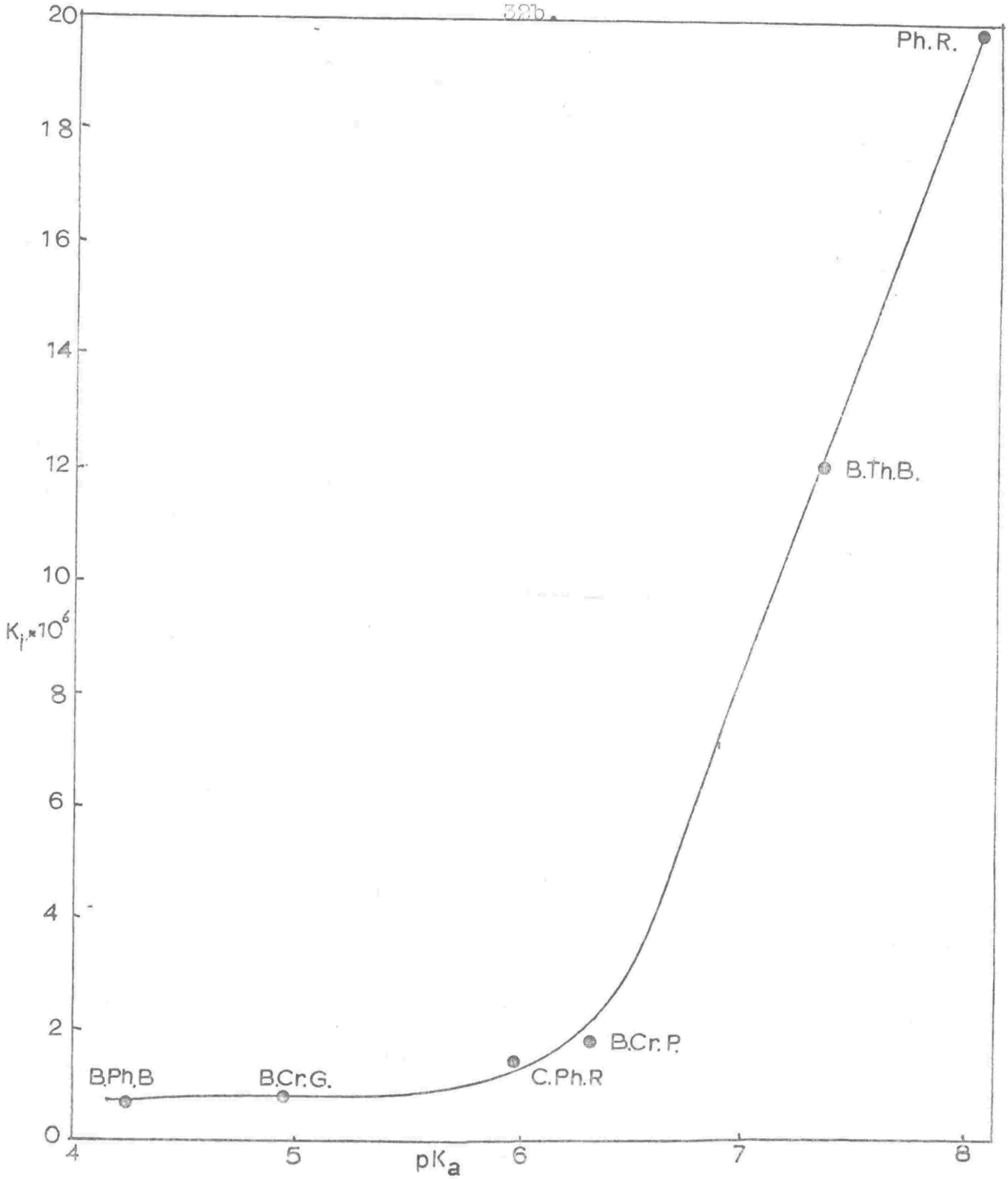


Fig.V.7: Relationship between Ki and pK_a for the sulphonphthaleins.

Thirdly, the observation that the state of ionization of various phthaleins and sulphonphthalein was irrelevant to the degree of inhibition produced by each demonstrates conclusively that a simple ionic mechanism cannot be involved.

The above conclusions have been drawn from data obtained from experiments with the grass-grub enzyme. It seems probable that the fly enzyme is similar with respect to its behaviour towards inhibitors. The sheep enzyme is unaffected by most of the inhibitors tested, which suggests that the nature of its active site is significantly different to that of the insect enzyme. This inference is borne out in the following section.

D.

Variation of Kinetics with pH (See Appendix B).

(i) Stability of enzymes towards extremes of pH.

A stock solution of the grass-grub acetone powder and a sheep-liver homogenate were prepared as previously described, using distilled water, and at five times the previously specified concentrations. One-ml. aliquots of each were pipetted into 4 ml. portions of 0.1 M buffers and incubated for three minutes. (This is three times the period of the average assay in which CDNB is used as substrate). After this time, 0.05 ml. of the enzyme solution was transferred to a cuvette containing 2.9 ml. of 0.1 M pyrophosphate buffer containing 2 mM GSH. 0.05 ml. of CDNB in ethanol was added to start the reaction, and the activity of the incubated enzyme was then assayed at 37°. The final pH of the incubation system was measured after the assay. The results are illustrated in Fig. V.10. It will be seen that the

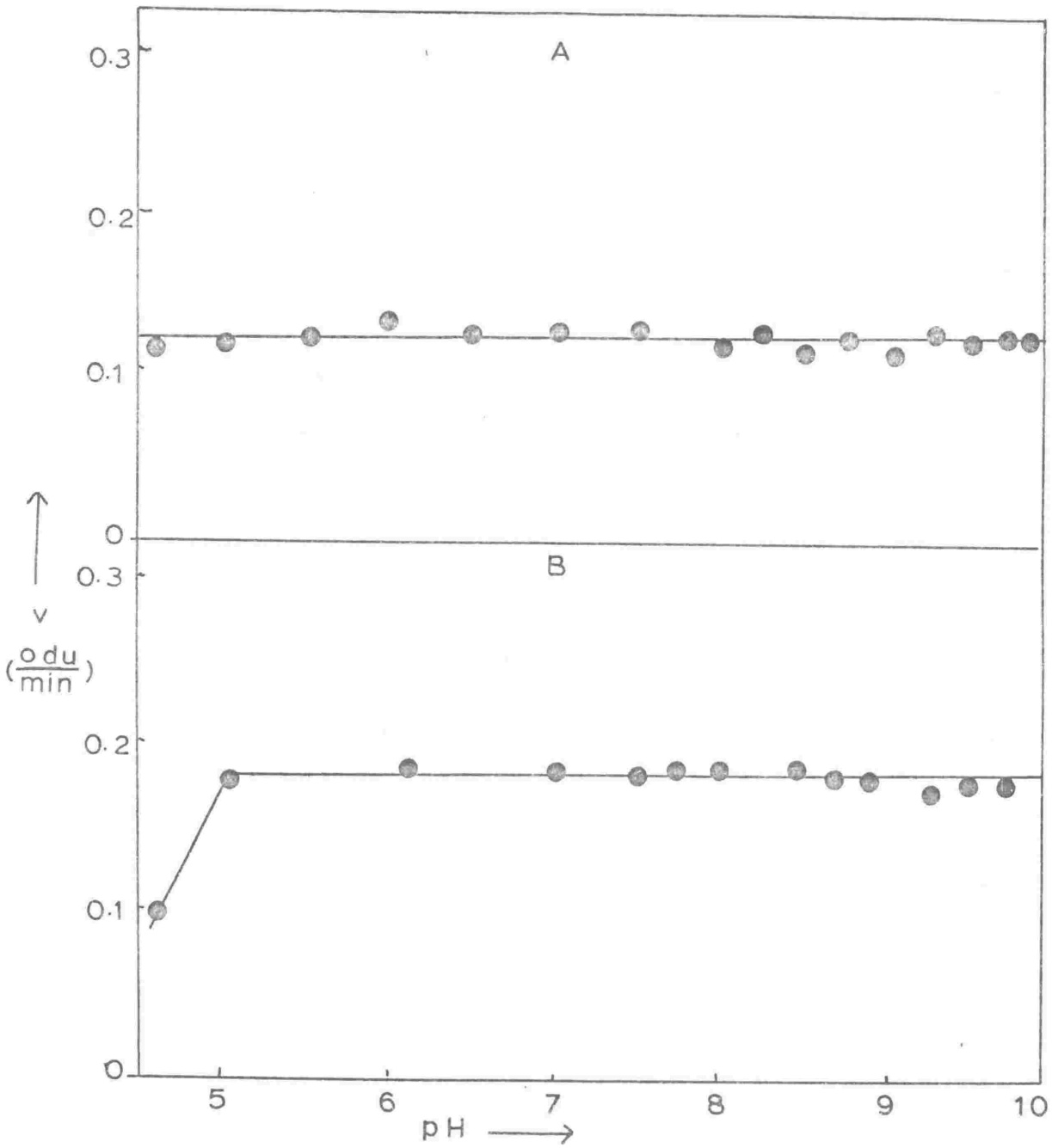


Fig.V.10: Stability of the aryltransferase of (A) Costelytra zealandica and (B) sheep liver to various pH's at 37°C.

activity of the grass-grub preparation was constant (± 5%) over the whole pH range studied, while that of the sheep liver was constant from pH 5 to pH 9.75. Below pH 5.0, the activity of the latter preparation fell off sharply, and above pH 9.75 it appeared to be starting to fall off. Thus, for both enzymes, over a working pH range of 5 to 9.75, denaturation during a one minute assay should be of little significance.

(ii) V_m, V_o, $K_{m_{GSH}}$ (0.1 mM CDNB) were determined for the grass-grub and sheep-liver enzymes over the pH range 4.5 - 10. V_m is the velocity at infinite GSH concentration, obtained by extrapolation of a Burke-Lineweaver plot; V_o the velocity at 0.4 mM GSH. At the same time, for the grass grub enzyme, the veocities obtained with a concentration of 0.8 μM bromcresol green were determined. From these, the K_i with respect to GSH was calculated from the slope and intercept of Burke-Lineweaver plots obtained at each pH. The results are shown graphically in Fig. V.8.

V_m, V_o and K_m for CDNB (2 mM GSH) have been determined for the grass-grub and sheep-liver enzymes at various pH values. V_o is the reaction velocity at 0.02 mM CDNB. These results are shown graphically in Fig. V.9.

The results can, to some extent, be interpreted using Dixon's rules (1953).

GSH Kinetics: (Fig. V.8.)

1. Grass-Grub enzyme.

Here we see, in the high pH region, changes of gradient in the pK_m curve, of -2 and +2 units at pH 9.3 (e_2) and at pH 9.75 (es_2).

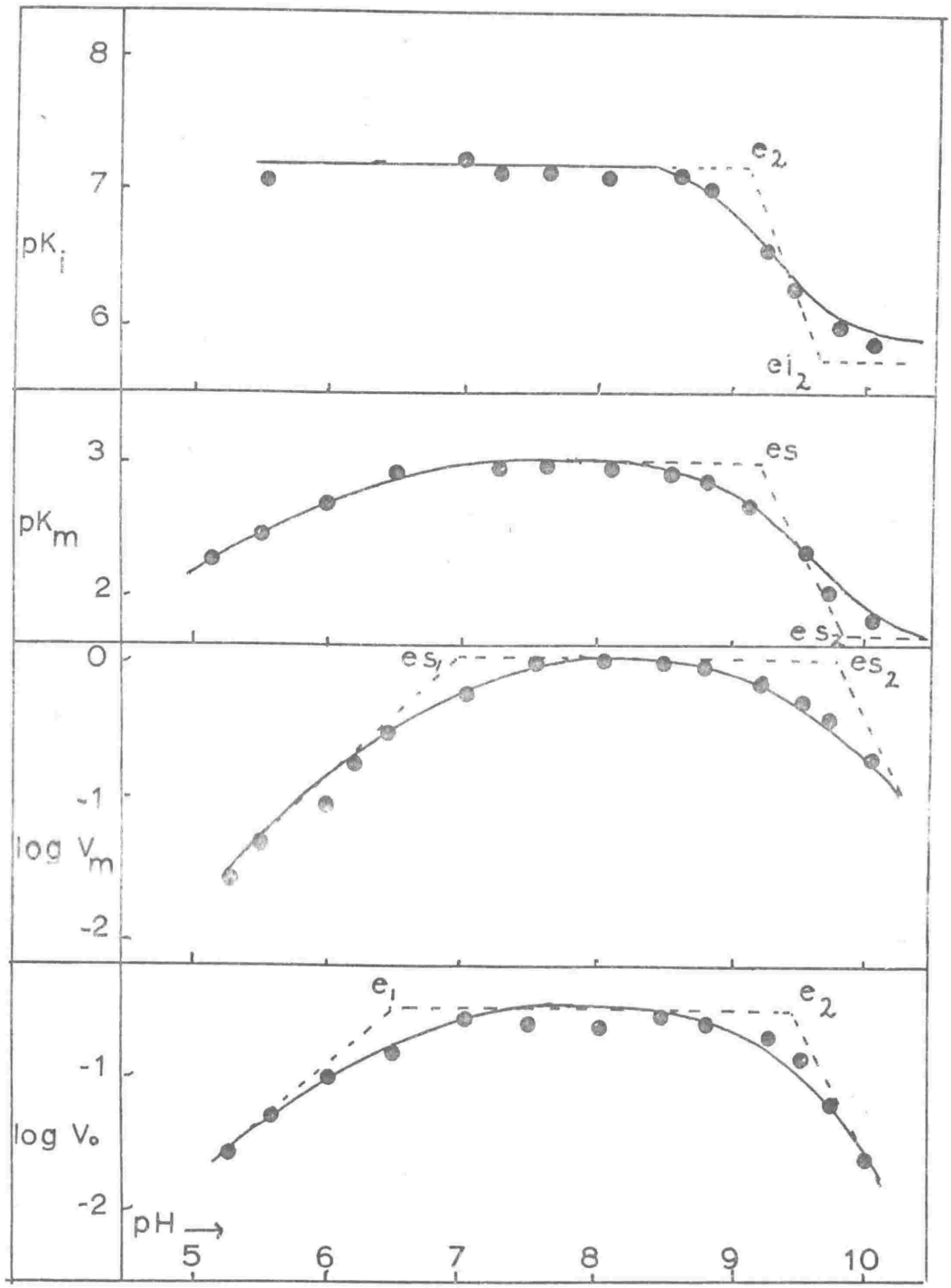


Fig.V.8(i): Variation with pH of the glutathione kinetic parameters of the C. zealandica aryltransferase.

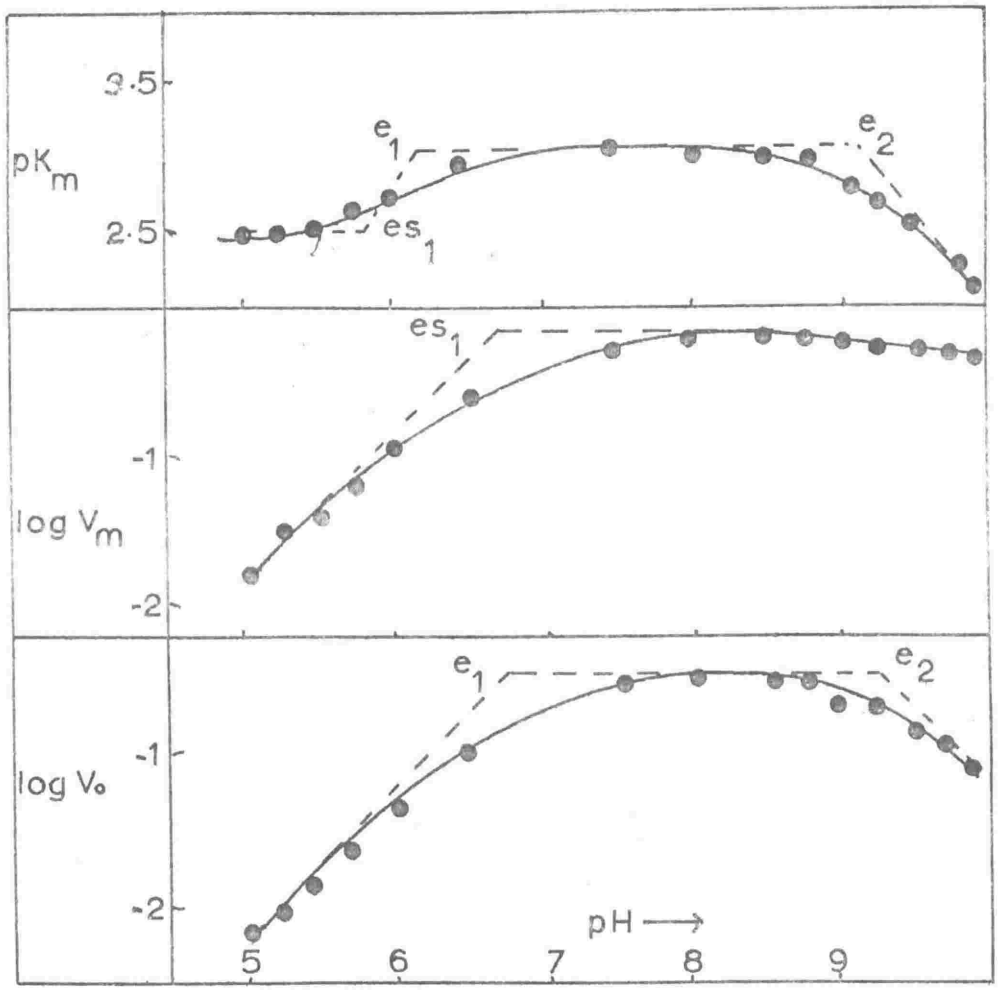


Fig.V.8(ii) : Variation with pH of the glutathione kinetic parameters of the sheep-liver aryltransferase.

These coincide with gradient changes in the log V_o and log V_m curves respectively, and indicate kinetically critical ionizations in the enzyme and enzyme-substrate complex. The changes in gradient show the changes in charge to be twofold. It will be noticed that ionization of the enzyme is not suppressed by the attachment of the substrate.

The e_2 ionization could have been due to ionization of the thiol group of GSH (pK 9.2 - Benesch and Benesch, 1955). However the plot of pK_1 against pH, which ideally is independent of substrate ionization (See appendix B), gives identical gradient changes. There is no question of these being due to the ionization of the bromcresol green (pK_a 4.92), so that they must represent changes in ionization of the enzyme.

Rather more tentatively, ionizations e_1 and es_1 are assigned pK values of 6.5 and 7.0. The inflection points in the log V_o and log V_m curves do not coincide with those in the pK_m curve, and the gradient change in the latter is not integral.

These factors make any rigid interpretation of the data impossible.

2. Sheep Liver Enzyme:-

Here, in the high pH region, only one gradient change occurs, in the pK_m and log V_o curves. This has a value of unity. We conclude that at this inflection point (pKe_2), a single ionization occurs which greatly reduces the affinity of the enzyme for GSH. If, however, GSH is attached to the enzyme, this ionization is suppressed and the E-GSH complex does not itself ionize into an inactive form.

Again, in the low pH region, well defined gradient changes occur

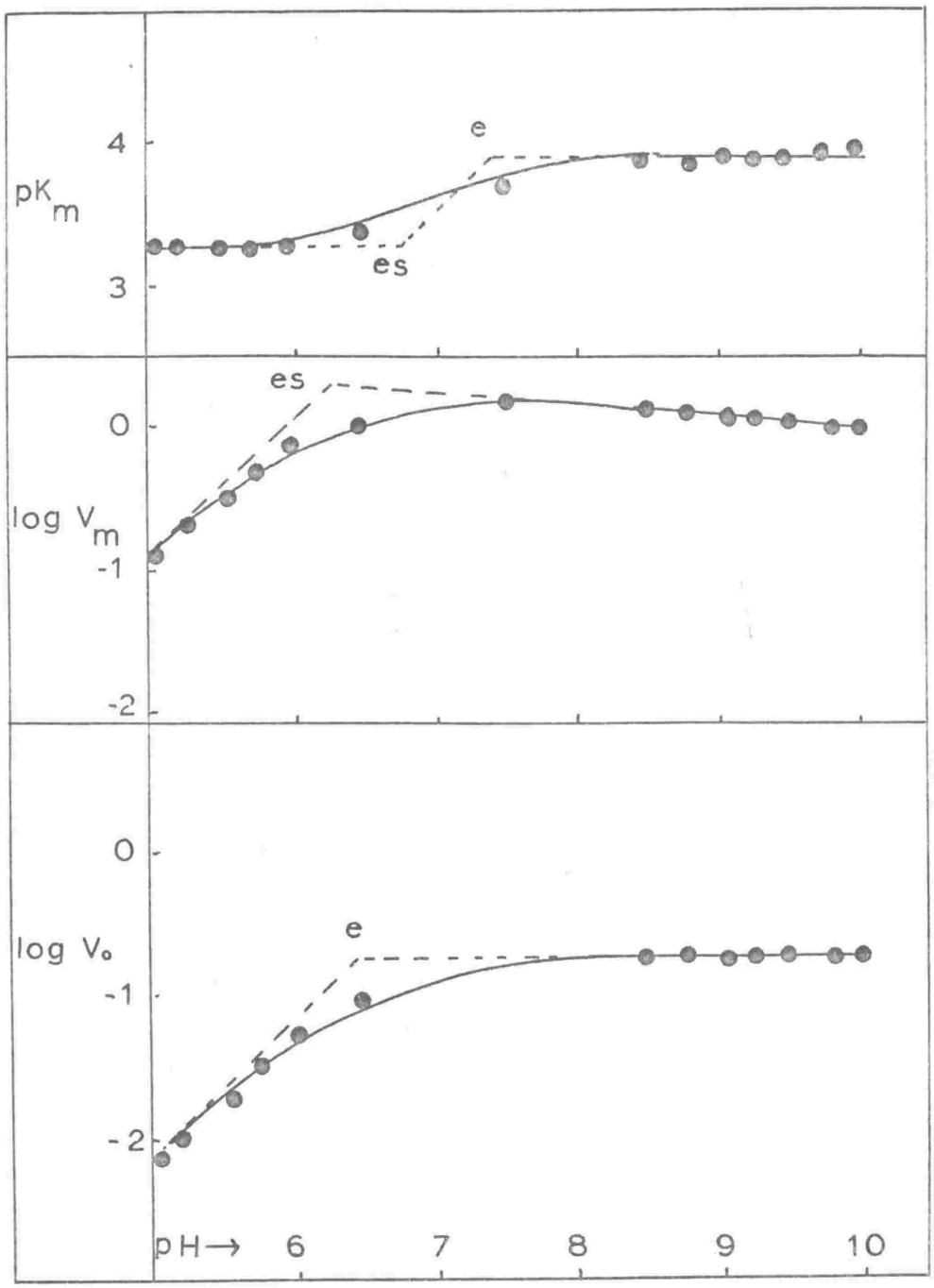


Fig.V.9(i): Variation with pH of the CDNB kinetic parameters of the C. zealandica aryltransferase.

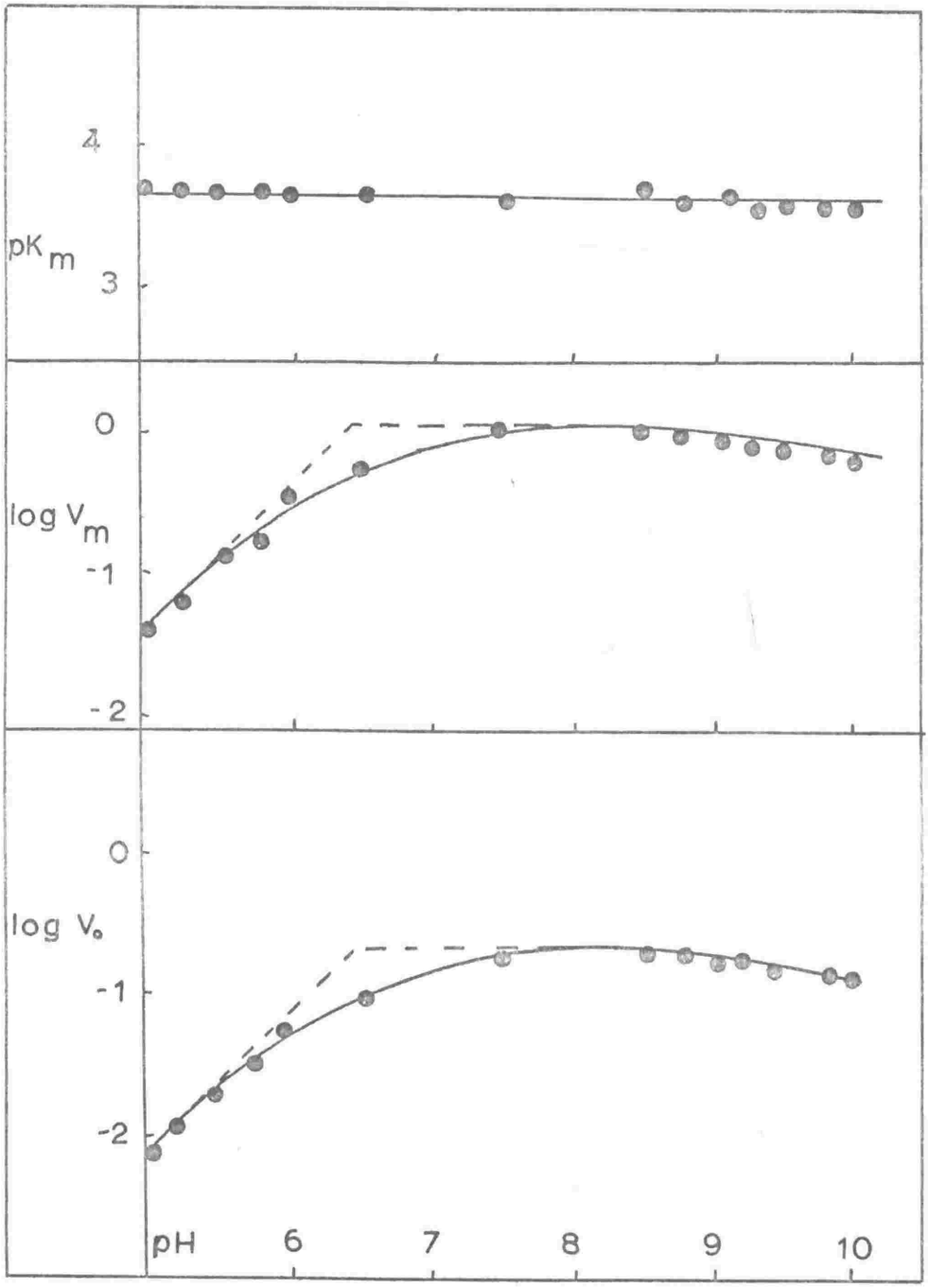


Fig.V.9(ii): Variation with pH of the CDNB kinetic parameters of the sheep liver aryltransferase.

in the log V_o and log V_m curves at pH 6.7, but these show little correlation with changes in the pK_m curve.

CDNB Kinetics (Fig. V.9.):-

1. Grass-Grub Enzyme.

In this case, it is plain that no groups ionizing between pH 8 and pH 10 are involved in the binding of CDNB to the enzyme.

Again, in the pH region 6-7, it seems that a critical ionization occurs, but again, lack of correspondence between the different curves (pK_m, e_1 = 7.4, es_1 = 6.75; log V_m, es_1 = 6.35; log V_o, e_1 = 6.5), makes interpretation difficult.

2. Sheep-Liver Enzyme:

Here, the picture is quite coherent. No critical ionizations occur between pH 7 and pH 10. The e_1 and es_1 ionizations are coincident at pH 6.5 - both involving unit changes in ionic charge. They show only on the log V_o and log V_m curves and cancel out each other in the pK_m curve. Thus attachment of aromatic substrate to this enzyme has no effect on the ionization at all.

(iii) Discussion:-

Between the sheep-liver enzyme and the grass-grub enzyme there seems to be a clearly defined species difference. In the case of the latter, two cationic groups appear to be involved in the binding of both GSH and the phthalein-type compounds (as represented by bromcresol green). The binding of neither GSH nor inhibitor suppresses the ionization of these groups. Residues at the active site which might behave in this fashion are the ε-amino group of lysine, the thiol

group of cysteine and the phenolic group of tyrosine. The thiol group is unlikely to be involved as the enzyme is not susceptible to heavy metal ion inhibition (F.J. Darby, unpublished data).

The sheep-liver enzyme has a group with a similar pK involved in GSH binding. Only a unit change in charge is involved, and binding of GSH to the enzyme suppresses its ionization. It could well be that this point of difference is responsible for the lack of sensitivity of the sheep enzyme towards potent inhibitors of the grass-grub enzyme.

Binding of CDNB to the sheep-liver enzyme involves a group ionizing at pH 6.5 in both the enzyme and the E-CDNB complex. It is tempting to invoke the ubiquitous histidine residue, and indeed, this provides a plausible explanation of the binding of CDNB to the enzyme. In the unionized state (above pH 6.5) the imidazole residue has an aromatic structure due to the participation of an imine nitrogen lone pair in a delocalized π orbital system. This could be expected to bind an aromatic substance by interaction of the π orbitals in the so called London, or dispersion, forces. Below pH 6.5, the addition of a proton to either of the imidazole ring nitrogens will abolish this aromaticity.

Binding of CDNB to the grass-grub enzyme appears to involve a similar mechanism. If so, the anomalous results for the GSH kinetics in the low pH region could, in both cases, be reflections of a decreasing amount of E-CDNB complex. This will affect the apparent K'g. for the system, which, if the concentration of CDNB is not saturating, must affect the experimentally determined $K_{m_{GSH}}$. This is discussed more fully in Appendix B.

It must be emphasized here that the ambiguity in the interpretation of these results is due to the fact that saturating concentrations of the co-substrate have not been used. This allows the possibility of irrelevant

ionizations affecting the logarithmic plots. In view of the high reaction velocities, the use of saturating concentrations would only be feasible if the experiments were performed at a lower temperature.

CHAPTER 6

In Vivo Metabolism of γ- and δ-PCCH in Mouse & Guinea-pig.

1) Materials and Methods:-

γ- and δ-PCCH were synthesized in 10 and 20% yields by alkaline monodehydrochlorination of γ- and δ-HCH (Hughes et al., 1953).

The δ-PCCH was distilled from the reaction mixture at 125-30°/10 mm. The crystalline solid was twice recrystallized from acetone, (m.p.68.5°).

The γ-isomer was distilled from the reaction mixture, again at 125-30°/10 mm. as a viscous, colourless liquid. This froze to a glass in liquid air, but became crystalline after being held at -15° for 1-2 months. Recrystallization from acetone took a further one month, but subsequent recrystallizations could be carried out in 2-3 days; m.p. 34.5°.

S-(2,4-Dichlorophenyl)-L-cysteine was prepared in 10% yield by the method of Parke and Williams (1951) (Parke, 1955) involving the coupling of 2,4-dichlorobenzenediazonium sulphate with L-cysteine cuprous mercaptide; m.p.180° (decomp.).

S-(2,4-Dichlorophenyl)glutathione was prepared by the same method, adapting the technique of Booth, Boyland and Sims (1960); m.p. 214-5° (decomp.).

D-Glucose (Analar)	m.p. 146°
Sucrose (Analar)	m.p. 183°
D-Glucurone	m.p. 177°
D-Glucuronic acid	m.p. 156°
p-Bromophenol glucuronide	
p-Nitrophenol glucuronide	

The glucuronides were kindly donated by Professor J.N. Smith.

Chromatography:-

Extracts were chromatographed on 250 μ. thin-layer plates of Kieselgel G; on Whatman's No.4 paper; and, for high loadings, on Whatman's No.31, using butanol-acetic acid-water (4:1:5) as solvent.

R_f values for reference compounds on the thin layer plates are as below.

Compound	Rf.
S-(2,4-dichlorophenyl)-L-cysteine	0.80 - 0.85
S-(2,4-dichlorophenyl)glutathione	0.55 - 0.60
2,4-dichlorophenylmercapturic acid	0.90 - 0.95
glucose	0.31
sucrose	0.60
glucurone	0.57
p-bromophenol glucuronide	0.20 - 0.25
p-nitrophenol glucuronide	0.73
inorganic chloride	0.10 - 0.20

Location reagents:

For thiols and thioethers, the Toennies and Kolb (1951) iodoplatinate reagent was used. This gives white or yellow spots on a pink background.

For α-amino acids: 0.25% ninhydrin in ethanol was used. For sugars and glucuronides, 0.1% Naphthoresorcinol in ethanol containing 10% HCl and 10% H_2SO_4 was used.

Sugars gave purple spots; glucuronic acid and glucurone, turquoise spots; and glucuronides red and purple spots.

With sugars, 1% aniline phthalate in butanol (water saturated), gave pink, yellow and brown spots.

With labile chlorinated hydrocarbons, Mitchel's (1952) silver nitrate reagent gave blue-grey spots on a white background after exposure to U.V. light at 254 mμ.

Quantitative Estimations:-

Glucuronic acid was estimated by the naphthoresorcinol method (See Mead, Smith and Williams, 1958).

Ethereal sulphates were estimated by turbidimetric estimation of inorganic sulphate by the method of Sperber (1948), before and after acid hydrolysis.

Thioethers were estimated by titration of iodine against thiols produced by alkaline hydrolysis of the thioethers (Parke and Williams, 1951).

Experimental:-

1) Three white mice were injected subcutaneously with 10 mg. of δ-PCCH (0.5 g/Kg.), a fourth being used as a control. The urine was collected over four days. One half of that from each mouse was evaporated down to dryness under reduced pressure, and made up to 2 ml. with distilled water. The other half was acidified and continuously extracted with ether for 12 hours. The ether layer was concentrated down to 2 ml. Both fractions were then chromatographed as described above and examined for reactivity towards the reagents mentioned.

2) Two guinea-pigs were kept on a constant diet (30g. "protein-enriched"

bread + 50 g turnip per day) for seven days and normal urinary output of ethereal sulphates, thioethers, glucuronic acid, and glucuronides were determined. Each animal was then injected subcutaneously with 0.25 g of δ-PCCH (0.5g/Kg) in 0.5 ml. of soy-bean oil. The urine (δ-urine) was collected and assayed daily for 9 days, by which time the urinary levels of the group of compounds being estimated had returned to normal. The animals were then fed on cabbage for one day, and returned to the standard diet for two days, to allow urinary output to stabilize. They were then injected with 0.25 g of γ-PCCH in 0.5 ml. of soy-bean oil. Urine (γ-urine) was again collected daily and assayed. The experiment ended on the seventh day after the second dosing, with the death of one of the guinea-pigs.

Portions of urine not used in these assays were stored at -15°. They were later pooled for chromatography. Chromatograms were run with the whole urines and their chloroform and ether extracts, and with the extracted aqueous layers.

Results:-

1) Chromatograms of the whole mouse urines and ether extracts of mouse urines showed no difference between those of dosed and undosed mice when treated with sugar and glucuronide reagents.

Ether extracts contained compounds running at R_f 0.92 - 0.96, giving yellow spots with the Toennies and Kolb (1951) reagent.

The whole δ-urines gave spots at R_f 0.50 and 0.85, giving white spots with the Toennies and Kolb reagent. These took three days to develop to full contrast. They did not appear in the control.

It is tempting to think of these three compounds as being a

mercapturic acid, glutathione conjugate, and cysteine conjugate respectively. It seems unlikely, however, that the last two compounds would be excreted in the urine. No further confirmatory tests were carried out.

2) Quantitative results from the guinea-pigs are shown in Fig. VI,1. These indicate that the PCCH's are excreted as glucuronides and as thioethers. Of the one millimole injected, a mean of 0.17 mmole appeared as the thioether, and a mean of 0.38 mmole as the glucuronide. On administration of the δ-PCCH, ethereal sulphate excretion fell drastically. It is possible that this could be related to the excretion of sulphur as thioethers. On administration of the γ-isomer, ethereal sulphate levels appeared to rise sharply and then to drop as before.

These results must, however, be regarded as being suspect, at least quantitatively. Increased urinary excretion of inorganic chloride, bile pigments, and glucose (identified as glucosazone) was observed during the second half of the experiment, suggesting kidney damage. Quantitative Benedict's assays for reducing sugars in the urine gave a peak value equivalent to a massive 1.2 g. of glucuronic acid per day. It was found that glucose in high concentrations (10-20 mg./ml.) obscured the end-point of the iodine titrations to a marked degree.

Chromatography yielded little useful information. Both γ- and δ-whole urines contained glucose and two other sugars (R_f 0.23 and 0.17) which were not present in the control urines.

Both γ- and δ-urines contained a naphthoresorcinol- and aniline phthalate-positive compound (red and pink colour reactions) of R_f 0.59 not present in the control.

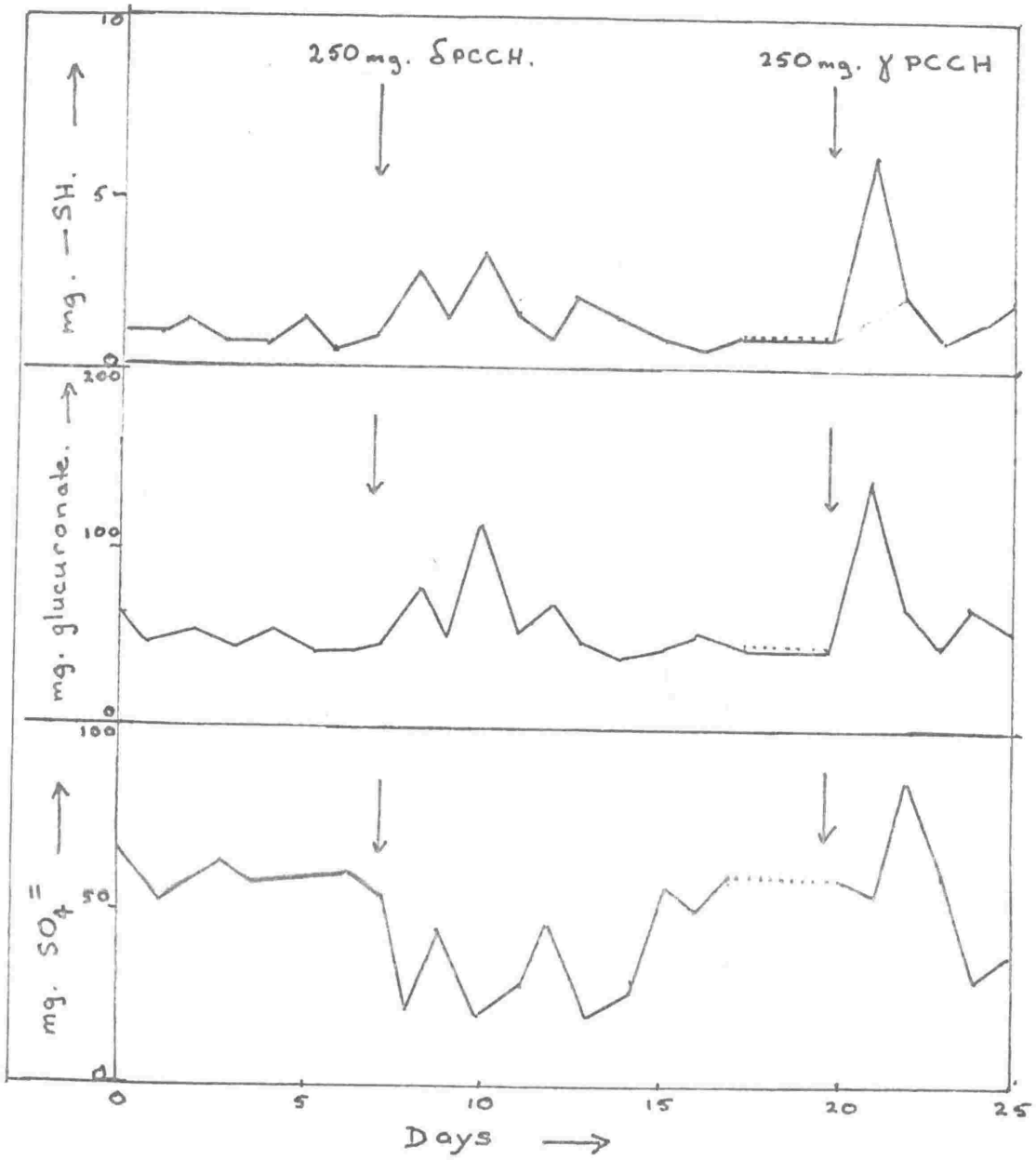


Fig.VI.1: Determination of thioethers, ethereal sulphates and glucuronides excreted by Guinea pigs dosed with δ- and γ-PCCH.

No significant differences were found between chromatograms run with γ- and δ- and control urines when sprayed with the Toennies and Kolb (1951) reagent.

Chromatograms of ether and chloroform extracts showed no significant differences between control and test urines when sprayed with any of the specified reagents.

CHAPTER 7.

Uptake of δ-HCH and δ-PCCH by Musca domestica and Costelytra zealandica

Materials:-

δ-HCH (Eastman Chemical Co.) m.p. 129-131°.

δ-PCCH m.p. 68.5°.

Reduced glutathione (Sigma)

Bromphenol blue

Phenol red

Tetrabromophenolphthalein ethyl ester (TBPOEt)

Methods:-

The chlorinated hydrocarbons were estimated by gas-liquid chromatography. A Perkin-Elmer '811' gas chromatograph fitted with an electron-capture detector was used. A 30 cm. glass column packed with 80 mesh Celite loaded with 0.5% (w/w) "Embaphase" silicone oil provided the stationary phase; oxygen-free nitrogen, with a flow rate of 85 ml/min. the mobile phase. Injector temperature: 200°; detector temperature 160°. Column temperatures were for δ-PCCH 90° (retention time, 45 sec.); for δ-HCH 140° (retention time, 85 sec.).

Grubs or flies were dosed with the chlorinated hydrocarbon by topical application or by injection, in 1µl. of 50% (v/v) aqueous acetone, and kept singly in stoppered homogenizer barrels for the required period at 25°. 10 mg. of 'Perlite' 15µ glass beads helped to prevent grubs from sticking to the walls of the barrel, and assisted disruption of the epidermal tissues during subsequent homogenization. After the required period, the grubs were killed by washing in 1 ml. of acetone.

These washings were diluted 100-fold into sulphur-free toluene, and the solution was used for estimation of 'outside' insecticide. 'Outside' insecticide still remaining was removed with two further washings with 1 ml. of acetone. The animal was then homogenized in a fourth one-ml. portion of acetone. The homogenate was gently warmed, and evaporated down to about half its volume. One ml. of sulphur-free toluene was added and the mixture was shaken vigorously with 15 ml. of distilled water for one minute. The layers were separated by spinning at 1000 g for 15 min, and aliquots of the toluene layer were removed and diluted 100-fold for assay.

Recovery from grass grubs killed and assayed immediately after dosing (topically) or from dead grubs dosed after killing (by freezing) and kept in stoppered tubes at 25° for 48 hr., was 96-100%.

Each determination was made on five animals. The disappearance of insecticide from each of a set of animals, varied by up to 40% of the mean.

Experimental:-

1) Grass grubs were dosed topically with 1 or 10 μg. doses of δ-HCH and its disappearance was followed for 36 hr., assays being made for 'outside', 'inside' and 'total' δ-HCH (See Fig. VII,1).

2) Grass grubs, in batches of five, were injected with 10^{-7} mole of either phenol red or bromphenol blue in 10 μl. of insect saline, adjusted to pH 7.4. (This is intended, assuming the total volume of the grub to be about 0.1 ml. to give an internal concentration of 10^{-3}M.). They were dosed 15 minutes later with 10 μg. of δ-HCH by topical application. 'Inside', 'outside' and total δ-HCH were estimated over a period of 36 hr. (see Fig. VII.2.).

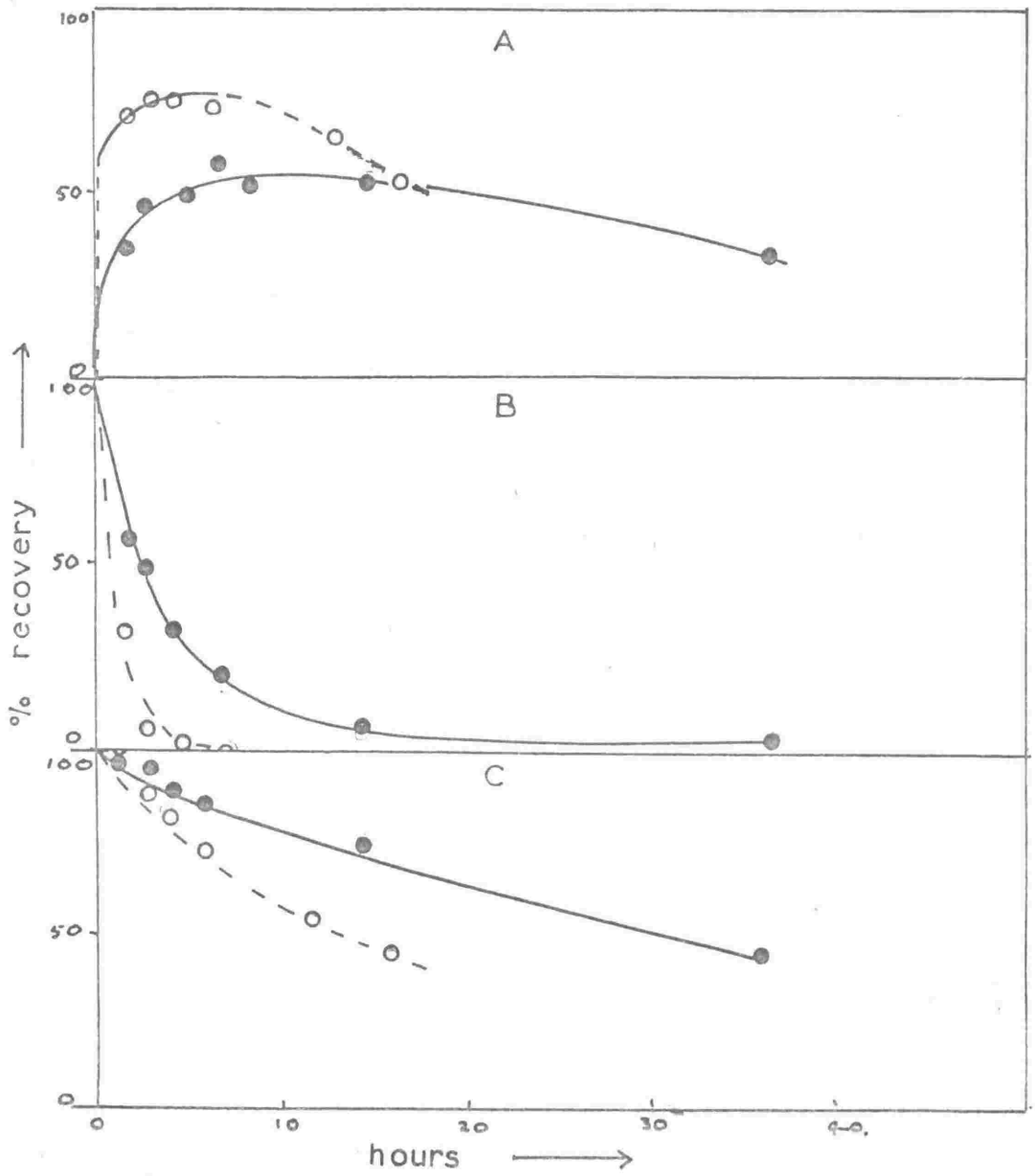


Fig.VII.1: Uptake of δ-HCH by C.zealandica topically dosed with 1 μg. -o-- and 10 μg —●—
A. 'Inside' HCH
B. 'Outside' HCH
C. Total HCH

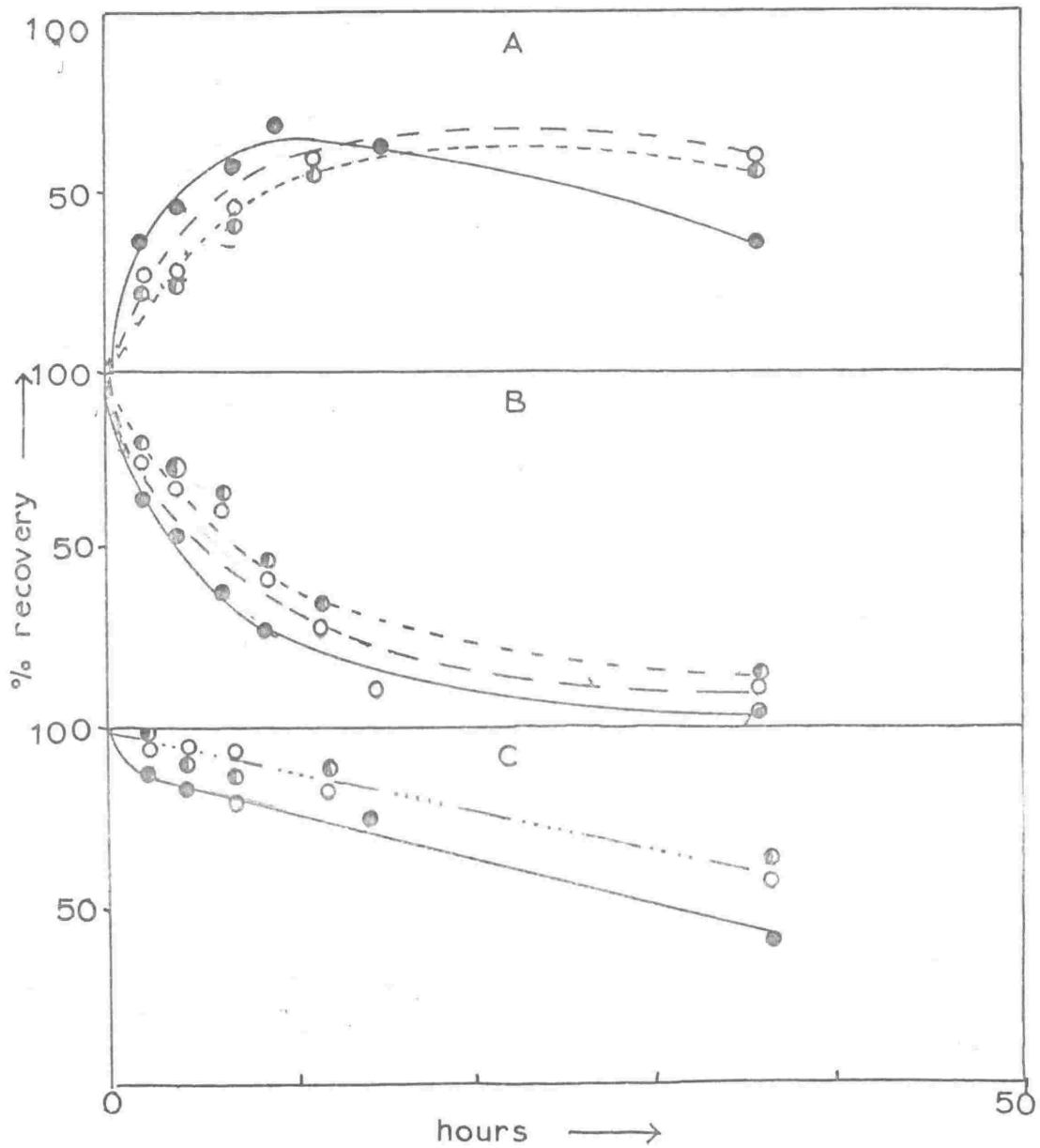


Fig.VII.2: Uptake of δ-HCH by C. zealandica topically dosed with 10 µg. HCH: ——●——, Untreated; ---O-----; previously injected with 10^{-7} moles of brom phenol blue; .....●..... previously injected with 10^{-7} Moles of phenol red.

A. 'Inside' HCH

B. 'Outside' HCH

C. Total HCH

3) Batches of grass grubs were dosed with 10^{-7} mole of TBPOEt by topical application or by injection, 10 minutes prior to the topical application of 10 μg. of δ-HCH. Disappearance of δ-HCH was followed over 36 hr. (see Fig. VII.3.).

4) 10 μg. of δ-PCCH was injected into untreated grubs, and into grubs which had been injected 10 min. previously with 10^{-7} mole of bromphenol blue. Disappearance of total PCCH was estimated over 16 hr. (see Fig. VII.4.).

5) 1 μg. of δ-HCH was topically applied both to untreated flies, and to flies topically dosed 10 minutes previously with 10^{-7} mole of TBPOEt (see Fig. VII.5.).

Results:-

1) It will be seen from Fig. VII.1 that in grass grubs, while the rate of δ-HCH uptake is greater for the 1 μg. dose than the 10 μg. dose, the rate of metabolism, in terms of % of the applied dose, is much the same. In both cases, the rate of disappearance of total δ-HCH is approximately constant.

With flies, the rate of uptake of 1 μg. of δ-HCH is lower than for the grubs (hardly surprising in view of the greater surface area of the latter) and, perhaps because of this, the total metabolic rate is also somewhat lower.

2) When the grubs had been previously dosed with phenol red or bromphenol blue by injection, there did appear to be a slight long-term inhibition. (In view of the massive doses of the dyes, the 30-40% inhibition can only be regarded as slight). As the rate of absorption of δ-HCH is also slowed down during the first few hours, this inhibition

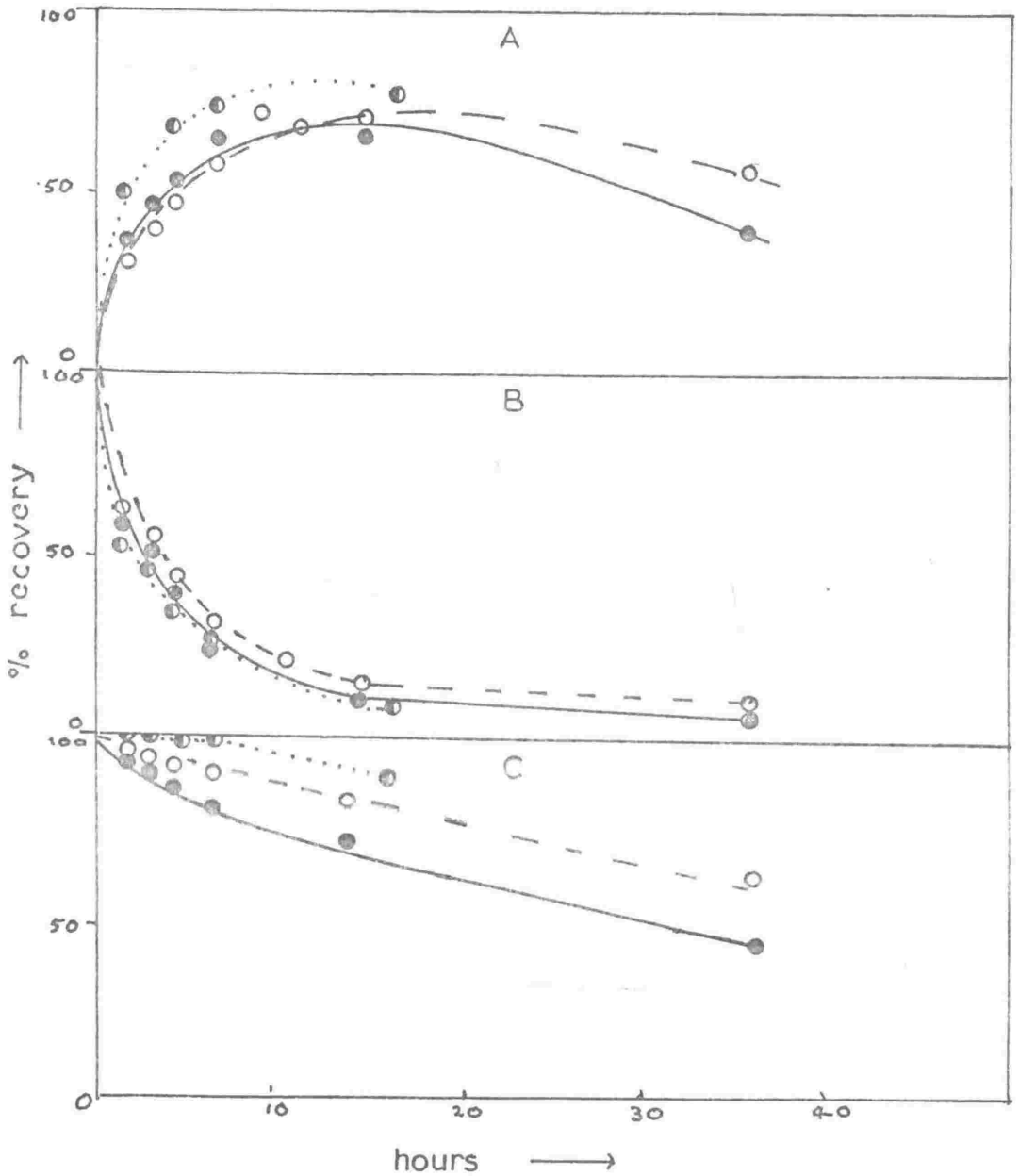


Fig.VII.3: Uptake of δ-HCH by C.zealandica dosed with 10 µg HCH:
—●— Untreated; – –○– – previously topically dosed with 10^{-7} Moles of TBPOE+; ···●··· previously injected with 10^{-7} Moles of TBPOE+.

A. 'Inside' HCH

B. 'Outside' HCH

C. Total HCH

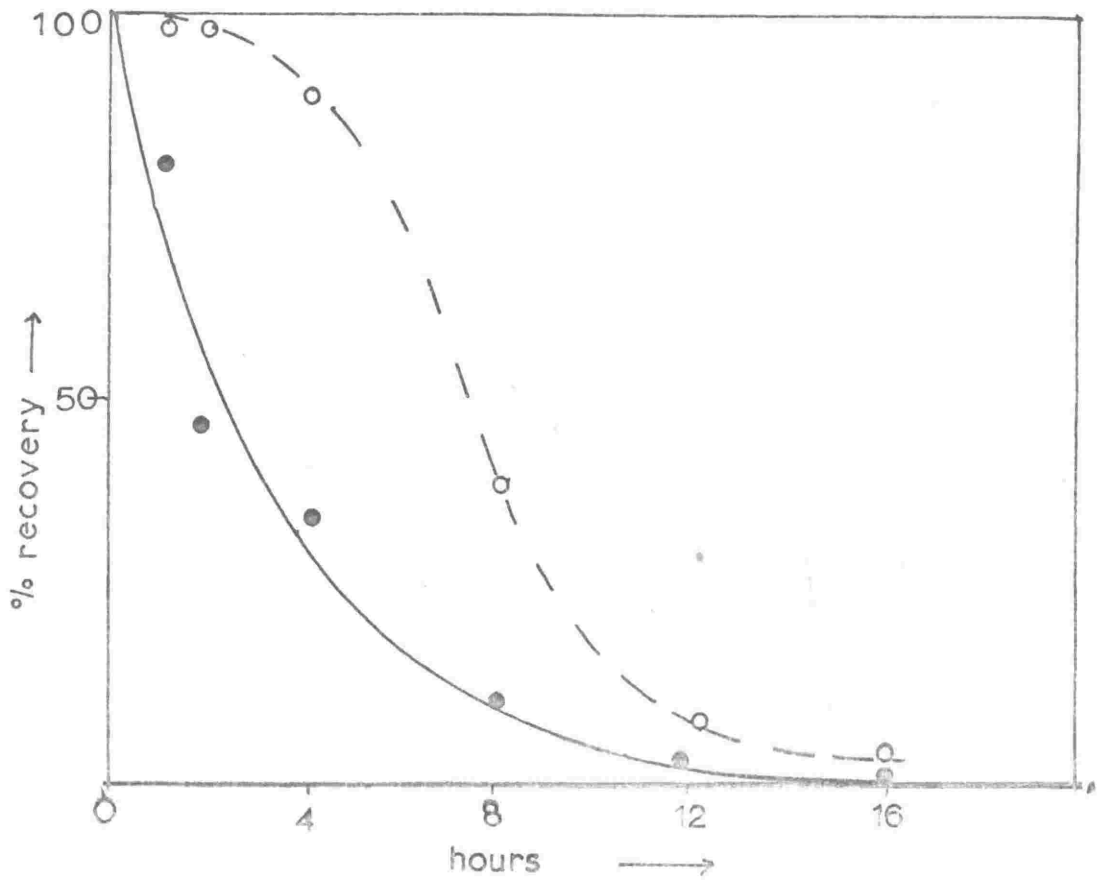


Fig.VII.4: Metabolism of δ-PCCH by C. zealandica injected with 10 μg of PCCH —●— ; and injected previously with 10^{-7} moles of bromophenol blue: - -o- - .

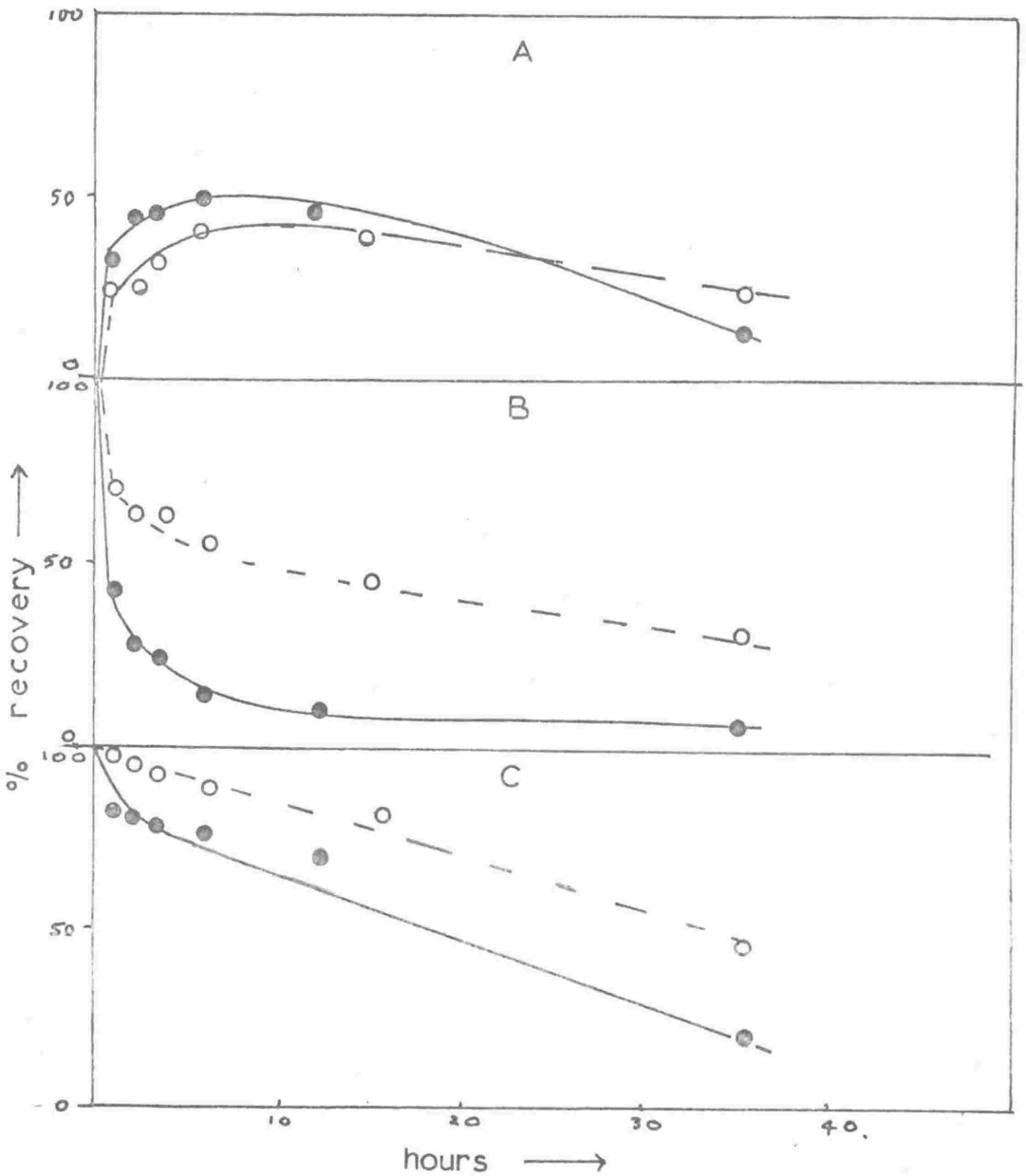


Fig.VII.5: Uptake of δ-HCH by M.domestica dosed topically with 1 μg. of HCH: —●— , and previously dosed topically with 10^{-7} moles of TBPOE$_t$. - -o- —

A. 'Inside' HCH

B. 'Outside' HCH

C. Total HCH

may only be apparent (Fig. VII.2.).

The phenol red is excreted within 4-6 hours, a process easily followed by watching the passage of the dye into the hind-gut. This also argues against the idea that phenol red exerts an inhibition at the enzymic level, for a period of 36 hours. TBPOEt does not appear to be excreted in this fashion, and it has not been determined whether phenol red is excreted as the free dye, or as a conjugate.

Whether injected, or applied topically, TBPOEt has a similar marginal, long-term effect on the disappearance of δ-HCH, and, when injected, appears to cause complete inhibition for about 8 hours. Of course, in view of the slow rate at which the δ-HCH is metabolized, even this effect is marginal (Fig. VII.3).

With flies, prior topical application of TBPOEt cuts down absorption of δ-HCH by about 50%, with a consequent apparent inhibition of the overall metabolic rate (Fig. VII.5).

δ-HCH has been used here because of its low toxicity, which enables comparatively large doses to be used and (as opposed to the faster-metabolized α-HCH) because it is capable of producing a stable PCCH, as does the γ-HCH. However, in none of the above experiments, inhibited or uninhibited, was the production of δ-PCCH detected.

3) δ-PCCH is metabolized at a much greater rate than is its parent compound. The effect of bromphenol blue is pronounced: over four hours, about 70% of the δ-PCCH was metabolized in the uninhibited case; in the inhibited case, only 10% (Fig. VII.4).

CHAPTER 8

Metabolism of [^{14}C] γ-HCH and γ- and δ-PCCH in Musca domestica and Costelytra zealandica.

Materials:-

γ-PCCH m.p. 34.5°.

δ-PCCH m.p. 68.5°.

^{14}C-labelled γ-HCH (Amersham Radiochemical Centre).

This was an old sample, discoloured and obviously impure. Isotope dilution showed it to be only 81% pure. When required, samples were purified by thin-layer chromatography as detailed below. The eluted material assayed by dilution analysis at 98% pure.

α-HCH (L. Light & Co.) m.p. 155-6°.

β-HCH (L. Light & Co.) m.p. 250°.

γ-HCH (B.D.H.) m.p. 111-112°.

δ-HCH (L. Light & Co.) m.p. 129-131°.

Phenoltetrabromphthalein disulphonate (BSP)

Bromphenol blue

Reduced glutathione

The six isomeric S-(dichlorophenyl)-L-cysteines were synthesized by the diazotization method of Parke and Williams (1951), and were obtained as the hydrochlorides. The melting points of the free bases and the hydrochlorides were taken. Free chloride was estimated mercurimetrically, using 0.01N mercuric nitrate with diphenylcarbazone as indicator. Free base was precipitated at pH 5 and filtered off to minimise errors due to its reaction with mercuric ion. $C_9H_{10}O_2NSCl_3$;

free chloride calculated = 11.6%.

Isomer	m.p. HCl	m.p. Free base	Chloride found
2,3-	187-8° (decomp.)	184-5° (decomp.)	11.9 ± 0.3%
2,4-	184-5° "	180° "	11.6 "
2,5-	180-1° "	179° "	11.9 "
2,6-	170-1° "	175° "	11.75 "
3,4-	185-6° "	177° "	11.8 "
3,5-	185° "	180-1° "	11.5 "

The pKa's of these compounds were determined approximately by titrating 0.1 N-NaOH against a 10 mM solution of the hydrochloride (for the pK_1) and 0.1 N-HCl against a 10 mM solution of the sodium salt for the pK_2. The titration was followed on a 'Radiometer' automatic titrator, and the mid-point of the titration curve (corrected for the dilution of the titrant) was taken to approximate to the true pK_a. The accuracy of this estimation is affected by supersaturation of the solution with respect to the neutral form of the amino acid, as its subsequent sudden precipitation during the titration causes some distortion of the titration curves. The values obtained in this manner are shown below

Isomer	pK_1	pK_2
2,3	2.4 ± 0.1	9.0 ± 0.1
2,4	2.2 "	8.9 "
2,5	2.25 "	8.65 "
2,6	2.0 "	8.70 "
3,4	1.9 "	8.85 "
3,5	2.0 "	8.80 "

Each isomer was examined for fluorescence at concentrations of 10,1 and 0.1 μg./ml. in 0.1 N-HCl and 0.1 N-NaOH. None of them showed any signs of fluorescence at 25°.

The synthesis of an S-(pentachlorocyclohexyl)glutathione has been attempted without success. The following methods have been tried:

1) A sodamide-type synthesis (Cohen and Smith, 1964), using reduced glutathione and α-, β-, γ- or δ-HCH. The product from the γ- and δ-isomers was identical in melting point, u.v. absorption, and chromatographic properties with the S-(2,4-dichlorophenyl)glutathione prepared by the diazotization method. No conjugate was isolated from the α- or β-isomers, but an aqueous solution of the reaction products showed an ultraviolet absorption maximum at 258-9 mμ. typical of the S-(dichlorophenyl)glutathione. Obviously, in this strongly basic medium dehydrochlorination can occur concurrently with substitution, giving the aromatic conjugate.

2) The Theodoropoulos (1951) reaction, similar in principle to the previous one except that the reaction medium is ethanol instead of liquid ammonia, was attempted with the α-, β-, γ-, and δ-HCH's and with L-cysteine. Conjugates were not formed in isolable amounts, but again, aqueous solutions of the reaction products had the characteristic absorption maxima at 258-9 mμ.

3) The silver mercaptide of L-cysteine was stirred with molten γ-HCH at 115°C. for one hour. There was no evidence of any reaction having taken place.

4) The attempted addition of GSH to δ-PCCH in a homogenous solution in 55% aqueous acetone (5 mM in each reagent) irradiated at 254 mμ. for

one hour yielded no detectable products. This is not unexpected, as absorption of ultra-violet light by the acetone would probably render the surface of the solution the only region where a reaction could take place.

5) The synthesis of bis-(pentachlorocyclohexyl)disulphide from γ-HCH and sodium disulphide (Dupire, 1947) appears to be a promising line. The disulphide could be reduced to the thiol and then, perhaps, added to -aminoacrylic acid, or condensed with serine, to give the substituted cysteine. It has not, however, yet proved possible to repeat Dupire's synthesis.

Separation Methods:-

1) Chromatography of the HCH's and PCCH's.

The method of Waldi (1965) was used. The chlorinated hydrocarbons were chromatographed on 0.5 mm thin-layer plates of Keiselgel G which had been made up in 0.4% aqueous disodium fluorescein and activated for 1 hour at 120°, using 80% cyclohexane/chloroform (8:2 v/v) as solvent. R_f values were as below:-

Compound	R_f
α-HCH	0.60
β-HCH	0.37
γ-HCH	0.50
δ-HCH	0.25
γ-PCCH	0.77
δ-PCCH	0.70

2) Separation of S-(2,4-dichlorophenyl)glutathione from the S-(dichlorophenyl)-L-cysteines:-

Four partition systems were used. Solvents were

A. Pyridine-water-butan-1-ol (1:1:1 by vol.).

B. Butan-1-ol-acetic acid-water (4:1:5 by vol.).

C. Butan-1-ol-Benzene-2 N-ammonia (1:1:2 by vol.).

D. Water-saturated o-cresol in an atmosphere containing ammonia (2 ml. 0.88 ammonia/100 ml. of solvent).

R_f values of the reference compounds in these solvents:-
(The substituted cysteines run together in all solvents).

Solvent	Compound	
	S-(2,4-dichlorophenyl)glutathione	S-(dichlorophenyl)-L-cysteine.
A	0.65 - 0.70	0.80 - 0.85
B	0.70 - 0.75	0.85 - 0.90
C	0	0.45 - 0.50
D	0.20	0.65 - 0.70

3) Separation of the isomeric S-(dichlorophenyl)-L-cysteines:-

This proved to be an extremely difficult task. A large number of partition systems were tried both on paper, and on various thin-layer media. None was found capable of separating these isomers.

Separation of the 2,6-isomer from the rest can be achieved by high voltage electrophoresis. A 'Shandon', water-cooled, high-voltage apparatus was used, and the techniques of Atfield and Morris (1961) were adopted. The best separation was achieved on Whatman No.31 paper, using a buffer of pH 1.85 (200 g. acetic acid and 20 g. formic acid made up to 1 litre with distilled water). The mean mobility of the 2,6-isomer was 2.3×10^{-3} cm/volt/hr. $\pm$ 10%, that of the other isomers was 1.9×10^{-3} cm/volt/hr. $\pm$ 10%. The operating voltage was 8,000 volts.

Because of the low mobility of these compounds at this pH, electrophoresis had to be run for periods of up to 1 hour. This introduces some difficulties into the high-voltage technique, as evaporation and electro-osmotic effects affect the regularity of the bands, and the reproducibility of the results. In extreme cases, complete drying of the paper in isolated areas, particularly near the edges of the cooling platens, caused arcing through the paper itself.

4) Use of Ion-exchange Papers:-

The best separation, but still imperfect, was obtained on Whatman's cellulose phosphate paper. Before use, the buffer to be used was run down the paper, the paper was dried, and strips were cut out for use. This procedure was necessary not only to pre-equilibrate the paper, but also to remove some substance in the paper which trails behind the solvent front and distorts the flow of buffer. A range of buffers was used between pH 2.0 (0.01 N-HCl) and pH 4.5 (0.02 N acetate buffer). There is some doubt as to the mechanism involved, as the R_f values obtained for these compounds remained constant over the whole pH range used. They were as below:-

Isomer	R_f.
2,3-	0.69
2,4-	0.75
2,5-	0.75
2,6-	0.87
3,4	0.80
3,5-	0.76

Again, there is a clear separation of the 2,6-isomer from the rest, which, in chromatograms of mixtures, tend to move as a single, elongated spot.

Measurement of Radioactivity:-

Radioactivity was measured in a Packard 'Tri-carb' 4000 scintillation counter. The scintillator used was 0.5% 2,5-diphenyloxazole plus 0.03% bis-(4-methyl-5-phenyloxazolyl)benzene (Packard scintillation grade) in toluene. Toluene-soluble radioactivity was estimated in a total volume of 20 ml. of scintillator. Quenching was estimated by use of the automatic external radium standard (A.E.S. system) with which the machine is equipped. In the absence of quenching, the efficiency of this system for ^{14}C radiation is 81%.

Radioactivity on chromatograms was measured by cutting the strip into 1 cm. sections, and counting each in 2 ml. of scintillator. The A.E.S. system cannot be used to estimate quenching when the activity is on paper and, as the count-rate was often too low to provide an accurate channels-ratio value within a reasonable time, quenching had not been compensated, and could not be taken into account when constructing histograms from the chromatographic data.

Experimental:-

Ten Musca domestica (DDT-resistant strain SP2AB42, kindly supplied by Dr. Spiller of the Fruit Research Division, D.S.I.R.) were each dosed by topical application of 0.009 µg. of [^{14}C] γ-HCH (1×10^{-9} Curie) in 1 µl. of acetone, and kept at 25° overnight in a stoppered flask. By morning, all were dead. The flask was washed out with two 5 ml. portions of distilled water, and the flies were homogenized in this water, using a ground-glass Potter homogenizer. The flask was then washed out with two

5 ml.-portions of toluene, and the homogenate was extracted with this toluene by vigorous stirring in a stoppered 20 ml. flask. The layers were separated by centrifuging at 2000g for 10 minutes, and the toluene layer removed. The recovered toluene-soluble activity amounted to 65% of the applied dose.

Protein and inorganic salts were removed from the toluene-extracted homogenate by addition of 4 volumes of absolute ethanol, and centrifuging the mixture. The supernatant was concentrated by evaporating under reduced pressure. Two further ethanol precipitations were performed in the course of reducing the volume of the supernatant to 0.5 ml. The concentrate was applied in a narrow 30 cm.-band to Whatman's No.31 paper, and chromatographed in solvent A. A two-cm. strip was cut off the chromatogram, and radioactivity was measured. The only significant band of radioactivity occurred at R_f 0.45 - 0.55. The band from R_f 0.40 - 0.80 was cut out, and the radioactivity was eluted from it. The eluate was rechromatographed on Whatman's No.1 paper in solvents A,B,C and D with the synthetic S-(2,4-dichlorophenyl)glutathione and the six S-(dichlorophenyl)-L-cysteines. Reference compounds were located with ninhydrin and the radioactivity was measured. Histograms are shown in Fig. VIII.1. It will be seen that the radioactivity co-chromatographed with the S-(2,4-dichlorophenyl)glutathione.

Water-soluble activity located in this band accounted for approximately 20% of the applied radioactivity, leaving 15% unaccounted for. It is possible that some γ-HCH was adsorbed on protein, and was not successfully extracted.

2) Three grass grubs were topically dosed with 0.45 µg. of γ-HCH

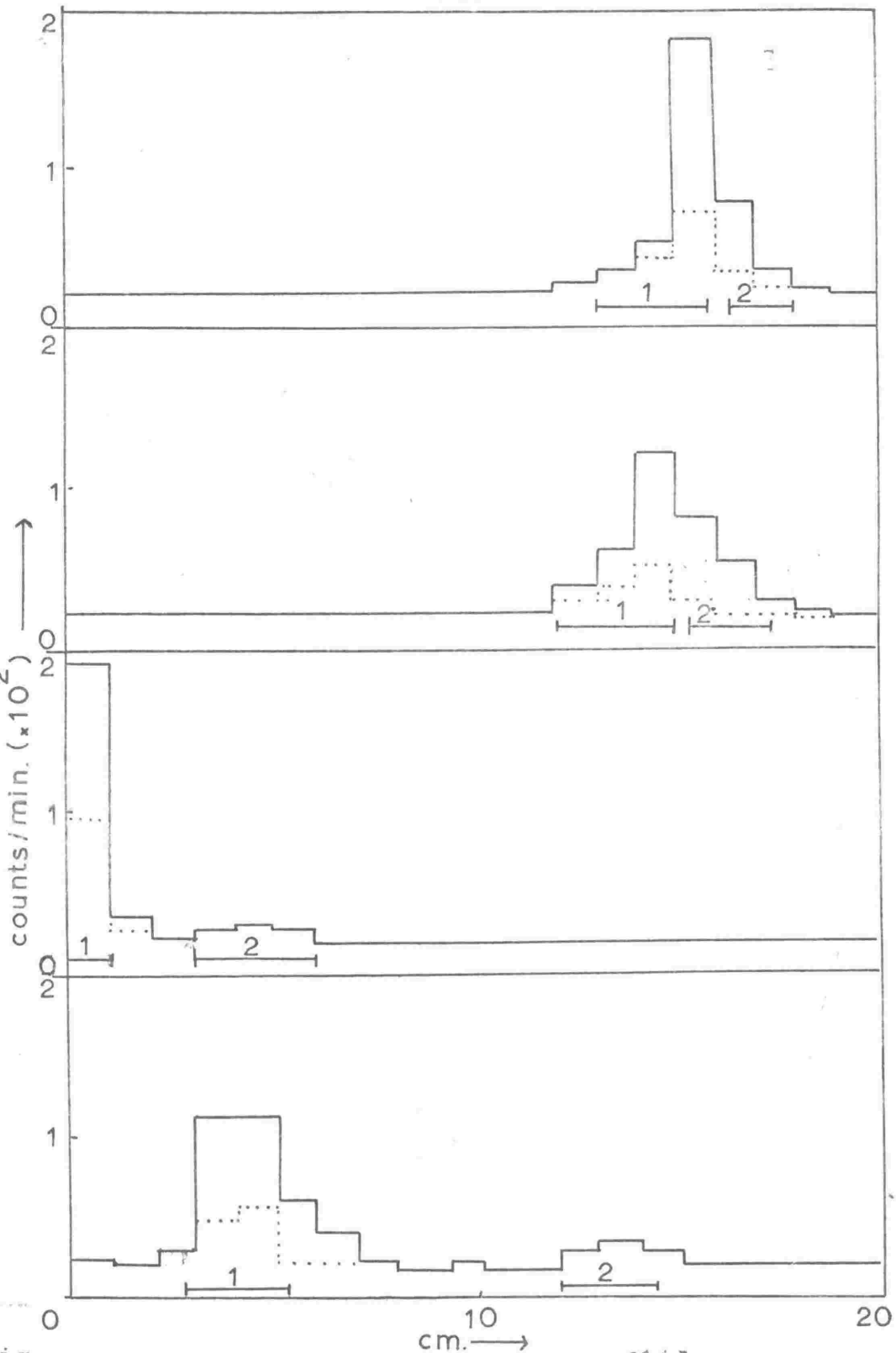


Fig. VIII.1: Distribution of metabolites of [^{14}C]γ-HCH produced by M.domestica *in vivo*: ---- and *in vitro* : ——— Numbered bars indicate the position of (1)- authentic S-(2,4-dichlorophenyl)glutathione and (2)- the S-(2,4-dichlorophenyl)-L-cysteines. Chromatograms were run in solvents A,B,C,&D.

(1×10^{-8} Curie), and were kept overnight at 25° in a stoppered flask. They were then treated exactly as were the flies. Recovered toluene-soluble activity accounted for some 75% of the applied dose. Water-soluble activity represented another 15%.

Histograms of the analytical chromatograms are shown in Fig. VIII.2. Again, the radioactivity co-chromatographed with the S-(2,4-dichlorophenyl)-glutathione.

3) In vitro studies:-

a) Grass-grub and fly homogenates:-

Homogenates of 100 flies (SP2AB 42) and of 20 grass grubs were prepared in 10 ml. of 0.1 M phosphate buffer, pH 7.4, and were centrifuged at 10,000g for 30 minutes. The supernatant was made 2 mM with respect to GSH and 0.05 mM to [^{14}C] γ-HCH (145 µg.containing 1×10^{-8} Curie of ^{14}C). Blanks were made up in an identical fashion, in 10 ml. of buffer. The systems were incubated for six hours at 37°.

At the end of this time, the incubation mixtures were extracted with 10 ml. of toluene, subjected to ethanol precipitations, concentrated, and prechromatographed on Whatman's No.31 paper in solvent A. Recovery of activity was as below:-

	Fly	Grass grub	Blank
Toluene-soluble	35%	48%	99%
Water-soluble	38%	40%	0
Missing	27%	12%	1%

Again, in both cases, the major part of the radioactivity co-chromatographed with authentic S-(2,4-dichlorophenyl)glutathione. A minor portion co-chromatographed with the S-(dichlorophenyl)-L-cysteines,

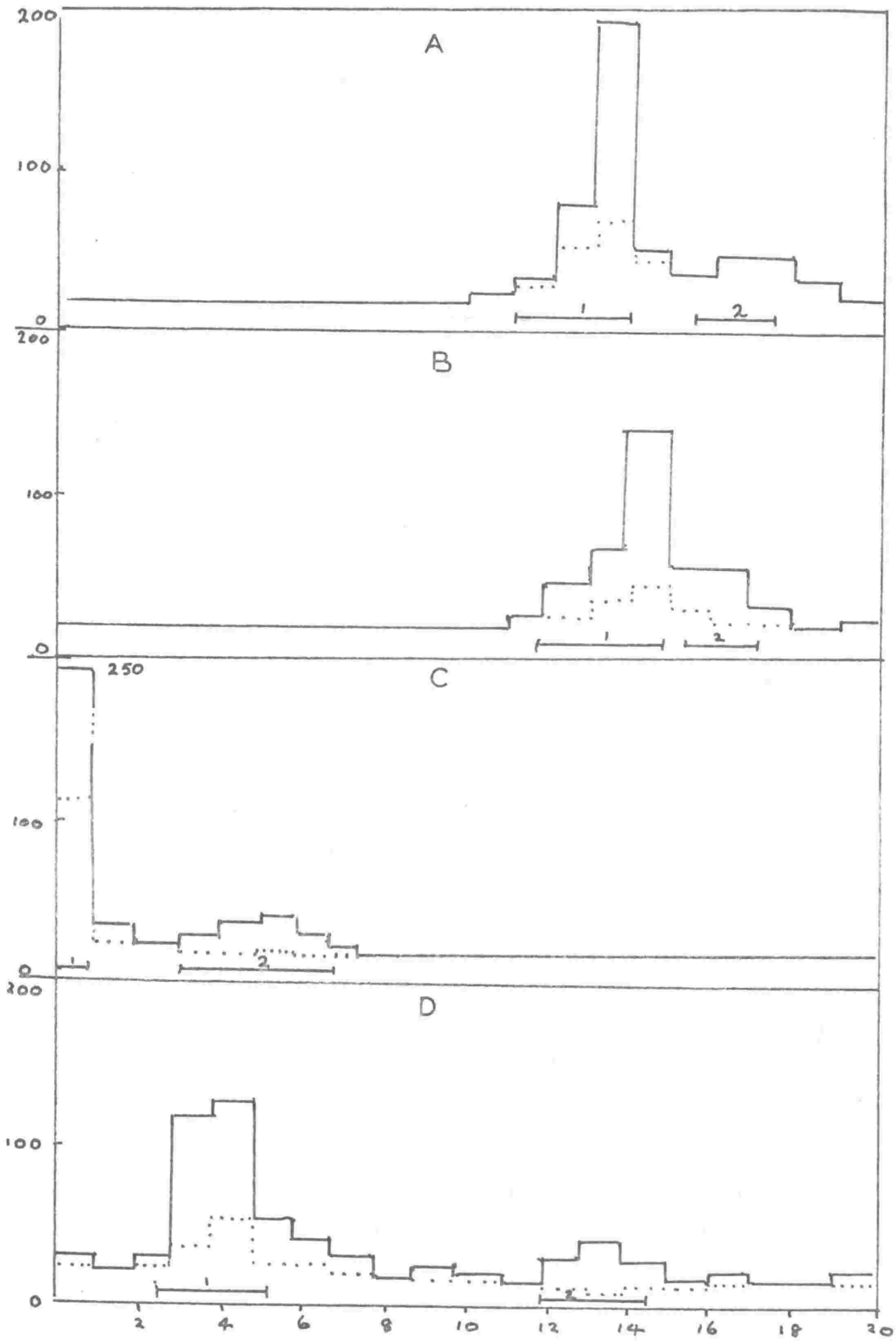


Fig. VIII.2: Distribution of metabolites of [^{14}C] γ-HCH produced by C.zealandica in vivo: ----- and in vitro ———. Numbered bars indicate the position of (1)-synthetic S-(2,4-dichlorophenyl)-glutathione and (2) the S-(dichlorophenyl)-L-cysteines. Chromatograms were run in solvents A, B, C & D.

though in the case of the flies, this was almost negligible. See Figs.VIII,1 and 2.

b) A series of fly homogenates was prepared as above. One was left as it was, while the second had irrelevant protein removed by precipitation at pH 5.0, after which the pH was returned to 7.4 by addition of micro-drops of 2 N-NaOH. Both series were made 2 mM with respect to GSH, and 0.05 mM with respect to [^{14}C] γ-HCH as previously described. The third series was set up, identical with the first except that [^{14}C] γ-HCH was added on a small strip of filter paper. Recovery of activity was as below.

System	Toluene-Soluble	Water-Soluble	Missing
1	38%	42%	20%
2	30%	55%	15%
3	20%	78%	2%

These results support the idea that the γ-HCH is being adsorbed on protein from solution, which affects both the rate of reaction, and the ease with which it is extracted. The effect is, of course, less marked where a large part of the inert protein had been removed. It is not obvious why extraction and the reaction rate are enhanced in the third system. It may be that, having to dissolve off the paper, the γ-HCH is metabolized before being trapped by inert protein.

In each case, the only significantly labelled metabolite co-chromatographed with the synthetic S-(2,4-dichlorophenyl)glutathione.

c) Fly and grass-grub homogenates were prepared as described in (3a), and were made 2 mM with respect to GSH, 0.05 mM with respect to γ-HCH

(1×10^{-8} Curie of ^{14}C), and 10^{-4} M with respect to B.S.P, and to bromphenol blue. The extents of inhibition were found to be (by estimation of water-soluble radioactivity) 93% and 96% for the fly homogenates, and 92% and 97% for the grass-grub systems.

Where the extraction procedure was aspreviously described, water-soluble radioactivity was found to co-chromatograph with the synthetic S-(2,4-dichlorophenyl)glutathione in all solvent systems. However, in one experiment, an attempt was made to extract bromphenol blue (which chromatographs very close to the synthetic S-(dichlorophenyl)-L-cysteines in solvent A (R_f 0.85 - 0.90)) by acidifying the extract and extracting with benzene. This was partially successful, but it was found, on preliminary chromatography, that the radioactivity did not run with either of the reference spots, but ran at an intermediate R_f. 0.70. On analytical chromatography in solvents A,B,C and D, it became clear that two labelled compounds were present (see Fig. VIII.3). The faster moving substance co-chromatographed with the synthetic S-(dichlorophenyl)-L-cysteines. The slower moving compound was eluted and refluxed with 5 N-HCl for four hours. On rechromatography, the radioactivity behaved identically with the S-(dichlorophenyl)-L-cysteines. On this evidence, it is suggested that this unknown compound may be S-(dichlorophenyl)cysteinylglycine, produced as an artifact by hydrolysis of the corresponding glutathione during the acid extraction.

d) One thousand M.domestica (SP2AB 42) were homogenized in 50 ml. of 0.1 M phosphate buffer. The homogenate was centrifuged at 10,000g for 30 minutes, and the supernatant was retained. It was made 2 mM with respect to GSH, and 290 µg (2.5×10^{-7} Curie) of [^{14}C]γ-HCH spotted on

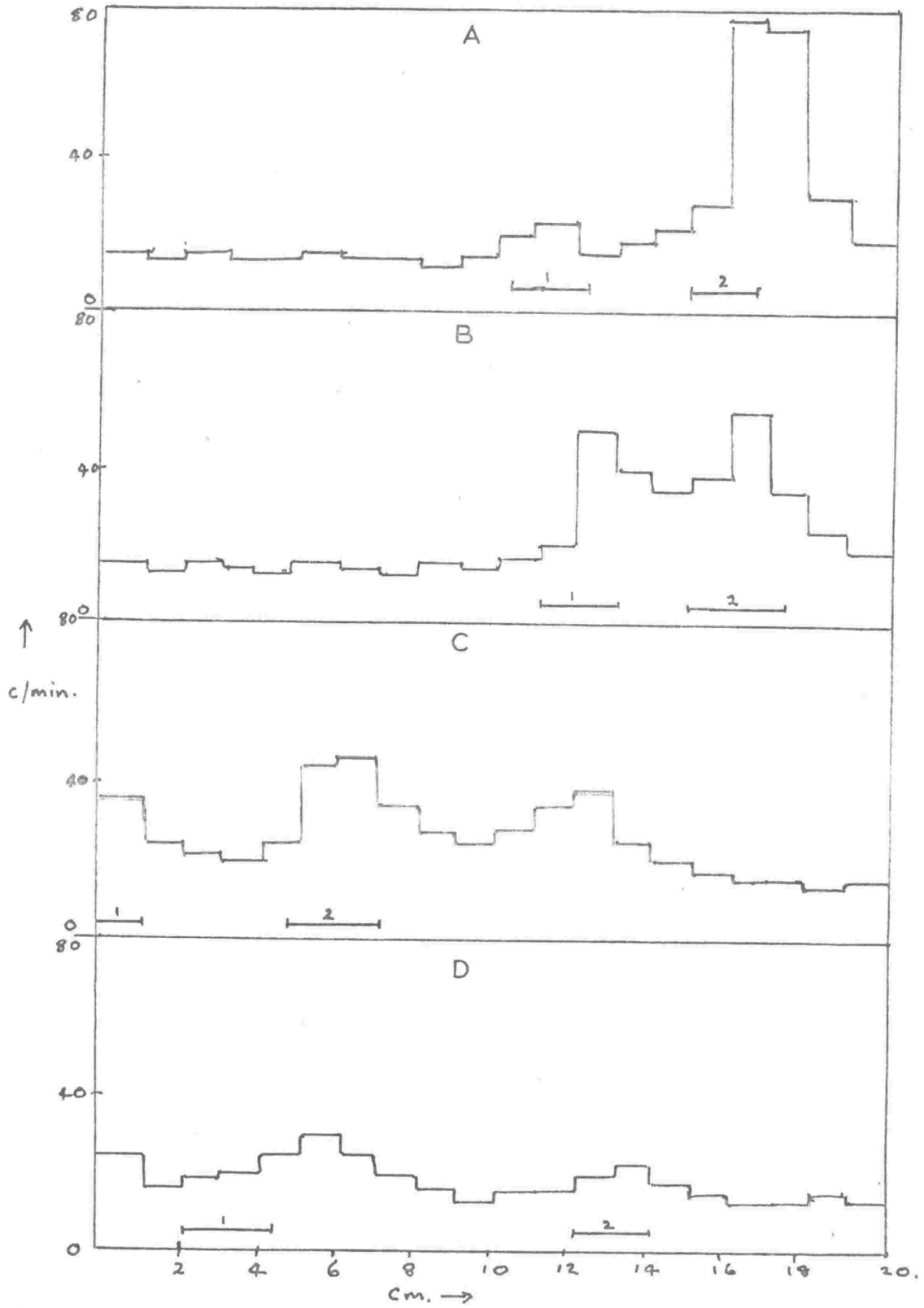


Fig.VIII.3: Distribution of radioactive compounds obtained by acid treatment of metabolites of [^{14}C] γ-HCH. Numbered bars indicate position of (1) synthetic S-(2,4-dichlorophenyl)-glutathione and (2) the S-(dichlorophenyl)-L-cysteines. Chromatograms were run in solvents A,B,C,&D.

filter paper was placed in the solution. The system was incubated for 3 hr. at 37°. After this time, the mixture was extracted with 50 ml. of toluene, and the aqueous layer was deproteinized and de-salted as before. Five per cent of the added radioactivity was recovered in the toluene layer, and 90% was located in the aqueous phase. Preliminary chromatography was carried out in solvent A, on Whatman's No.31 paper. A histogram of this is shown in Fig.VIII.4, which demonstrates that, even with this high-activity preparation, there is only one significantly labelled metabolite produced *in vitro*.

The radioactive material was eluted from the chromatogram, and half refluxed with 5 N-HCl for five hours. Eluted with the radioactivity is a yellow pigment. The hydrolysis mixture rapidly turned black, and a bulky, black precipitate was produced. It is assumed that this resulted from decomposition of the yellow pigment.

The hydrolysis mixture was filtered, evaporated to dryness several times to remove free HCl, and finally chromatographed on Whatman's No.31 paper in solvent A. A histogram is shown in Fig.VIII.5.

Some 30% of the total radioactivity ran at the same R_f as the synthetic *S*-(dichlorophenyl)-L-cysteines. This band reacted strongly with ninhydrin, and obviously contained a number of amino acids of similar R_f.

The major part of the activity ran ahead of the reference compounds, and appeared to be associated with yellow, ninhydrin-negative material. About 30% of the activity in this peak was extractable in benzene. It is thought that this may consist of thiophenols produced in a side-reaction during the hydrolysis. The nature of the benzene-insoluble activity is not known.

Fig.VIII.4: Distribution of metabolites of [^{14}C] γ -HCH produced *in vitro* by *M. domestica* on a preliminary chromatogram run on Whatmans No. 31 paper in solvent A.

Fig.VIII.5: Distribution of products of the acid hydrolysis of the principal γ -HCH metabolite, on a chromatogram run on Whatmans No. 31 in solvent A.

Fig.VIII.6: Electrophoretic behaviour of the material obtained by the acid hydrolysis of the major metabolite of [^{14}C]-γ -HCH.

Fig.VIII.7: Distribution of radioactivity on ion-exchange chromatogram of partly purified hydrolysis product of major metabolite of [^{14}C] γ -HCH.

Numbered bars represent (1) - the *S*-(2,4-dichlorophenyl)glutathione and (2) - the *S*-(dichlorophenyl)-L-cysteines. Where separation of the isomers occurs (Fig.VIII.7) the bars are numbered accordingly.

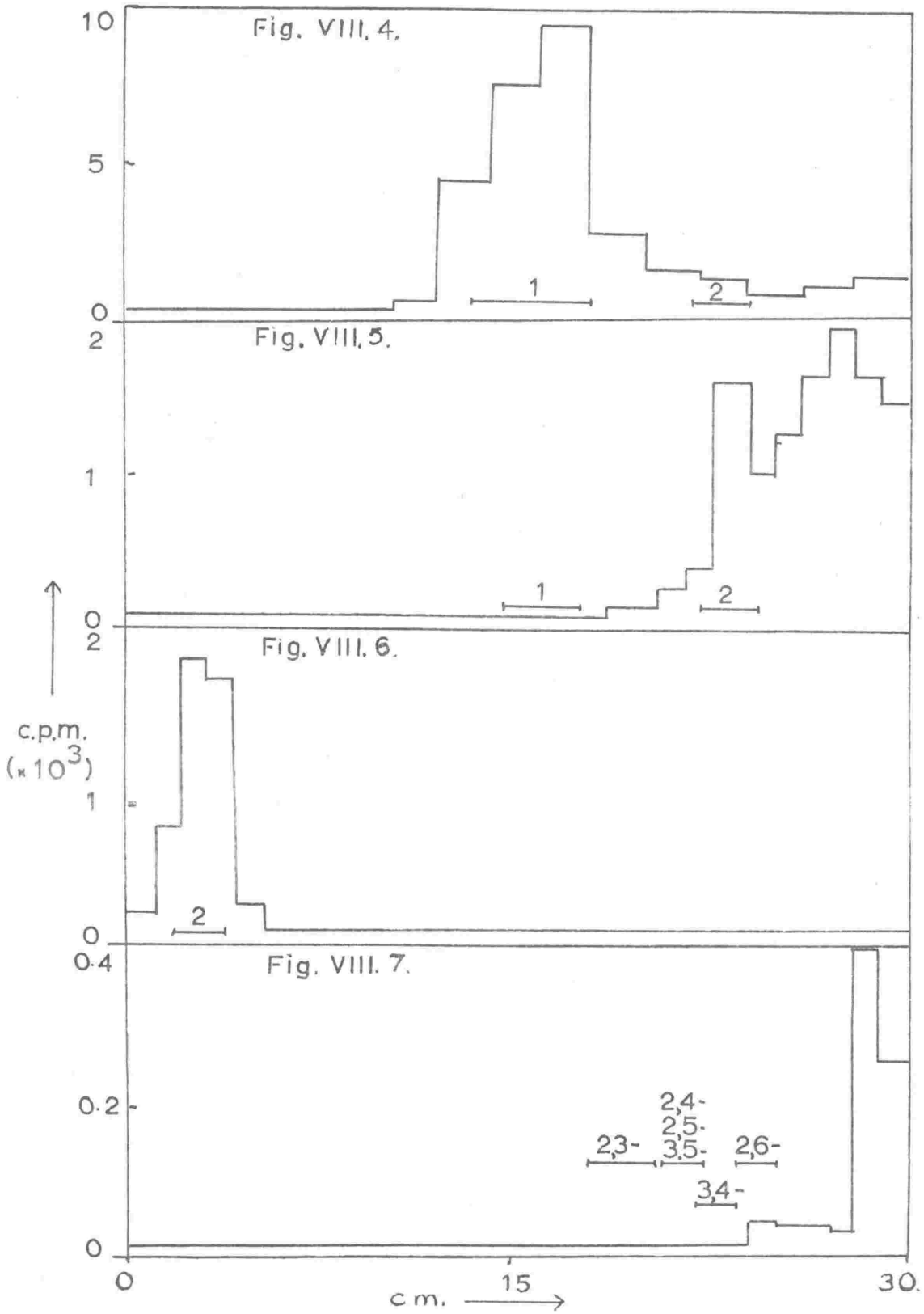


Fig. VIII. 4.
10
5
0
1
2
Fig. VIII. 5.
2
1
0
1
2
Fig. VIII. 6.
c.p.m.
(× 10³)
2
1
0
2
Fig. VIII. 7.
0·4
0·2
0
2,4-
2,5-
2,3-
3,5-
2,6-
3,4-
0
15
30.
cm.

The radioactivity running with the S-(dichlorophenyl)-L-cysteines was eluted, concentrated and electrophoresed in 1 N-acetic acid for 15 minutes at 8,000 volts. It was found that the peak of the radioactivity was identical in mobility with authentic S-(dichlorophenyl)-L-cysteines. Also found on the electrophorogram were six other ninhydrin-positive bands, containing no radioactivity, plus a yellow pigment which fluoresced yellow under u.v. light (see Fig. VIII,6).

The radioactivity was eluted from the electrophorogram, concentrated, and chromatographed with the synthetic S-(dichlorophenyl)-L-cysteines on Whatman's cellulose phosphate ion-exchange paper in 0.02 M-acetic acid (pH 3.35). It was found that the radioactivity ran close to the front, and did not coincide with any of the reference compounds (see Fig. VIII.7). The same results were obtained in buffers from pH 2.0 - 4.5, and in preparations which had not been cleaned up by electrophoresis.

e) A homogenate of 100 flies in 10 ml.of 0.1 M phosphate buffer, pH 7.4 was made as described in section (3a), and was made 2 mM with respect to GSH. To this was added a solution of γ-PCCH on egg-lipovitellin (prepared as described for DDT by Lipke and Kearns, 1959) to give a final concentration of 1 mM, and 145 µg of γ-HCH was added in 0.05 ml. of acetone. The γ-HCH contained 1×10^{-8} Curie of ^{14}C. The system was incubated for 1 hour at 37°. The blank was identical, except that the homogenate was replaced by buffer. After the incubation, each system was extracted into 2 x 10 ml. of toluene. This fraction was assayed for γ-PCCH by gas-liquid chromatography, and for radioactivity in the scintillation counter. 25% of the PCCH, and 80% of the radioactivity were found in this fraction. Recovery from the blank was 65% and 100%

for the PCCH and HCH, respectively. The toluene extracts were concentrated and aliquots were chromatographed on thin-layer plates as specified above. 0.5 cm. bands were removed and assayed for radioactivity (see Fig. VIII.8). Radioactivity moving at the same R_f as γ-PCCH accounted for 0.1% of the total radioactivity.

f) Conjugation of γ- and δ-PCCH:-

Homogenates of 100 flies and of 20 grass grubs were made in 10 ml. of 0.1 M phosphate buffer, pH 7.4, as detailed in section (3a) and were dialysed overnight against 0.1 M phosphate buffer at pH 7.4. Precipitated material was spun off at 10,000g for 15 minutes, and the supernatant was made 2 mM with respect to GSH, and 1.6×10^{-5} M with respect to γ-PCCH or δ-PCCH. Two types of blank were used: one containing enzyme and GSH but no PCCH, and the other containing GSH and PCCH, but with the enzyme replaced by buffer. These systems were incubated at 37° for six hours.

After this time, PCCH was extracted into 10 ml. of toluene by vigorous stirring for 10 minutes in a stoppered flask. Recovery from PCCH-containing blanks was 94% ± 3%, and from complete systems in all cases less than 5%.

The aqueous layers were concentrated as described in section (3a), and subjected to preliminary chromatography on Whatman's No.31 paper in solvent A. Strips were cut off the chromatograms and examined for u.v. quenching, and reactivity towards ninhydrin and Toennies and Kolb (1951) reagent. No significant difference was detected between enzyme-containing blanks and complete systems. The blanks still gave faint amino-acid spots - the dialysis was obviously not exhaustive.

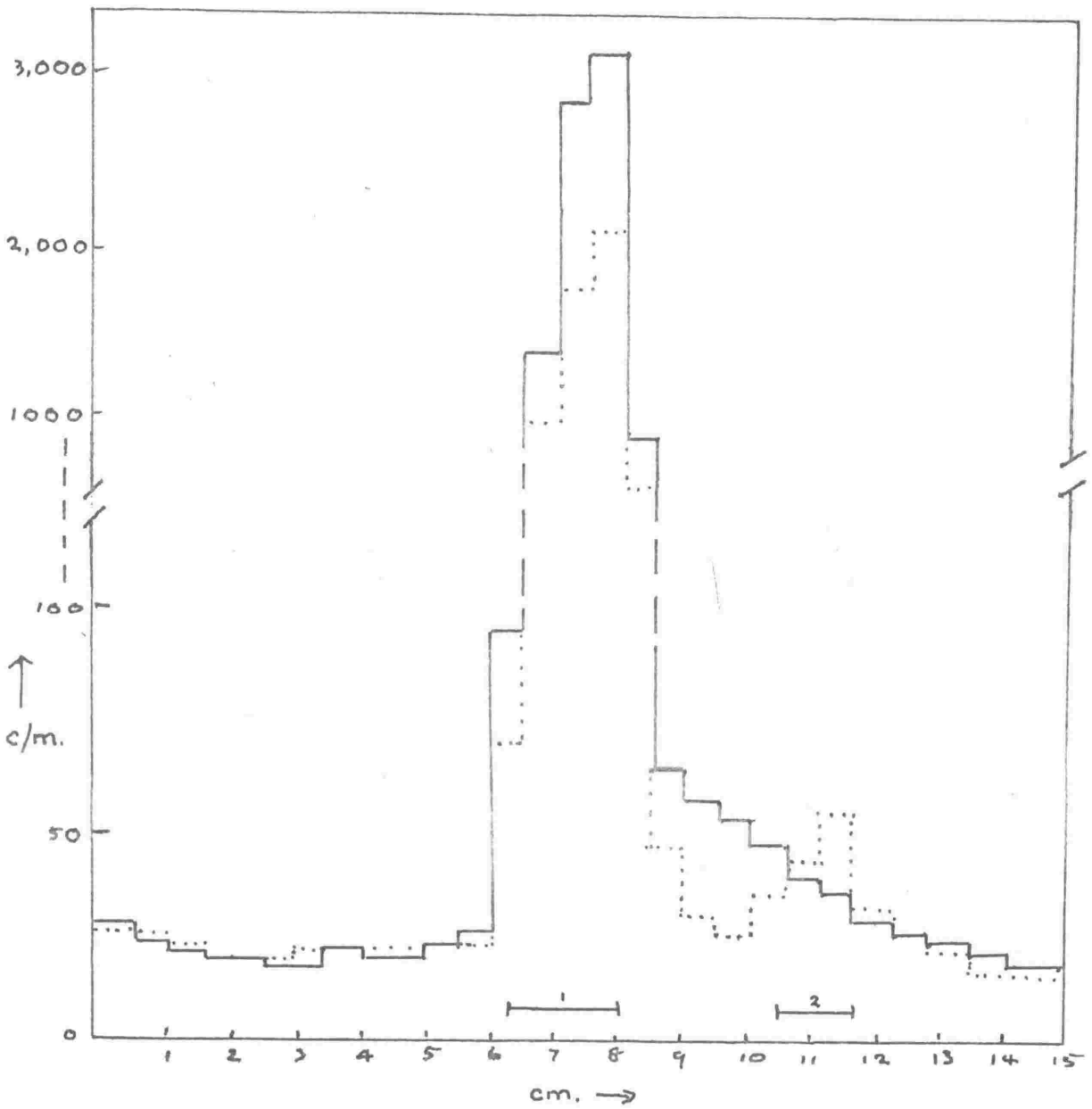


Fig.VIII.8: Labelling of diluant γ-PCCH by PCCH produced metabolically from [^{14}C] γ HCH.
Numbered bars indicate the R_f of (1) γ-HCH and (2) γ-PCCH.

_____ Blank containing γHCH only.

····· Reaction system (containing diluant γ-PCCH).

The band from R_f 0.40 - 0.75 was cut out of all the chromatograms, eluted, and the eluate from each was applied in equal quantities to four strips of Whatman's No.1 paper, which were then run in solvents A,B, C and D, respectively. In each case, one definite, but not very strong, ninhydrin spot was observed at R_f 0.70, 0.70, 0.05 and 0.25 in chromatograms run in solvents A,B,C and D, respectively, and this spot did not appear in either blank. This spot did not appear when chromatograms were treated with Toennies and Kolb (1951) reagent, which is less sensitive than ninhydrin, and it is probable that even if this material does react with this reagent, there was insufficient of it to produce a perceptible spot.

The identity of the R_f values of this material in the different solvents with those of S-(2,4-dichlorophenyl)glutathione, as well as the glutathione-dependence of the reaction, suggest strongly that this compound is a glutathione conjugate.

CHAPTER 9

PCCH Metabolism and GSH-S-Aryltransferase

Materials:-

δ-PCCH m.p. 68.5°.

δ-HCH (L. Light & Co.) m.p. 129-130°.

Reduced glutathione

Bromphenol blue

Enzyme Preparations:-

Homogenates of 100 flies (SP2AB 43) and of 20 grass grubs in 10 ml. of 0.1 M phosphate buffer, pH 7.4, were spun at 10,000g for 30 minutes, and portions of the supernatant were taken for the experiments.

Acetone powders were prepared from one-day-old DDT-resistant flies (SP2AB 43) and from DDT-susceptible grass grubs, as previously described. The yield from 49.5 g. of flies (3000) was 3.85 g. of a light pink powder, and from 38.3 g. (309) of grass grubs the yield was 1.65 g. of a dark brown powder. Solutions of 128 mg. of fly acetone powder (equivalent to 100 flies) or of 106 mg. of grass-grub acetone powder (equivalent to 20 grass grubs) were made up in 10 ml. of buffer, centrifuged at 10,000g for 10 minutes, and portions of the supernatant used in the assay, as required.

Methods:-

Aryltransferase activity was assayed spectrophotometrically at pH 7.4, as described previously. The GSH concentration was 2 mM, and that of the CDNB, 0.1 mM.

δ-PCCH-metabolising activity was assayed as follows:-

Systems, of a final volume of 5 ml., were set up, containing GSH 2 mM, δ-PCCH 1.6×10^{-6} M (added in 0.05 ml. of acetone), and enzyme solution in amount sufficient to maintain a linear reaction curve for 5 min.; the buffer was 0.1 M phosphate, pH 7.4. The tubes were incubated at 37°, and 0.05 ml. aliquots were removed at intervals, and shaken vigorously with 1 ml. of ice-cold toluene for 2 minutes. Recovery from blanks containing no enzyme was 100% $\pm$ 3%.

It was hoped that this technique could be used for kinetic studies of the enzyme involved in δ-PCCH solubilization. The method is, however, not sufficiently accurate. At best, the experimental error in a single assay is $\pm$ 2%, which becomes disproportionately great when initial reaction rates are to be determined. To maintain a linear reaction curve, less than 25% of the substrate may be allowed to be metabolized. Thus, when determining the amount of substrate that has disappeared, the total error may amount to 4%, which, when related to only 25% of the initial substrate concentration, becomes 16-20% of the actual reaction velocity. An example is given. Five identical systems were set up, each containing 4.8 mg. of fly acetone powder, and were incubated for 15 minutes. δ-PCCH recovery was 86%, 83%, 84%, 88%, and 87%; mean = 85.6 $\pm$ 2.5%. The mean amount of δ-PCCH metabolized in this period is therefore 14.4 $\pm$ 2.5% - an actual percentage error of 17% of the initial velocity.

It is possible that, working with a number of replicates, one could get a reasonably accurate mean. For prolonged kinetic studies, this would be very tedious.

Acetone powders from both flies and grass grubs exhibited no

perceptible activity towards δ-HCH, even when 200 mg of powder was used.

DDT dehydrochlorinase activity was kindly assayed by Mr. T. Cundell using the following technique. Enzyme solution, GSH (2 mM) and DDT (2×10^{-5}M) added in 0.02 ml. of dimethylsulphoxide were added to 0.1 M phosphate buffer, pH 7.4, to give a total volume of 2 ml. The mixture was incubated with gentle shaking, in ground glass-stoppered test-tubes for 30 minutes at 37°. At the end of this time, the mixture was extracted by vigorously shaking with 2 ml. of toluene. DDE produced was assayed by gas-liquid chromatography, as described, with a column temperature of 170°.

Gel Filtration:- A column of 4.5 x 40 cm. of Sephadex G-100 was used, being set up in the cold room at 4°C. Fractions of 200 drops (12.9 ml.) were collected with an L.K.B. 'Ultrorac' fraction-collector. U.V. absorption at 280 mμ was monitored continuously with an L.K.B. 'Uvicord' recording spectrophotometer. A constant hydrostatic head of 60 cm. was used, which gave a flow rate of 54 ml./hour.

The experiments recorded here are primarily preparative, the active fractions being freeze-dried. The use of tap-water avoids the possibility of denaturation due to high salt concentration when freeze-drying, or when redissolving the product. Tap water was used in preference to distilled water, in the hope that ions present would minimise adsorption effects due to the presence of carboxyl groups on the Sephadex gels.

The gel bed was topped with filter paper. Before application of the solution to be filtered, the water level was drained to just below the filter-paper. After application, the solution was allowed to drain to the same point before the elution reservoir was connected.

The bed-volume of the column was 640 ± 10 ml. and the void volume 170 ± 5 ml. The inner volume, by calculation, was 470 ± 15 ml. In fact, low molecular weight pigments were being eluted in up to 580 ml. of eluant. The rather high void volume, and the high apparent inner volume suggests that a considerable amount of adsorption did occur in this system.

Experimental:-

a) Fly and grass-grub homogenates, and solutions containing an equivalent weight of fly and grass-grub acetone powders, were assayed for aryltransferase and δ-PCCH-metabolizing activity. For the fly preparations, aryltransferase activity was 0.12 m-mole/fly/min. and 0.05 m-mole/fly equivalent weight of acetone powder/min. for the homogenates and acetone powder solutions respectively, while δ-PCCH activities were 1×10^{-5} m-mole/fly/min. and 0.58×10^{-5} m-mole/fly equiv./min. For Costelytra, aryltransferase activities were 7.8 m-mole/grub/min. and 3.4 m-mole/grub equiv./min., and δ-PCCH activities 0.08×10^{-5} m-mole/grub/min. and 0.05×10^{-5} m-mole/grub equiv./min.

Ratios of aryltransferase activities in homogenate to acetone powders used were 2,35:1 and 2.29:1 for fly and grass grub respectively, while the corresponding ratios for δ-PCCH activity were 1.73:1 and 1.60:1.

An experiment was made with two fly acetone powder systems, one uninhibited, and the other made up to 5×10^{-6}M in bromphenol blue. A mean inhibition of 36% was observed.

Time courses for the reaction in fly homogenates and acetone powder solutions are shown in Fig. IX.1.

The inhibited (B) run was conducted some six weeks after the uninhibited runs shown in (A). It will be noticed that, in this period,

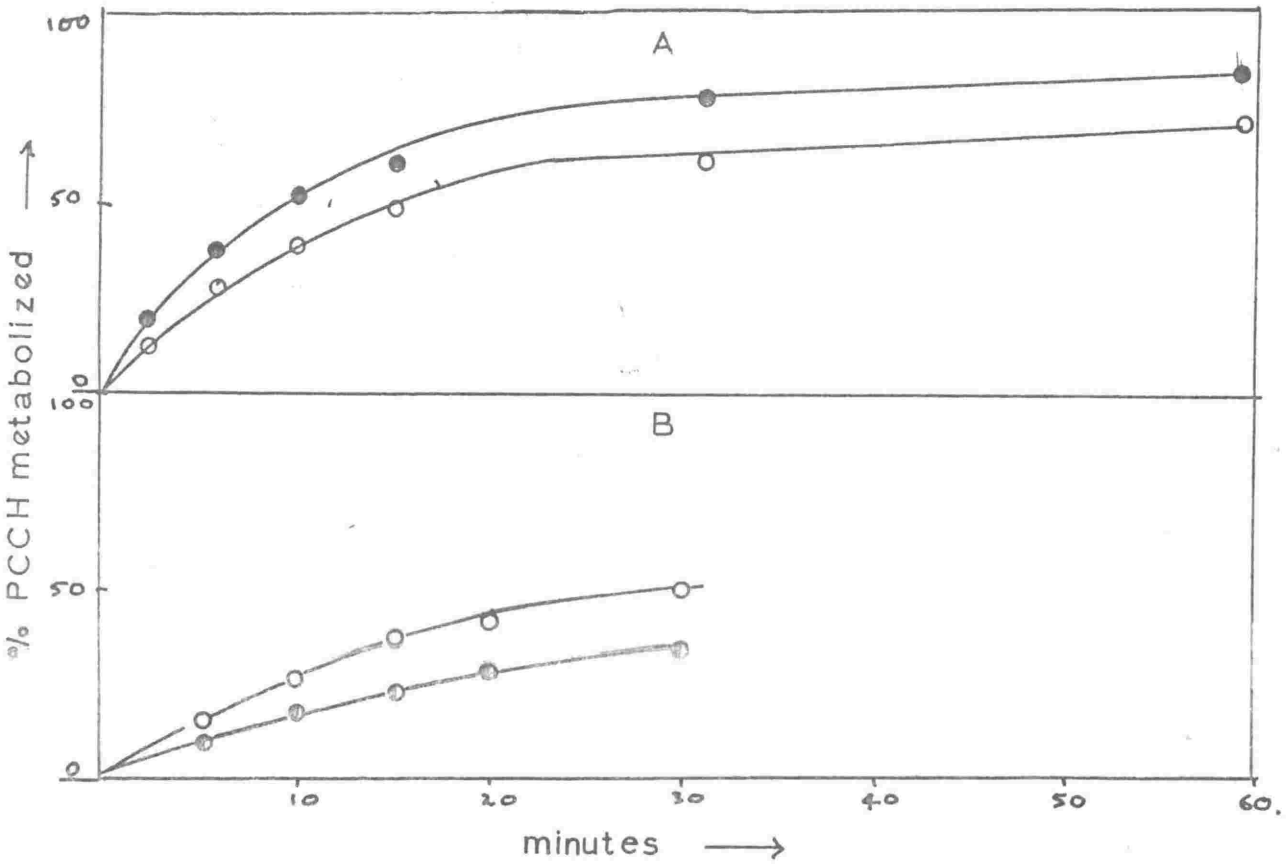


Fig.IX.1: In vitro metabolism of δ-PCCH by : —●— M.domestica homogenate; —○— M.domestica acetone powder solution; and —◐— acetone powder solutions 5×10^{-6}M in bromophenol blue.

Results in (A) were obtained 2.12.66.
Results in (B) were obtained 20.1.67.

the acetone powder used for both runs had lost a considerable amount of its activity.

b)(i) Gel filtration was carried out on a solution of 1 g. of the M.domestica acetone powder in 10 ml. of 0.1 M phosphate buffer, pH 7.4. The solution was centrifuged at 10,000g for 10 minutes before application. The effluent was examined for aryltransferase, DDT dehydrochlorinase and δ-PCCH-metabolizing activity. The results are shown graphically in Fig. IX.2.

It will be seen that all three activities ran close together. The aryltransferase moved in a comparatively narrow band with a peak at fraction 25. The DDTase activity appeared to run in two bands (a separation obtained in each of two experiments), the first with a broad peak at fractions 25-26, and the second with a peak at fraction 28. The δ-PCCH activity reached a peak at fraction 26, with a shoulder at fractions 27-28.

b)(ii) The 10,000g supernatant of a solution of 500 mg. of C.zealandica acetone powder in 10 ml. of 0.1 M phosphate buffer, pH 7.4, was subjected to gel-filtration. This acetone powder, assayed one month prior to the experiment, contained all three enzyme activities. When assayed immediately before the experiment, the preparation exhibited only aryltransferase activity. It will be seen from Fig. IX.3 that this activity runs with a peak at fraction 21, significantly faster than that of M.domestica preparations. This does not, however, necessarily imply a great difference between the two enzymes. In this system, where adsorption appears to play a significant part in determining the elution pattern, it is possible that differences in irrelevant components of the

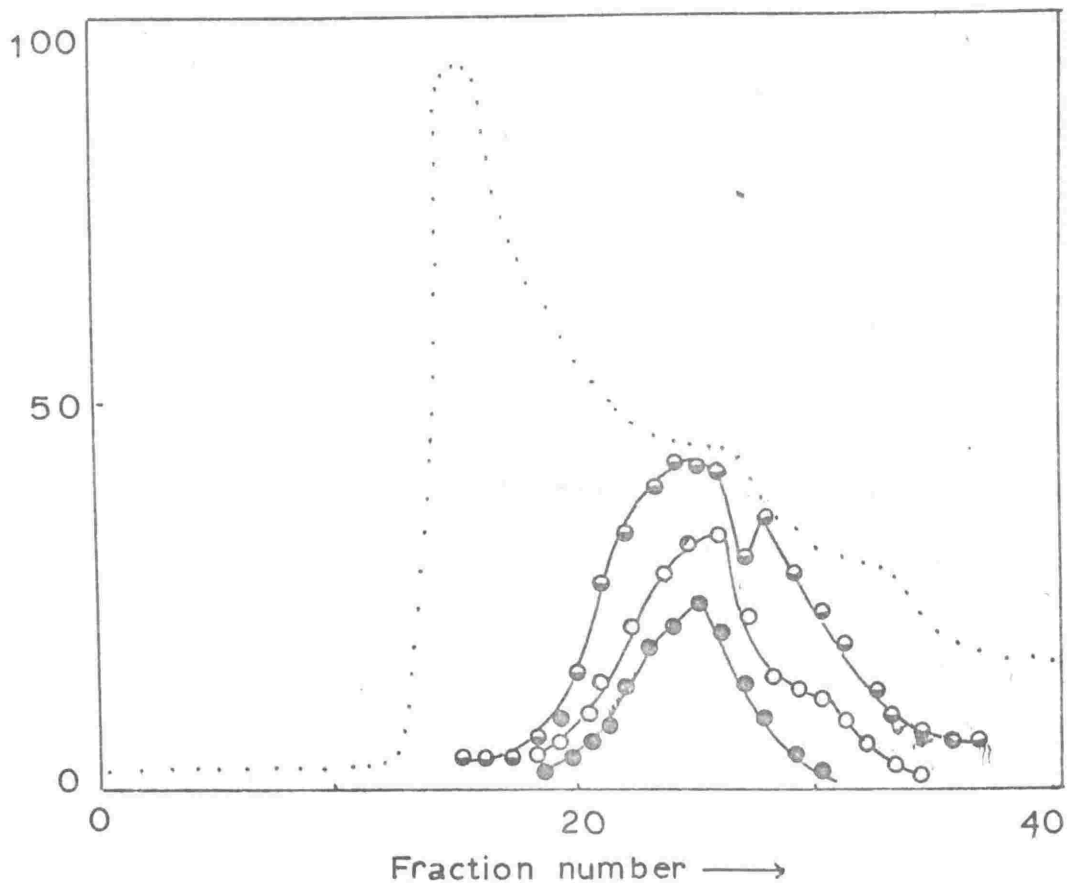


Fig. IX.2: Elution of M. domestica aryltransferase, DDT dehydrochlorinase and δ-PCCH-metabolizing activity from Sephadex G-100.

.....% Absorption at 280 mμ.

—●— Aryltransferase activity - unit = 10^{-5} moles/min./ml. of eluate.

—○— PCCH activity - unit = 10^{-9} moles/min./ml. eluate.

—○— DDT dehydrochlorinase activity - unit = 10^{-10} moles/hour/ml. eluate.

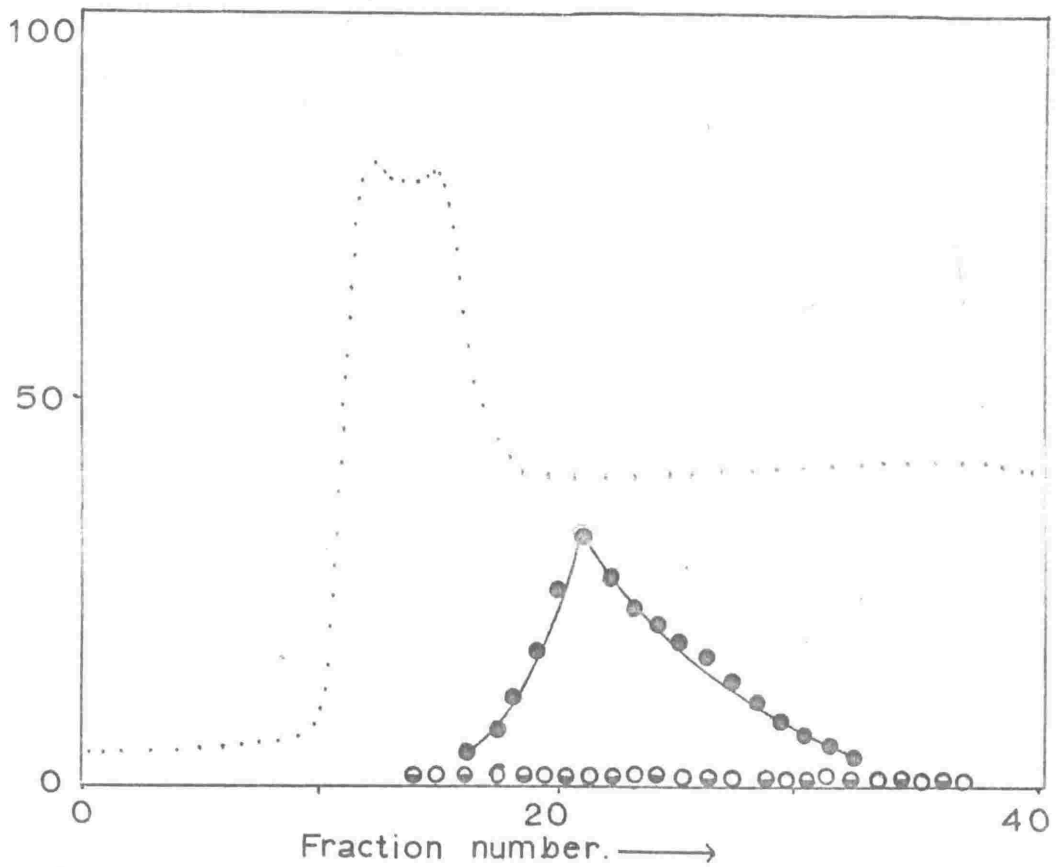


Fig.IX.3: Elution of C. zealandica aryltransferase from Sephadex G-100

.....% absorption at 280 mμ.

———●——— Aryltransferase activity - unit = 10^{-5} moles/min./ml. of eluate.

———o——— PCCH activity - unit = 10^{-9} moles/min./ml. eluate.

———⊖——— DDT dehydrochlorinase activity - unit = 10^{-10} moles/hour/ml. eluate.

two systems may determine the elution behaviour of the enzymes in which we are interested. For instance, the grass grub solution appears to have a much higher protein concentration than the fly preparation. It might thus be predicted that adsorption (assuming a fixed number of adsorption sites) will retard any one component in the grass-grub preparation to a lesser extent than the corresponding component in the fly preparation.

Addendum to Experimental Work

The Chromatography of Various Glutathione Conjugates

Materials:-

1. S-(2,4-Dichlorophenyl)glutathione m.p. 215° (decomp.)
2. S-(2,4-Dinitrophenyl)glutathione
3. S-(p-Chlorobenzyl)glutathione m.p. 202° (decomp.)
4. S-(p-Nitrobenzyl)glutathione
5. S-(Phenylethyl)glutathione m.p. 211° (decomp.)
6. S-(2-Methylnaphthyl)glutathione

All these, with the exception of the S-(2,4-dichlorophenyl)glutathione were kindly lent by Professor J.N. Smith.

Compounds 2,4, and 6 were crude preparations containing chromatographically detectable impurities.

Experimental:- The above compounds were chromatographed on Whatman's No.4 paper, in solvents A,B,C and D. The R_f values obtained are recorded below.

Compound	Solvent			
	A	B	C	D
1	0.65	0.59	0.0	0.18
2	0.59	0.38	0.0	0.20
3	0.63	0.56	0.0	0.16
4	0.60	0.42	0.0	0.20
5	0.59	0.52	0.0	0.16
6	0.63	0.58	0.0	0.20

It will be seen that in only one of the above solvents does any

significant separation of these conjugates occur and that even then only the nitrated compounds separate. In conjunction with the results of Booth et al. (1961) (see discussion), these results point to the conclusion that, if a number of GSH conjugates are formed in the metabolism of the HCH's, it is probable that even if they contain differing numbers of chlorine atoms, of differing orientation, chromatographic separation is unlikely to be achieved.

CHAPTER 10

Discussion

The significance of the kinetic behaviour of the aryltransferase has been discussed in Chapter 5. For lack of methods of sufficient accuracy, it has not been possible to compare the kinetic characteristics of the solubilization of the HCH's and PCCH's with those of the aryltransferase activity. The metabolic work falls into several sections and, while the findings in each section have relevance to those in the others, the results will be discussed, for convenience, section by section.

1) Metabolism of the PCCH's in vertebrates:-

This work has not yielded satisfactory results. In the case of both mice and guinea pigs no metabolites have been positively identified

fluctuations in urinary excretion (Fig. VI.1) suggest that biliary excretion and intestinal resorption of metabolites occurs.

If the vertebrate metabolism of the HCH's proceeds principally through oxidation of trichlorobenzene to the phenols, as proposed by Grover and Sims (1965) (see Fig. X.2), metabolites which might be expected to be present in the bile include the S-(dichlorophenyl)-glutathiones, the corresponding cysteinylglycine and cysteine conjugates, the mercapturic acids and the glucuronides, as well as possibly the ethereal sulphates, of the trichlorophenols. If, however, a different pathway to that proposed by these authors is followed, possibly involving GSH conjugation of an epoxytrichlorobenzene, one might expect, in addition to the above compounds, various acid-labile pre-mercapturic acids. Such compounds have been thoroughly studied in the metabolism of various aromatic hydrocarbons, particularly naphthalene, phenanthrene (Booth, Boyland and Sims, 1960, 1961) and benzanthracene (Boyland and Sims, 1964). The appearance of an acid-labile compound yielding chlorobenzene (Bridges, 1959) is suggestive of a premercapturic acid being involved in the metabolism of γ-HCH in flies. Clarification of this point may well help to define in what manner the vertebrate metabolic pathway differs from that in insects.

Another useful experiment might be to examine the *in vitro* metabolism of the HCH's and PCCH's in microsomal oxidation systems. Ishida and Dahm (1965a) report that γ- and δ-PCCH are metabolized in such a system, but have not identified the metabolites. Koransky, Portig, Vohland and Klempau (1963) report that the *in vivo* metabolism of the α- and γ-HCH's is markedly stimulated by microsomal activators such as phenobarbitone, and conversely, that α-HCH stimulates

Fig. X.1

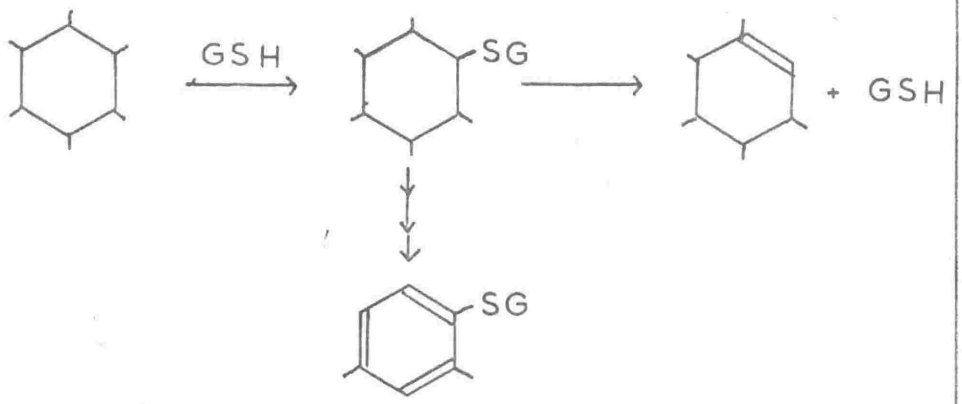


Fig. X.2

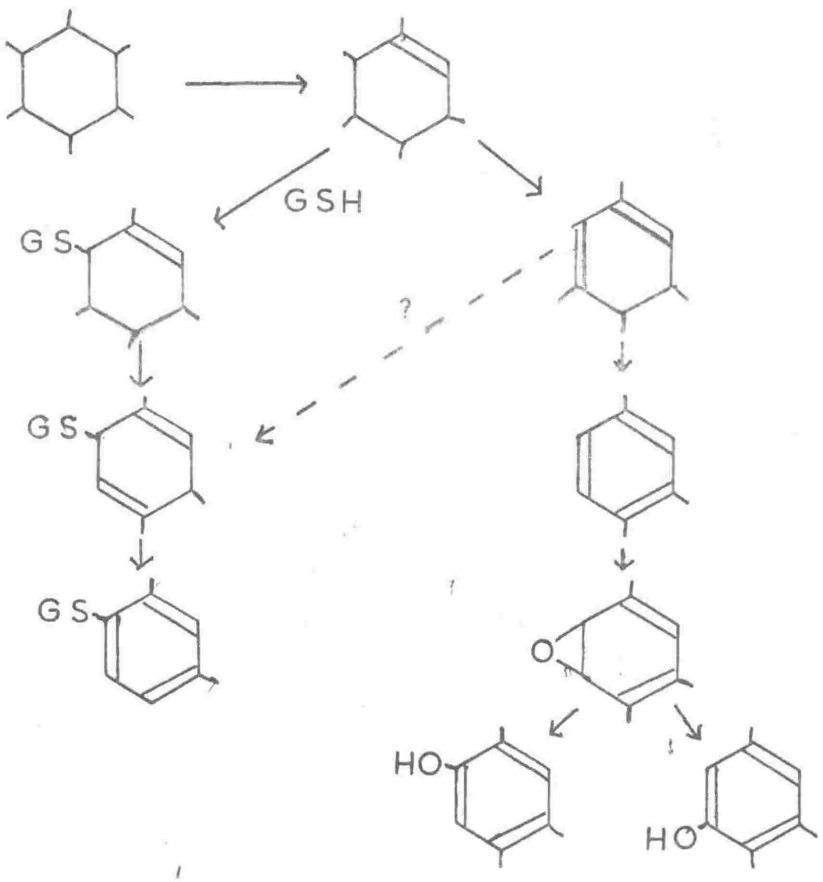


Fig.X.1: The scheme of Bradbury & Standen (1957) for the metabolism of Gammexane in M. domestica.

Fig.X.2: Grover & Sims' scheme for the metabolism of Gammexane in rats.

microsomal activity towards hexabarbital and reduces the narcotic effects of a number of barbiturates. It is possible that this effect is due only to the microsomal oxidation of trichlorobenzene produced by dehydrochlorination of the HCH, but it is conceivable that microsomal dechlorination of the HCH itself occurs. Microsomal dechlorinations have been reported, e.g. the conversion of Halothane (1,1,1 trifluoro-2-bromo-2-chloroethane) to trifluoroacetic acid, (Van Dyke, 1966). If such a mechanism is involved in the metabolism of the HCH's, existing concepts of the metabolic pathway would have to be radically changed.

2) Uptake of δ-HCH and δ-PCCH by C.zealandica and M.domestica

These experiments, while quite straightforward, yield little useful information. As stated, the δ-HCH was used because of its low toxicity, and because, like the γ-HCH, it produced a chemically stable PCCH on monodehydrochlorination. Unfortunately, next to the β-isomer, the δ-HCH is the slowest to be metabolized, so that the effects of the short-lived possible synergists are difficult to perceive. The experiments might be usefully repeated with the rapidly metabolized, non-toxic α-HCH, though this does not, unfortunately, yield an isolable PCCH.

As expected, the δ-PCCH is metabolized very rapidly (Fig. VII.4). Because of this, if δ-PCCH is produced as an intermediate in the metabolism of δ-HCH, it is unlikely to accumulate in significant amounts.

The in vivo metabolism of δ-PCCH is totally inhibited for 3-4 hours by injected bromphenol blue. This could suggest that the PCCH is metabolized by conjugation with GSH, mediated by an enzyme similar to the aryltransferase. The metabolism of δ-HCH is affected only slightly by bromphenol blue. This presumably, is a result of both the rapid removal of the dye, and the slow metabolism of the HCH.

3) The Metabolites of γ-HCH, γ- and δ-PCCH in M.domestica and C.zealandica:-

In vivo and in vitro experiments with M.domestica and C.zealandica yielded from [^{14}C] γ-HCH and from γ- and δ-PCCH a major metabolite which co-chromatographed with synthetic S-(2,4-dichlorophenyl)glutathione in four solvent systems. This cannot however, be taken as proof that the conjugate formed is, in fact a dichlorophenyl conjugate. Booth, Boyland and Sims (1961) observed that the R_f's of various GSH conjugates were remarkably similar in two solvent systems, the nature of the conjugated species varying quite markedly. Some of their results are reproduced below. Solvent I is butan-1-ol-acetic acid-water (12:3:5), and Solvent II is butan-1-ol-acetic acid-water (2:1:1).

Conjugated Species	Solvent	
	I	II
3,4-Dichloronitrobenzene	0.45	0.62
2,4-Dichloronitrobenzene	0.42	0.60
3,4,5,6-Tetrachloro-1,2-epoxycyclohexane	0.42	0.64
Phenanthrene	0.43	0.61
1,2-Epoxy-1,2,3,4-tetrahydronaphthalene	0.49	0.63
Bromopropane	0.44	0.62

(See also addendum to experimental work - p.70).

Thus, particularly in this work, identity of chromatographic properties of two glutathione conjugates cannot be taken as proof of the identity of the conjugates.

The radioactive conjugate obtained in this work yielded on acid hydrolysis a compound which co-chromatographed and co-electrophoresed in 1 N-acetic acid with the synthetic S-(dichlorophenyl)-L-cysteines.

However, on chromatography on cellulose phosphate ion-exchange paper, the radioactivity did not run with any of the synthetic arylcysteines. While the mechanism of separation here is somewhat uncertain, the fact that at all stages chromatograms and hydrolyses were run with the radioactivity diluted by the synthetic conjugates considerably reduces the chance of this result being spurious. The result leads inescapably to the conclusion that this radioactive hydrolysis product is not a S-(dichlorophenyl)-L-cysteine and, possibly, to the conclusion that the original conjugate obtained was not a S-(dichlorophenyl)glutathione.

That these compounds were expected is based on data presented by Bradbury and Standen (1957). They demonstrated that the in vitro metabolism of γ-HCH was associated with release of 4-5 atoms of chlorine/molecule of γ-HCH metabolized. This suggested that dehydrochlorination and aromatization were occurring. In 1959, the same authors demonstrated that metabolites of γ-HCH gave the 2,4-dichlorothiophenols on alkaline hydrolysis. This was taken to mean that the end-products of metabolism were dichlorophenyl thioethers, obtained as in the scheme in Fig. X.1. The possibility that the intermediate S-(pentachlorocyclohexyl)thioether (which would be expected to yield a mixture of dichlorothiophenols on alkaline hydrolysis) might occur as a relatively stable intermediate was not considered. Bradbury and Standen's scheme requires that this conjugate be enzymically dehydrochlorinated (though this was not stated), as it would be expected to be at least as stable as γ-HCH to alkaline dehydrochlorination, and would not spontaneously yield the amount of chloride found. This, to the writer, seems an unnecessary complication.

Support for the idea of a non-aromatic conjugate, with a fairly long half-life, is gained from the observation by Sternberg and Kearns (1956) of a Schechter-Hornstein-positive metabolite, which might be a S-(pentachlorocyclohexyl)glutathione, of the type proposed by Bradbury and Standen. Treatment with zinc and acetic acid would dechlorinate the ring and cleave the thioether bond (Zincke and Glahn, 1907) to give, ultimately, the o- and p- chlorothiophenols. It has been found that these compounds nitrate to products including 1-chloro2,4-dinitrobenzene, and thus would give spurious values for PCCH.

Another scheme was advanced by Grover and Sims (1965), which accounts for the metabolites obtained from rats (see Fig. X.2). In this case, the S-(trichlorocyclohexadienyl)glutathione would be expected to give a positive Schechter-Hornstein reaction. However, the same criticsm as for the previous scheme can be made here; enzymes would be required for the further dehydrochlorination of both PCCH and the initial glutathione conjugate. Furthermore, this scheme implies that PCCH is present as a free intermediate. While the findings of Bridges (1959) demonstrate that only a fraction of the water-soluble, Schechter-Hornstein-positive material was actually PCCH, there is some doubt as to whether or not this small amount of PCCH is produced in a side reaction, or whether it constitutes the steady-state concentration of a key, rapidly metabolized intermediate. Superficially, the isotope-dilution experiments of Bridges and those presented in Chapter 8, appear to exclude the second possibility. However, the interpretation of both sets of experimental results is ambiguous. The work of Bridges is subject to the reservations stated in the introduction. In the work presented here, the fact that diluant

PCCH is added on lipoprotein might prevent the free mixing of diluant and metabolically produced PCCH. It seems probable that any other method of achieving the necessary high PCCH concentration will be subject to the same criticsm, and that the ambiguity of these results will not easily be resolved.

However, a scheme is presented, in Fig. X.3, which assumes that PCCH does not appear free, that dehydrochlorination and conjugation involve the same GSH-dependent mechanism, and that the whole process occurs on the same enzyme molecule, only minor amounts of intermediates being released. This scheme accounts for most of the observed phenomena and is basically simpler in its requirements than the previous two schemes.

Supporting evidence is presented below:

a) There is no evidence for the existence of an enzyme catalysing only the dehydrochlorination of the HCH's to the PCCH. Ishida and Dahm (1965a,b) present evidence for the existence of a number of enzymes catalysing the degradation of the HCH's and PCCH's, each enzyme favouring one particular isomer. To propose that these enzymes catalyse only the initial dehydrochlorination and, by implication, that other enzymes are required for further dehydrochlorination and conjugation, is to propose an uneconomical and, considering the non-physiological nature of the substrates, rather improbable situation. (Though admittedly, any metabolism at all of these compounds seems, <u>a priori</u> to be rather improbable).

b) Subject to the previously stated reservations, it appears that if PCCH is an intermediate in the metabolism of the HCH's, it is not produced in a form readily miscible with added PCCH.

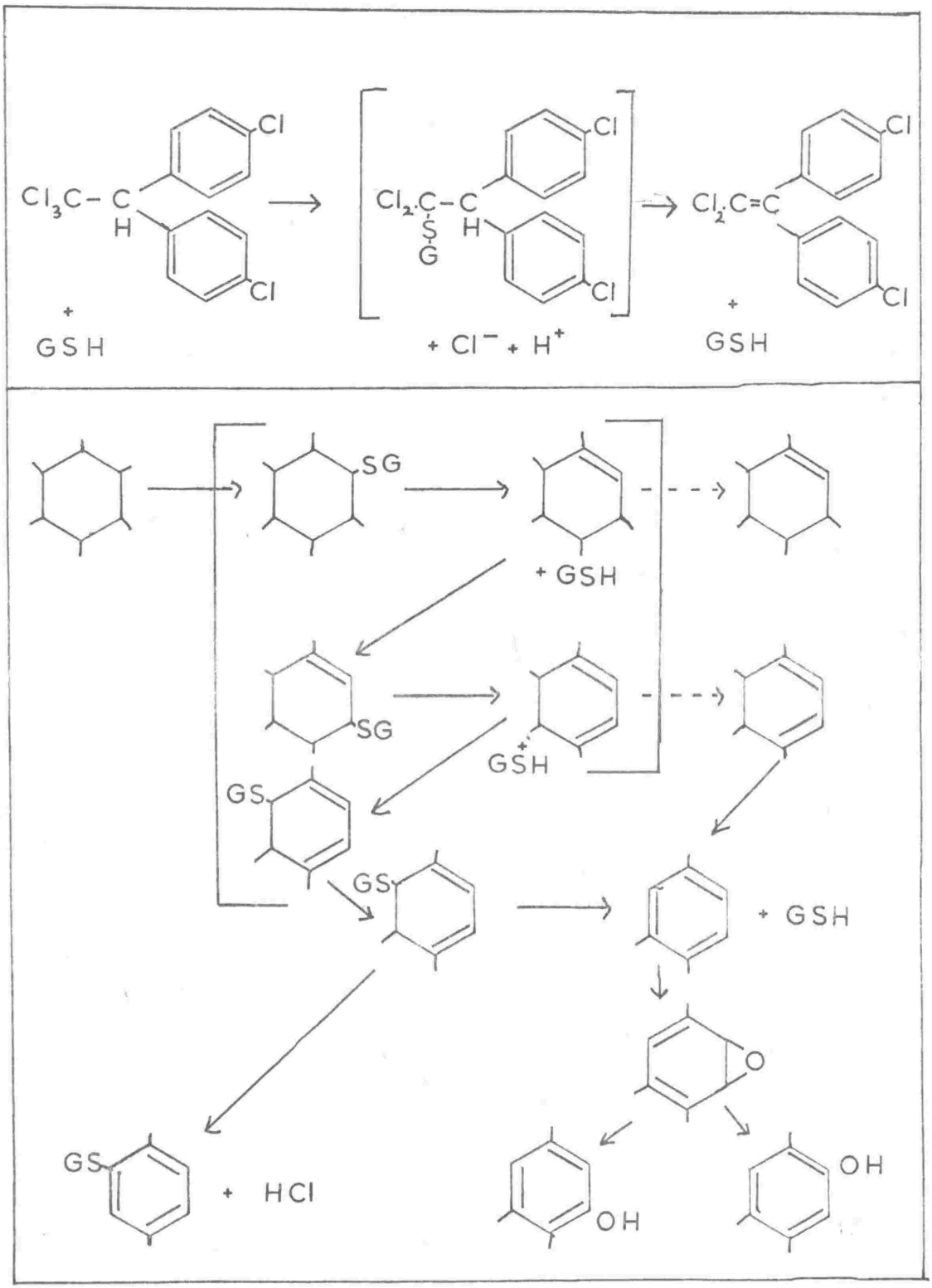


Fig.X.3: Proposed scheme for common means of metabolism of Gammexane and DDT.

c) The α-HCH, which does not produce an isolable PCCH, gives the same metabolites as the γ-HCH (Bradbury and Standen, 1960). This suggests that a free monodehydrochlorination product is not produced. If this were to be the case for the α-isomer, the PCCH produced could be expected to be rapidly and spontaneously dehydrochlorinated to the trichlorobenzene, thus giving metabolites different from those obtained from γ-HCH.

d) Ishida and Dahm (1965a) have shown that the existence of an enzyme catalysing both the metabolism of γ-HCH and the dehydrochlorination of DDT. This is taken to mean that dehydrochlorination and conjugation are both GSH-dependent, and that the same basic mechanism, involving a transitory GSH conjugate in the dehydrochlorination, is involved in both reactions.

The first free intermediate is proposed to be S-(trichlorocyclohexadienyl)-glutathione. This would be expected to give a positive Schechter-Hornstein reaction. It might also be expected, slowly, but spontaneously, to undergo aromatization, either by elimination of GSH to give the trichlorobenzenes, or by elimination of HCl to give a S-(dichlorophenyl)-glutathione. These assumptions are rather arbitrary, but they fit the observed facts, perhaps a little more plausibly than do the schemes involving S-(pentachlorocyclohexyl)- or S-(pentachlorocyclohexenyl)-glutathione.

This scheme should be capable of experimental verification. Isotope dilution of the radioactive glutathione conjugate with the S-(dichlorophenyl)glutathiones should unambiguously determine whether, and in what amount these compounds are present.

This would avoid the basic defect of experiments involving acid hydrolysis: that the possible existence of acid-labile premercapturic acids and related compounds is ignored. There is some reason to believe that such compounds could be formed during the metabolism of the HCH's. Possible pathways are shown in Fig.X.4.

The proposed S-(1-hydroxy-2,3,4,5,6-pentachloro-2-cyclohexyl)-glutathione would be unlikely spontaneously to undergo dehydrochlorination. It might be expected to lose a molecule of water in acid solution to give S-(2,3,4,5,6-pentachloro-2-cyclohex-1-enyl)glutathione. The S-(1-hydroxy-2,4,5-trichloro-6-cyclohexa-2,4-dienyl)glutathione and S-(6-hydroxy-2,4,5-trichloro-1-cyclohexa-2,4-dienyl)glutathione would be expected to yield the S-(2,4,5- and S-(3,5,6-trichlorophenyl)glutathiones. It seems uncertain whether or not these premercapturic acid-type compounds would give a spurious Schechter-Hornstein reaction for PCCH. This would depend on whether dehydration preceded or succeeded dechlorination.

Another line of research could be aimed, initially, at the isolation and study of the chromatographic and electrophoretic properties of Schechter-Hornstein-positive metabolites. It now seems important to determine whether these metabolites are glutathione conjugates, or whether they are compounds produced by an, as yet, unsuspected pathway.

4) The Aryltransferase and δ-PCCH Metabolism.

Sims and Grover (1965) postulate that only one enzyme is responsible for both DCNB and γ-PCCH conjugation in vertebrates. Ishida and Dahm (1965a,b) have isolated a fraction from fly homogenates, probably containing a number of enzymes with varying degrees of cross-specificity, which metabolizes the HCH's and PCCH's. This fraction contains an

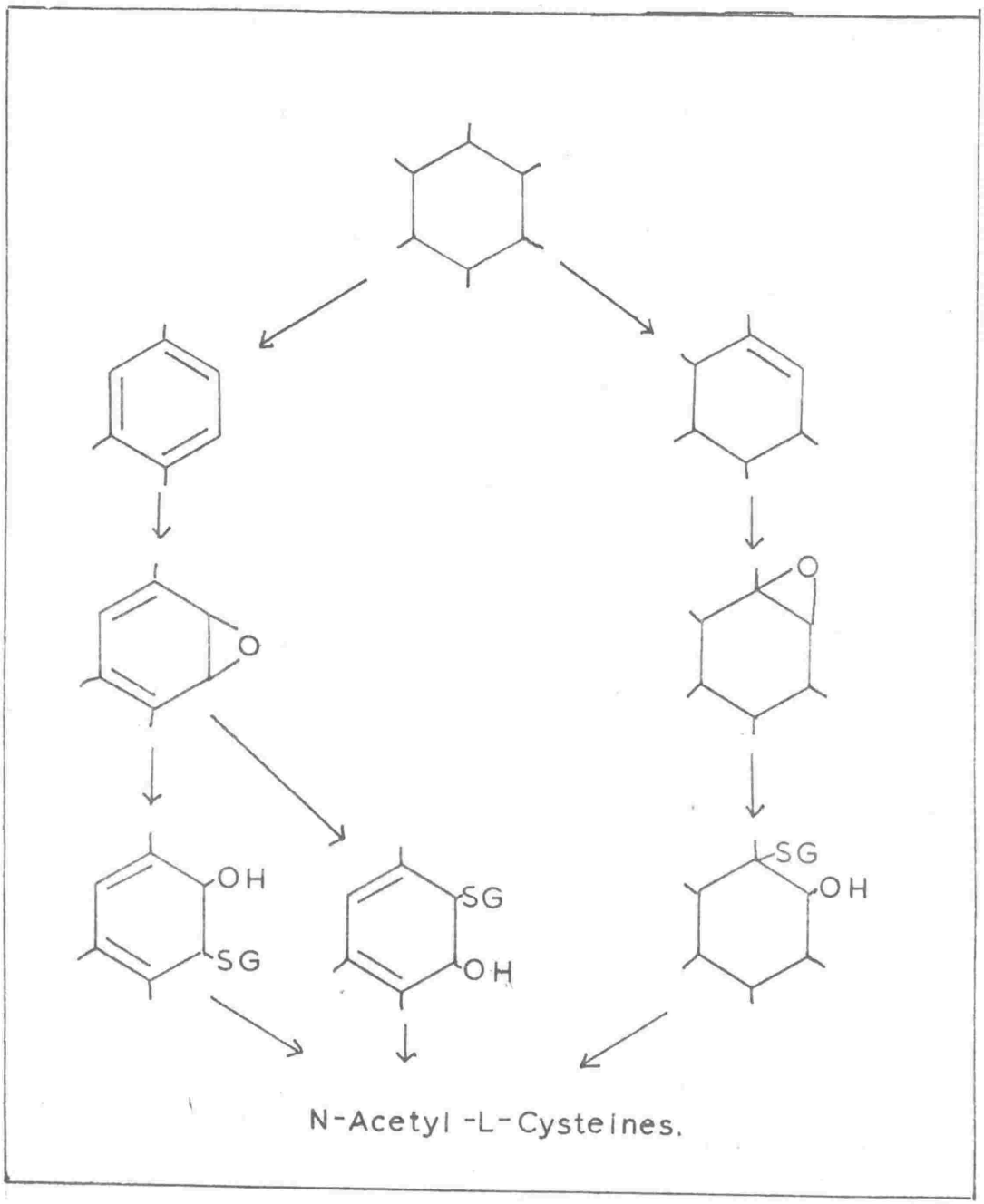


Fig.X.4: Proposed scheme for production of premercapturic acids from Gammexane.

enzyme capable of metabolizing both γ-HCH and DDT. In insects, aryltransferase, DDT dehydrochlorinase, and γ-HCH-metabolizing activities are all inhibited by bromphenol blue. From Sims and Grover's initial contention, if one ignores the species difference, extrapolation of the available data leads to the conclusion that a single enzyme is responsible for all activities. This seems sterically and mechanistically improbable.

In the work presented in Chapter 9, an attempt has been made, with some success, to differentiate, initially, between the aryltransferase activity and the PCCH-metabolizing activity.

In the first section it was found that fly and grass-grub homogenates and acetone powders had different ratios of aryltransferase to δ-PCCH-metabolizing activity. This strongly suggests that different enzymes were involved. It must, however, be pointed out that, as was shown in Chapter 8 for γ-HCH, the amount of inactive protein present in solution can affect the apparent activity of the enzyme. It is thus possible that the differences observed were due more to differing effects of impurities present in the solutions on the substrates than to differing amounts of enzyme.

Gel-filtration experiments, however, support the idea of at least two enzymes being involved. With the fly enzyme preparation the peak activities of aryltransferase and of the δ-PCCH metabolizing system are separated by 10 ml. and the peaks differ distinctly in shape.

DDT dehydrochlorinase appears to run in two distinct fractions. The faster appears to coincide with the δ-PCCH activity peak, and the slower with a shoulder of low δ-PCCH activity. The same results were obtained in each of two experiments.

It is suggested that three distinct enzymes were present. The first, and fastest moving, is the aryltransferase. The second, catalysing both the dehydrochlorination of DDT and the metabolism of δ-PCCH, is analogous to that found by Ishida and Dahm (1965a). The third, capable of dehydrochlorinating DDT, but having only a low activity towards δ-PCCH, is comparable with the DDT dehydrochlorinase isolated by Sternberg et al. (1954), which was found to be active towards DDT, but not towards γ-HCH.

The finding that the grass-grub preparation contains aryltransferase, but has lost all appreciable activity towards δ-PCCH and DDT, confirms the idea that these activities are due to different enzymes.

It would, however, be desirable to isolate fractions exhibiting only a single activity. This implies that future work should be with separation systems of greater resolving power. It is suggested that gels of a greater porosity, and the use of recycling systems, could be usefully investigated.

Appendix A - Inhibition Kinetics.

Little detailed work, theoretical or experimental, has been done on the inhibition of random-order-combination, two-substrate enzyme systems. Friedenwald and Mængwyn Davies (1954) have given a general equation for such a system which accounts for substrate-substrate interactions and inhibitor-substrate interactions for fully competitive, and fully and partially non-competitive types of inhibition. J. Webb (1963) has examined the plots obtained from this general equation for the competitive and non-competitive cases, and also for the case where combination of one substrate (or activator) is required before combination of the other can take place. The effect of substrate-substrate and inhibitor-substrate interactions are implicitly assumed by Webb to alter only the value of the K_i obtained. As will be seen, such interactions can alter the type of plots obtained, possibly leading to an erroneous interpretation. To quote Webb "The literature contains many examples of type A plots where the curves, although linear, do not meet at the axes; usually the inhibition is stated to be competitive or non-competitive depending upon the axis to which the interaction is closer. It is quite possible that these represent mixed inhibition". In fact, in two-substrate systems, such cases can be accounted for by mechanisms simpler than those involved in mixed inhibition.

All equations given here will be arranged in analogy with the form of the uninhibited reaction given by Haldane (1930).

i.e.

$$v_i = \frac{\frac{V_m . b}{b + k'b}}{1 + \frac{1}{a} \cdot \frac{KaK'b + bK'a}{b + K'b}}$$

$$= \frac{V_m . \frac{a}{a + K'a}}{1 + \frac{1}{b} \frac{KaK'b + aK'b}{d + K'a}}$$

here, the apparent V_m when $\frac{1}{v}$ is plotted against $\frac{1}{a}$, is

$$V_{ma} = \frac{V_m . b}{b + K'b}$$

and the apparent K_m,

$$K_{ma} = \frac{KaK'b + bK'a}{b + K'b}$$

The four substrate constants, and the true maximum velocity can be obtained with multiple Burke-Lineweaver plots in the manner of Florini & Vestling (1957).

The general case for inhibition in these systems is given in Fig. A, 1. Here, the extent of substrate-substrate interaction is represented by $\frac{K'a}{K_a}$, $(= \frac{K'b}{K_b})$ = Inhibitor-substrate interaction are represented by α and β respectively where $1 \leqslant (\alpha, \beta) \leqslant \infty$

The effect of the inhibitor on the rate of breakdown of the EAB complex is represented by γ, where $0 \leqslant \gamma \leqslant 1$.

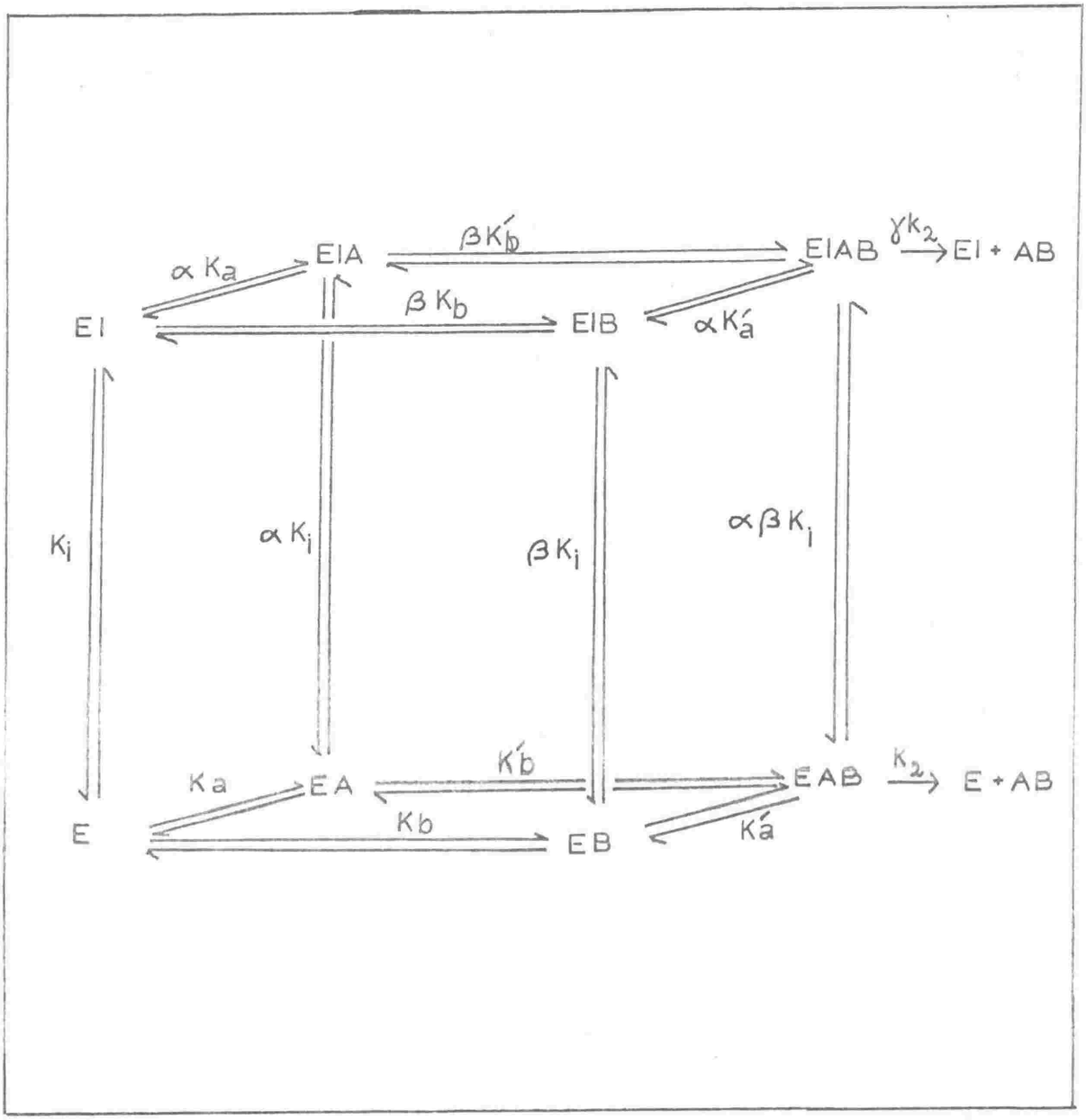


Fig.A.1: Generalised inhibition of a two-substrate enzyme system.

The equation for the general case is

$$v_i = \frac{\dfrac{V_{m.\,a}}{a\left(1+\dfrac{I}{\alpha\beta K_i}\right)+K'a\left(1+\dfrac{I}{\beta K_i}\right)}\cdot\dfrac{\alpha\beta K_i + I}{\alpha\beta K_i}}{1+\dfrac{1}{b}\,\dfrac{K'a\,Kb\left(1+\dfrac{I}{K_i}\right)+aK'b\left(1+\dfrac{I}{\alpha K_i}\right)}{a\left(1+\dfrac{I}{\alpha\beta K_i}\right)+K'a\left(1+\dfrac{I}{\beta K_i}\right)}}$$

The equation is, of course, quite symmetrical with respect to a and b. From it, by varying the values of α, β, and $\frac{K'a}{Ka}$ we can obtain equations to fit any mechanism of inhibition (or activation) involving the attachment of one molecule of inhibitor (or activator) to the enzyme. A few of the simpler cases will be examined.

Case I (a) Purely non-competitive: $\alpha = \beta = 1$, $\gamma = 0$.

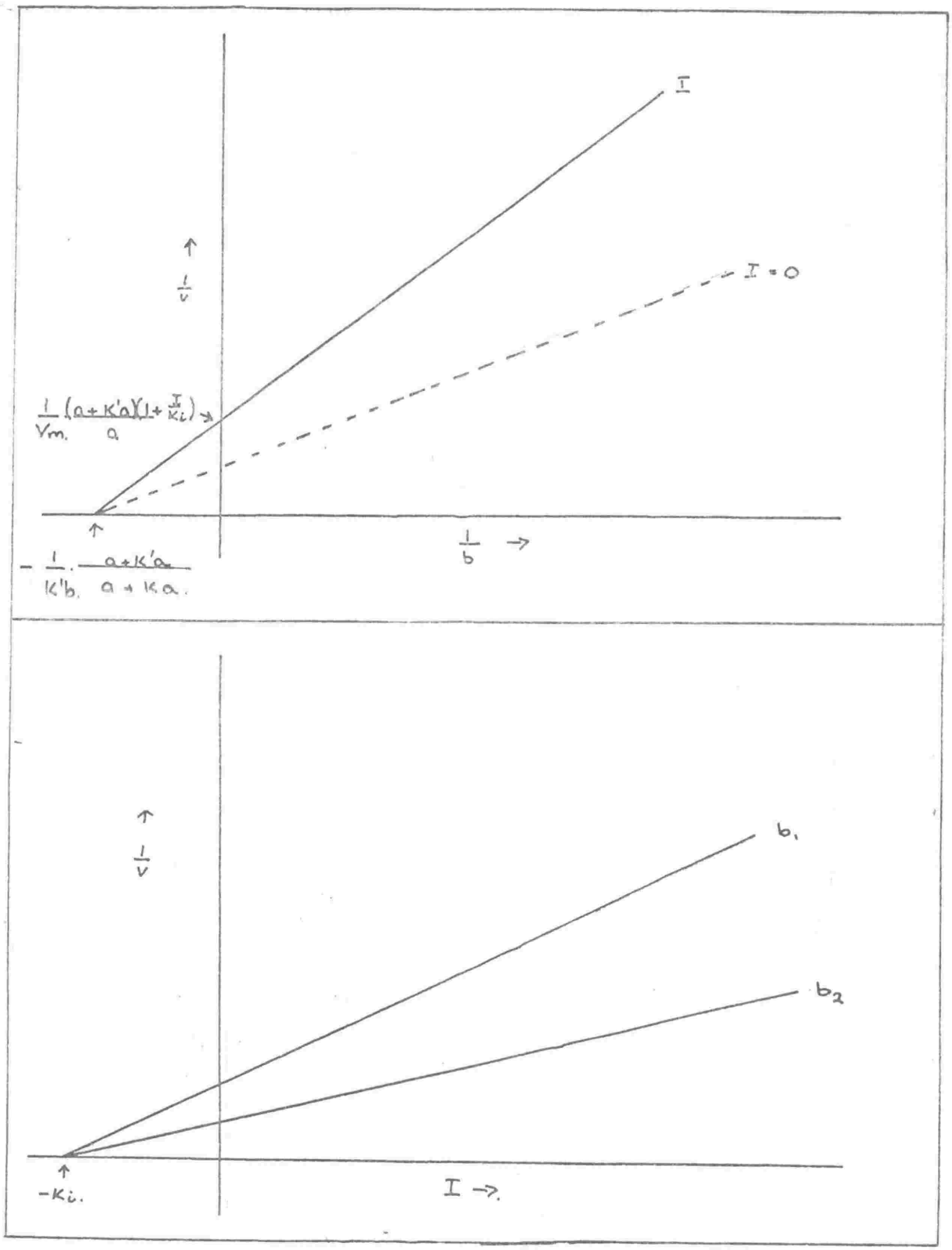
See graph A. 1a

$$v_i = V_m\,\frac{\dfrac{a}{(a+K'a)\left(1+\dfrac{1}{K_i}\right)}}{1+\dfrac{1}{b}\cdot\dfrac{KaK'b+aK'b}{a+K'a}}$$

(b) Partially non-competitive: $\alpha = \beta = 1$, $0 \leqslant \gamma \leqslant 1$.

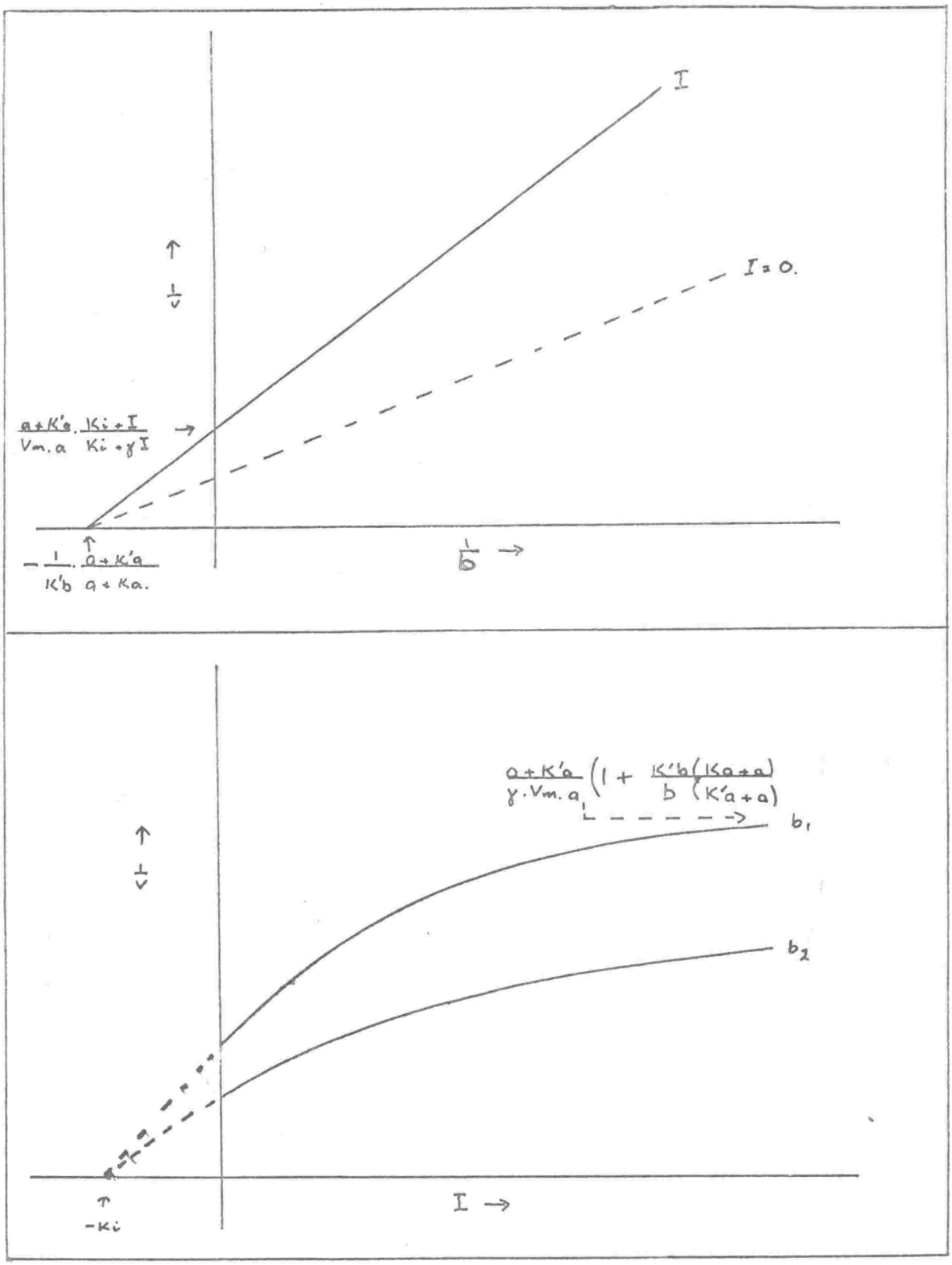
See graph A. 1b.

$$v_i = \frac{\dfrac{V_{m.a}}{a+K'a}\cdot\dfrac{Ki+\gamma I}{K_i}\cdot\dfrac{1}{1+\dfrac{I}{K_i}}}{1+\dfrac{1}{b}\cdot\dfrac{KaK'b+aK'b}{a+K'a}}$$



Graph A.1a: Complete non-competitive inhibition.

(iii) (b)



Graph A.1b: Partial non-competitive inhibition.

These two types are both analogous to the classical single-substrate cases. In, for example, a $\frac{1}{v}$ vs $\frac{1}{b}$ plot, in both cases, the lines intersect on the $\frac{1}{b}$ axis at the reciprocal of the apparent Km. In $\frac{1}{v}$ vs I plots, in both cases the lines intersect on the I axis at $I = -K_i$.

In the partial case, the gradient of the curve flattens off at $I = \infty$, at,

$$\frac{1}{v_i} = \frac{1}{\frac{\gamma V_m . a}{a + K'a}} \left(1 + \frac{1}{b} \cdot \frac{KaK'b + aK'b}{a + K'a} \right)$$

In effect, a new enzyme has been formed where the rate constant for the breakdown of the enzyme-substrate complex is $\gamma K + 2$.

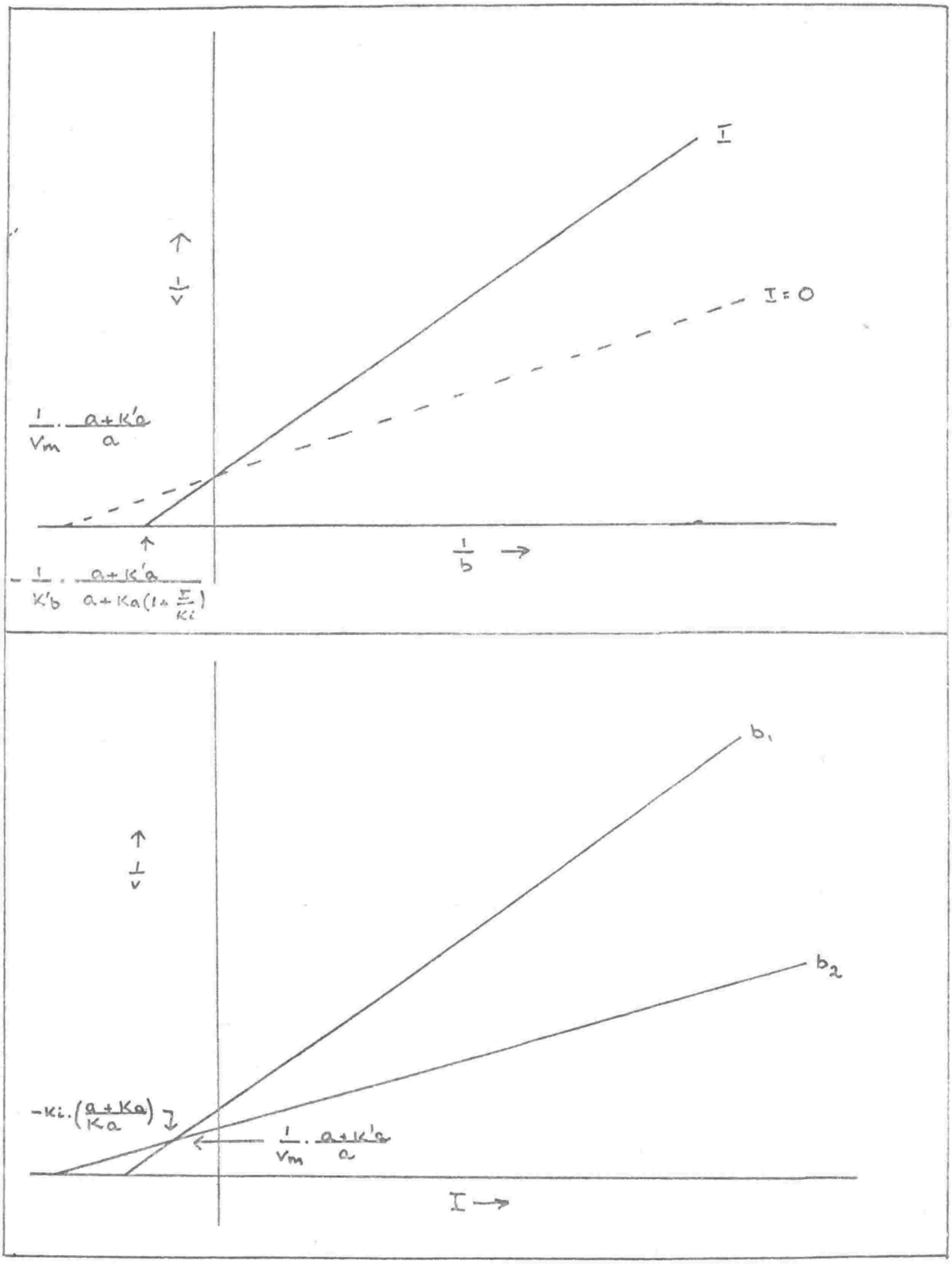
Case 2. Competitive types.

(a) Competitive towards both substrates $\alpha = \beta = \infty$, $\gamma = 0$

The equation is, (see graph A, 2a)

$$v_i = \frac{V_m . \frac{a}{a + K'a}}{1 + \frac{1}{b} \frac{K'aKb \left(1 + \frac{I}{K_i}\right) + aK'b}{a + K'a}}$$

As can be seen, the plots obtained for both substrates are very similar to those for the classical case. In the $\frac{1}{v}$ vs I plots, all lines intersect at a point the value of which is that of the K_i multiplied by the factor $\left(1 + \frac{a}{Ka}\right)$ or $\left(1 + \frac{b}{Kb}\right)$. The vertical coordinate of the intersection point is the reciprocal of the apparent maximum velocity i.e. $\frac{1}{V_m} \cdot \frac{a + K'a}{a}$.



Graph A.2a: Complete competitive inhibition with respect to both substrates.

2(b) Competitive inhibition with respect to one substrate only.

See Graph A. 2b $\alpha = \infty$, $1 < \beta < \infty$, $\gamma = 1$.

The equation in this case is:

$$v_i = \frac{V_{m,a}}{a + K'a\ (1 + \frac{I}{\beta K_i})} \Bigg/ \left(1 + \frac{\frac{1}{b}\left(KaK'b\ (1 + \frac{I}{K_i}) + aK'b\right)}{a + K'a\ (1 + \frac{I}{\beta K_i})}\right)$$

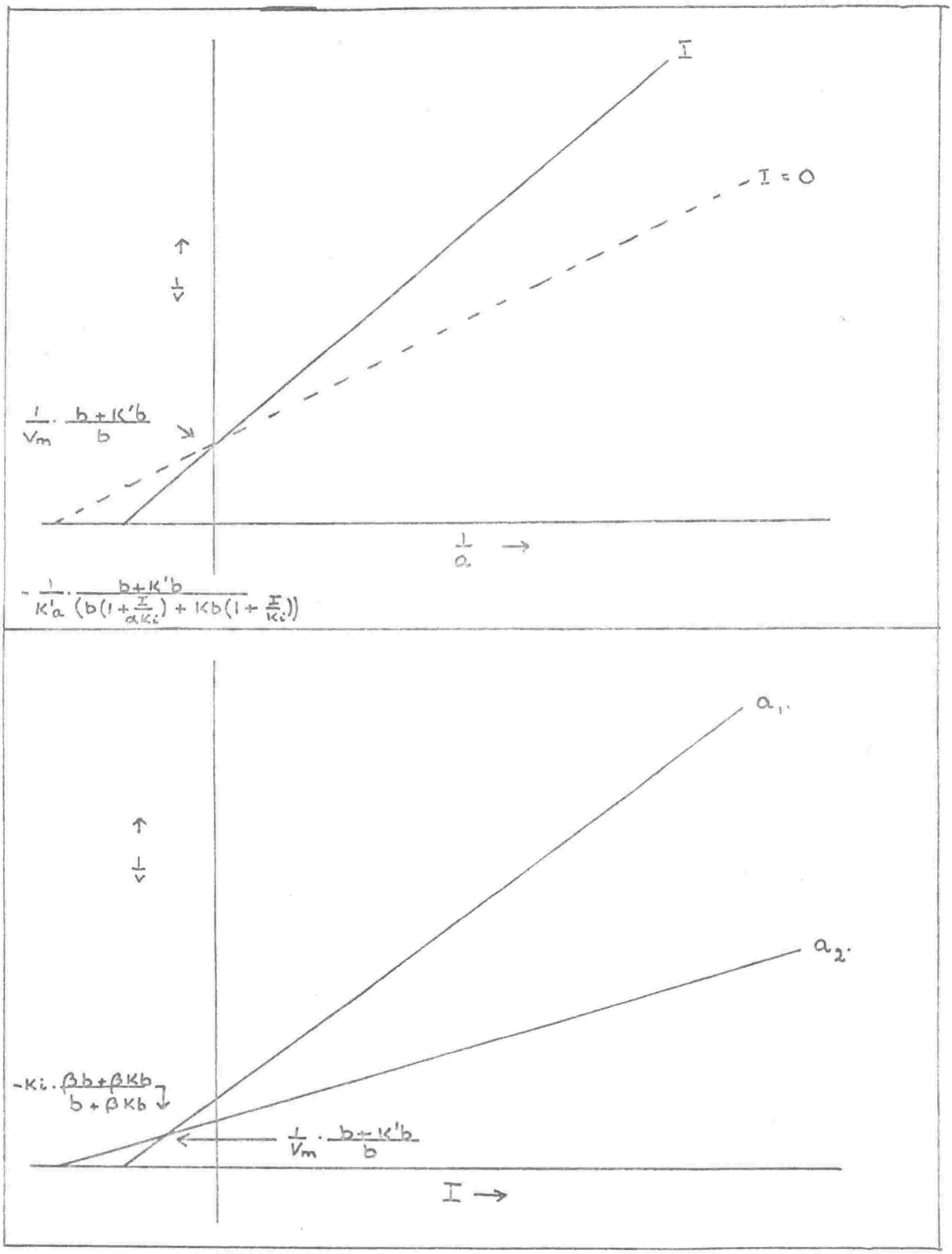
$$= \frac{V_{m,\,b}}{b + K'b} \Bigg/ \left(1 + \frac{1}{a}\left\{\frac{KaK'b\ (1 + \frac{I}{K_i}) + bK'a\ (1 + \frac{I}{\beta K_i})}{b + K'b}\right\}\right)$$

Here, the equations, and hence the plots, are unsymmetrical with respect to a and b.

Here, we see that the plots involving the competing substrate (a) give apparently classical plots, for the competitive case. In the $\frac{1}{v}$ vs $\frac{1}{a}$ plots, all lines intersect at the apparent maximum velocity. In the $\frac{1}{v}$ vs I plot, the lines intersect to give an apparent K_i of $\frac{\beta b + \beta Kb}{b + \beta Kb} \cdot K_i$; $\frac{1}{v} = \frac{1}{Vm}$ (app.)

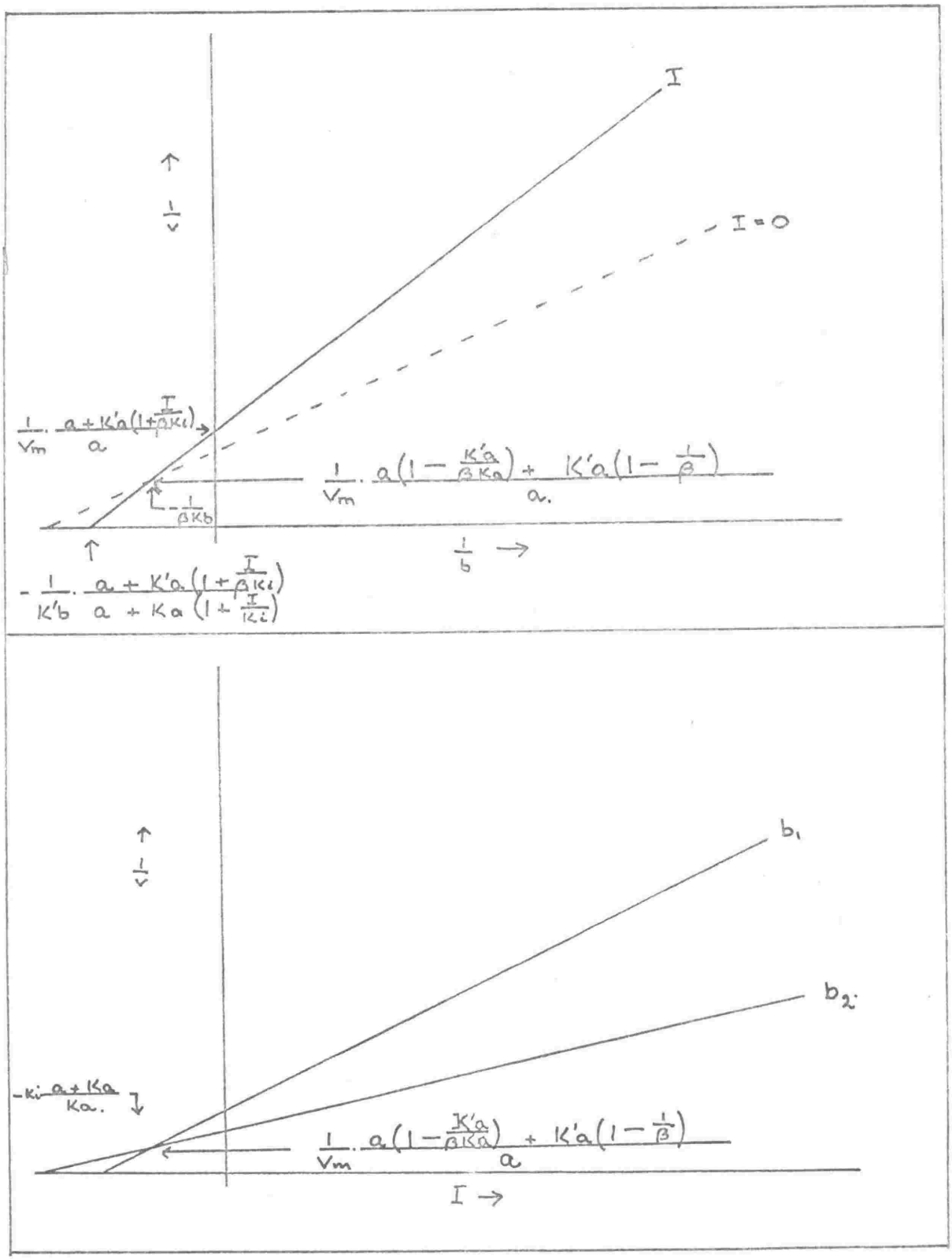
However, plots involving b give an anomalous plot - resembling that for mixed inhibition in the classical case. The $\frac{1}{v}$ vs $\frac{1}{b}$ plot gives lines intersecting at neither axis but at a point $\frac{1}{b} = -\frac{1}{\beta Kb}$, $\frac{1}{v} = \frac{1}{Vm_a}\left(a(1 - \frac{K'a}{\beta Ka}) + K'a\ (1 - \frac{1}{\beta})\right)$.
Also, for the $\frac{1}{v}$ vs I plots, the lines intersect at $I = -K_i\ (1 + \frac{a}{Ka})$, at the same reciprocal velocity coordinate as above.

(v) a



Graph A.2b(i): Complete competitive inhibition with respect to one substrate only.
Plotted with respect to competing substrate (a).

(v) b



Graph A.2b(ii): Complete competitive inhibition with respect to one substrate only. Plotted with respect to non-competing substrate (b).

Thus, an apparently mixed inhibition has arisen from a very simple mechanism. It could be argued that this is, in fact, analogous to a classical mixed inhibition by definition as, for b, γ effectively equals zero, and the inhibitor changes substrate-enzyme affinities. However, if $\beta = 1$, removing the latter effect, the points of intersection will still be non-axial being respectively:

(i) $\frac{1}{v} = \frac{1 - \frac{K'a}{Ka}}{V_m}$; $\frac{1}{b} = \frac{1}{Kb}$; and (ii) $\frac{1}{v} = \frac{1 - \frac{K'a}{Ka}}{V_m}$ and $I = -K_i.(1 + \frac{a}{Ka})$

This model fits the experimental plots for the inhibition of insect glutathione aryl transferase by the sulphonphthalein related compounds, with the stipulation that $\beta = 1$ and $\frac{K'a}{Ka} = \frac{K'b}{Kb} = 1$, where a is GSH and b the aromatic substrate. That $\frac{K'a}{Ka} = 1$ is borne out by the determination of the substrate constants where, within experimental error, the ratio $\frac{K'a}{Ka}$ is unity.

It will be also noticed, that when $\frac{K'a}{Ka} = 1$, $\beta = 1$ and $\alpha = \infty$; the K_i obtained from a $\frac{1}{v}$ vs I plot for different a, is the true K_i.

2 (c) (i) Partial competitive inhibition with respect to both substrates, where $\gamma = 1$, and $1 < (\alpha, \beta) < \infty$.

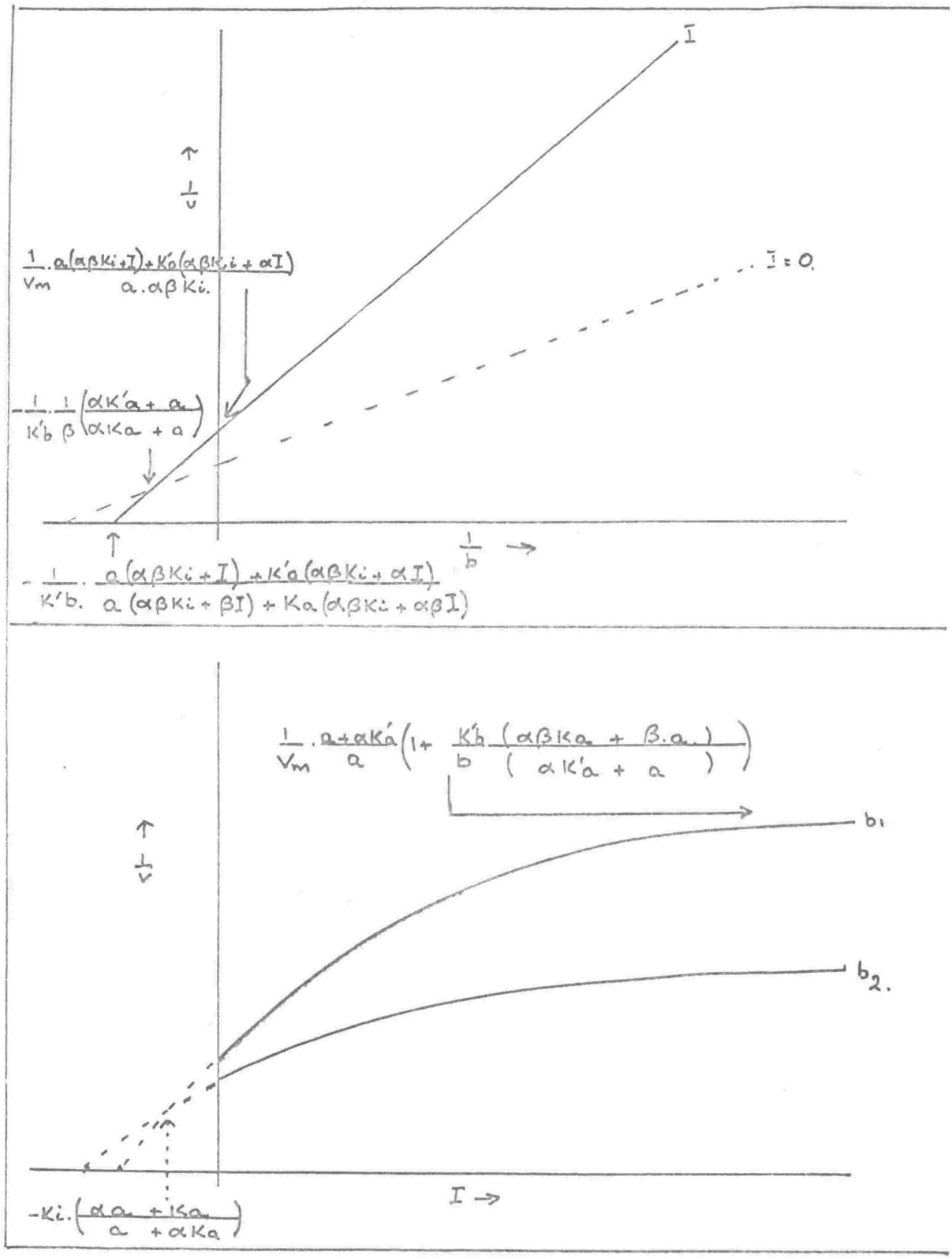
The equation is: (see Graph A, 2c, (i))

$$v_i = \frac{\frac{V_{m.\,a}}{a\,(1 + \frac{1}{\alpha\beta K_i} + K'a\,(1 + \frac{I}{\beta K_i})}}{1 + \frac{1}{b}\left\{\frac{KaK'b\,(1 + \frac{I}{K_i}) + aK'b\,(1 + \frac{I}{\alpha K_i})}{a\,(1 + \frac{I}{\alpha\beta K_i}) + K'a\,(1 + \frac{I}{\beta K_i})}\right\}} \cdot \frac{\alpha\beta K_i + I}{\alpha\beta K_i}$$

Similarly for the equation in terms of $\frac{1}{a}$.

As $I \rightarrow \infty$, the equation becomes

$$\frac{1}{v_i} = \frac{1}{V_m} \cdot \frac{a + \alpha K'a}{a}\left(1 + \frac{1}{b} \cdot \frac{\alpha\beta KaK'b + a\beta K'b}{a + \alpha K'a}\right)$$



Graph A.2c(i): Partial competitive inhibition with respect to both substrates.

i.e. we have in fact, a new enzyme with substrate constants of αK_a, $\alpha K'a$, βKb and $\beta K'b$.

As will be seen, $\frac{1}{v}$ vs $\frac{1}{a}$ or $\frac{1}{b}$ gives linear plots, intersecting at neither axis, while the $\frac{1}{v}$ vs I plot gives non-linear plots tending towards a minimum velocity.

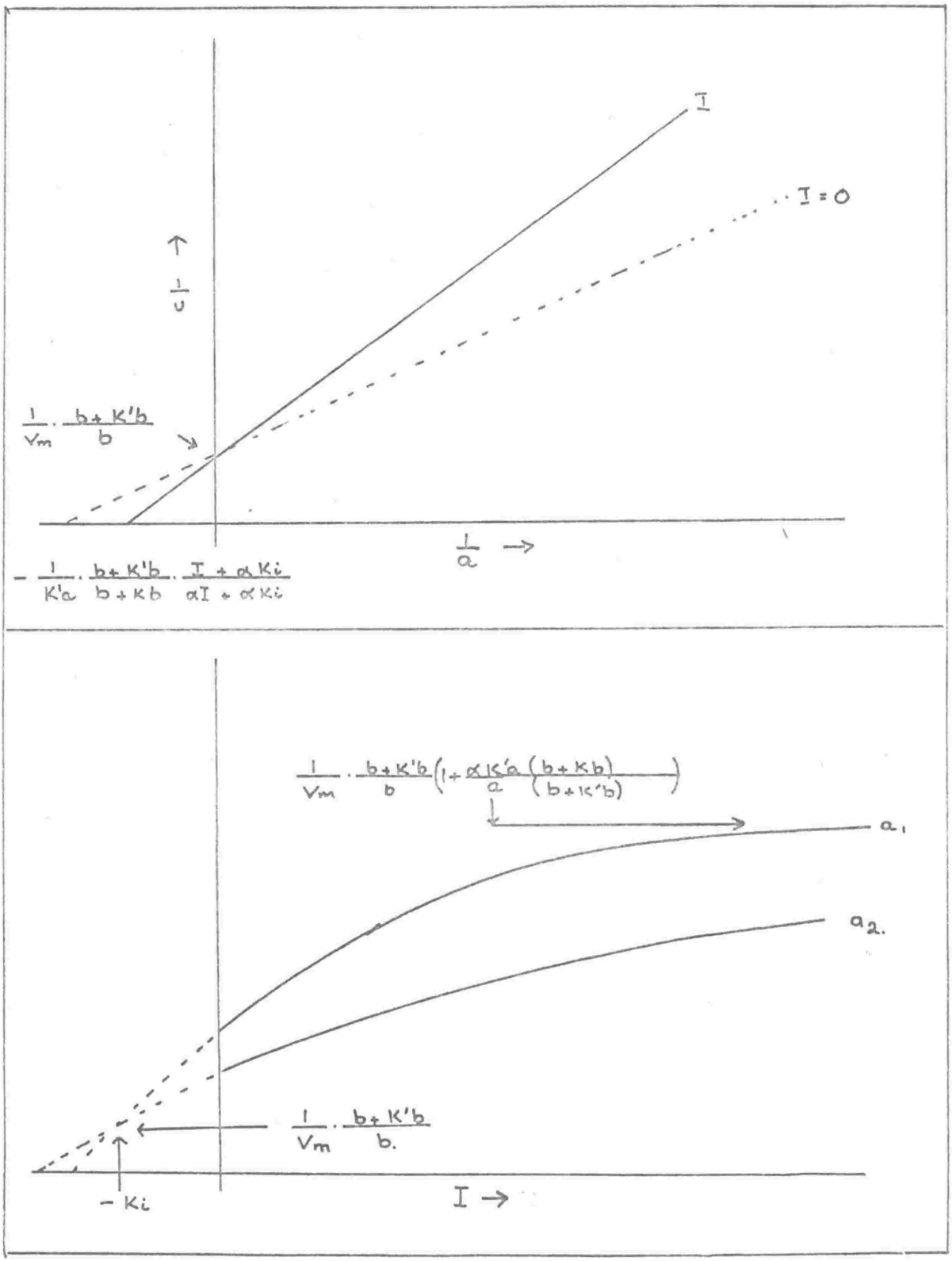
2c (ii) Partially competitive inhibition with respect to one substrate only, where $1 < \alpha < \infty$, $\beta = \gamma = 1$.

This is a restricted case of 2c (i) and gives us the non-symmetrical equation below.

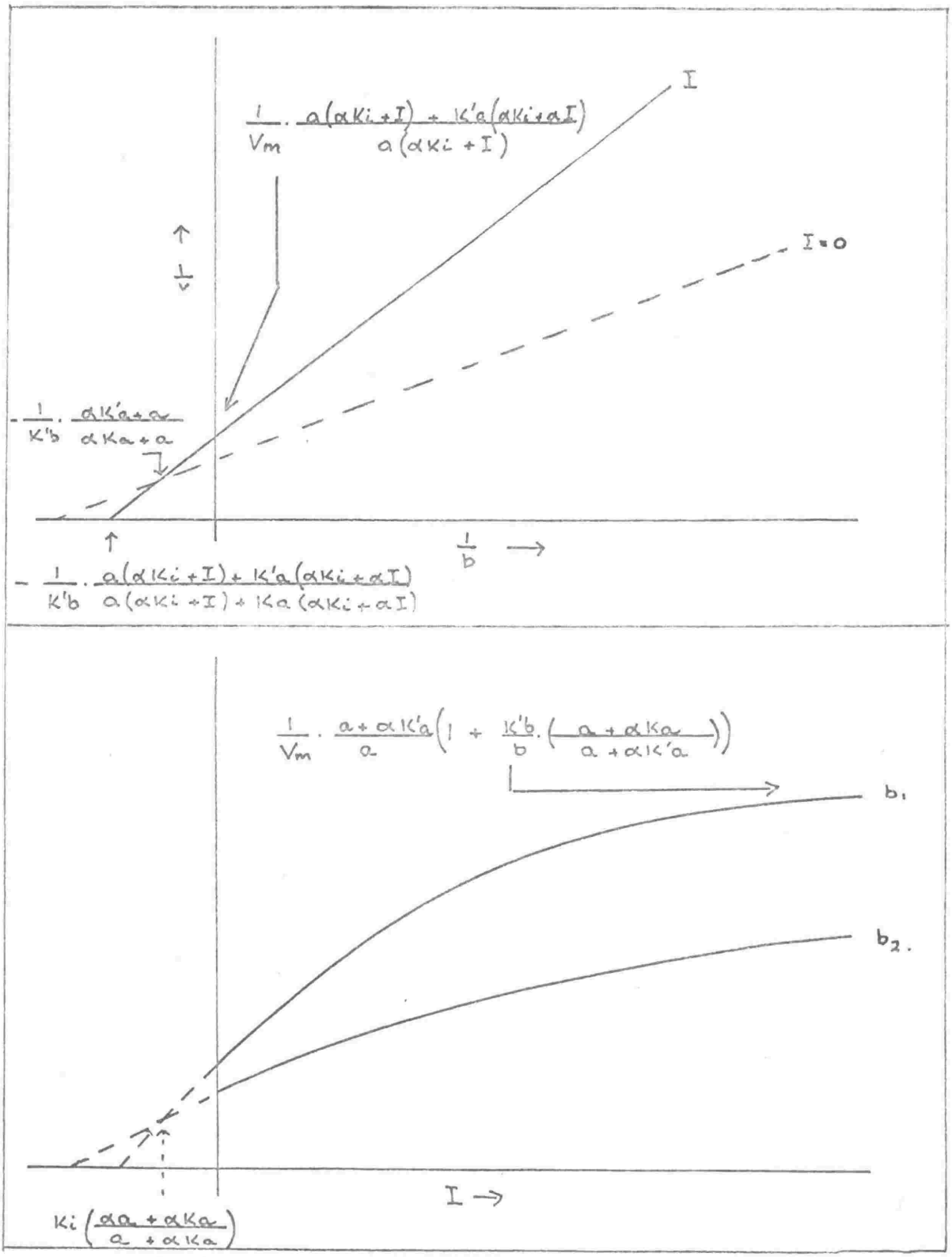
$$v_i = \frac{V_{m.\,a} \cdot (1 + \frac{I}{\alpha K_i})}{\frac{a\,(1 + \frac{I}{\alpha K_i}) + K'a\,(1 + \frac{I}{K_i})}{1 + \frac{1}{b}\left\{\frac{KaK'b\,(1 + \frac{I}{K_i}) + aK'b\,(1 + \frac{I}{\alpha K_i})}{a\,(1 + \frac{I}{\alpha K_i}) + K'a\,(1 + \frac{I}{K_i})}\right\}}}$$

$$= \frac{\frac{V_m}{b + K'b}}{1 + \frac{1}{a}\left\{\frac{KaK'b + aK'b}{b + K'b} \cdot \frac{\alpha I + K_i}{I + K_i}\right\}}$$

Thus, two differing sets of curves are obtained. $\frac{1}{v}$ vs $\frac{1}{a}$ gives us a classical plot for partially competitive inhibition: all lines intersect at the $\frac{1}{v}$ axis at the apparent maximum velocity. The intersection point of the curves in the $\frac{1}{v}$ vs I plot is $-K_i$ (though of course, this cannot be determined experimentally). The curves obtained for plots involving (b) are identical with those in A 2(c) (i), except of course, the values are altered, since $\beta = 1$.



Graph A.2c(ii): Partial competitive inhibition with respect to one substrate only. Plotted with respect to competing substrate (a)



Graph A.2c(ii): Partial competitive inhibition with respect to one substrate only.
Plotted with respect to non-competing substrate (b).

Case 3. Competitive inhibition at both sites by separate inhibitor molecules. See Fig A.2 See Graph A.3.

The equation is symmetrical:

$$V = \frac{V_{m,\,a}}{a + K'a\,(1 + \frac{I}{\beta K_i})} \Bigg/ 1 + \frac{1}{b}\left\{\frac{KaK'b\,(\frac{I}{K_{i_1}} + \frac{I}{K_{i_2}} + \frac{I^2}{K_{i_1} K_{i_2}} + 1) + aK'b\,(1 + \frac{I}{\alpha K_i})}{a + K'a\,(1 + \frac{I}{\beta K_i})}\right\}$$

The curves plot as in graph 3.

It will be seen that the Dixon plots ($\frac{1}{v}$ vs I) are parabolic.

This complicated model may be of more than merely academic interest with regard to the present kinetic work, e.g., BSP is a known substrate for the mammalian transferase - (this being the basis of the BSP test for liver function). Because it competes with GSH in the insect enzyme it cannot act as a substrate to any measureable extent. It seems likely, however, in view of the wide specificity of the enzyme for aromatic substrates, that were its inhibitory powers removed, it would become, in practical terms, a substrate for the insect enzyme. If we do not remove the inhibitory powers completely, and create an analogue that acts as a substrate and as a weak competitor towards GSH, then this type of kinetics will occur. A candidate for this type of inhibitor might be the desulphonated BSP analogue - phenoltetrabromphthalein.

<u>Determination of enzyme concentrations</u>.

(1) For purely non-competitive inhibition in two substrate enzyme systems, the method of Easson and Stedman (1936) can be used.

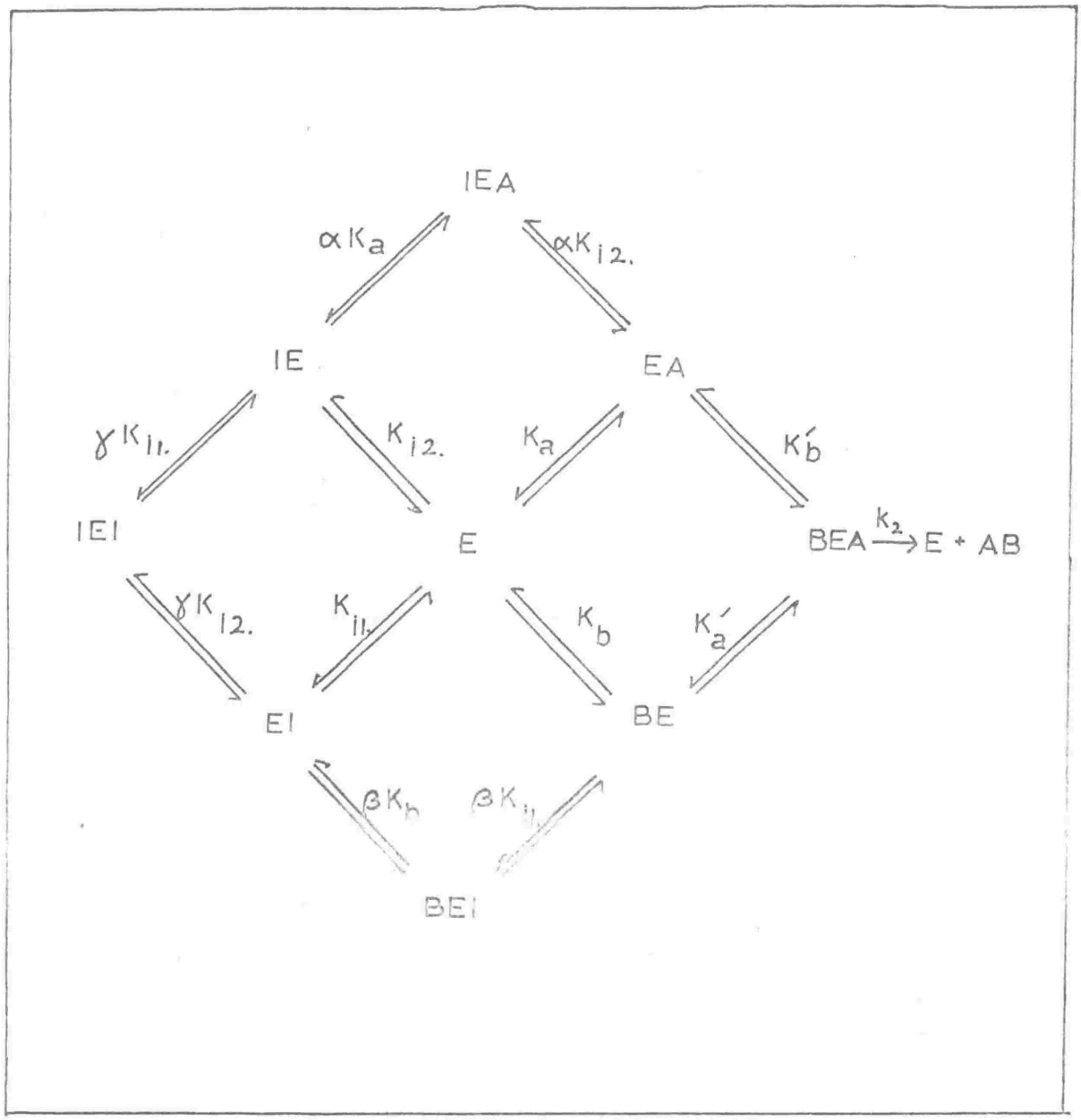
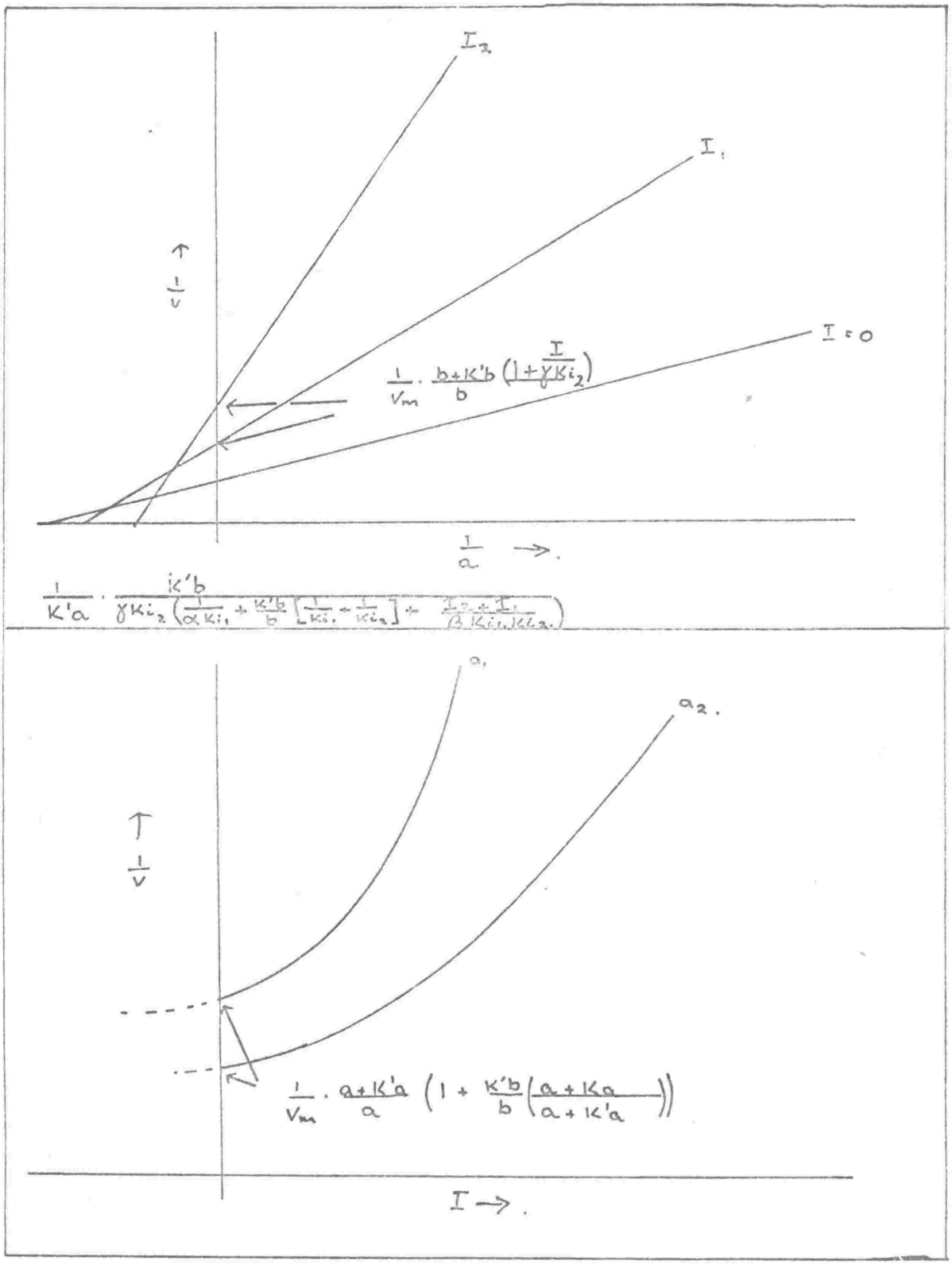


Fig.A.2: Scheme for fully competitive inhibition at either or both sites on the ensyme by one or more inhibitor molecules.



Graph A.3: Fully competitive inhibition at either or both sites on the enzyme by one or more inhibitor molecules.

i.e. when a significant proportion of the inhibitor is complexed with the enzyme, then

$$I_{total} = I_{free} + EI + EIS \quad (S = A \text{ or } B)$$

Now, i, the fractional inhibition, $= 1 - \frac{vi}{v}$

$$= \frac{1}{1 + \frac{Ki}{If}} .$$

$$\therefore \; I_f = K_i \cdot \frac{i}{1-i} .$$

Further,

$$\frac{El + ElA}{Et} = \frac{1}{1 + \frac{Ki}{I_f}} = i .$$

$$\therefore \; I_t = \frac{K_i \; i}{1 - i} + i . Et.$$

Thus, plotting $\frac{I_t}{i}$ vs $\frac{1}{1-i}$, Et is obtained at i = 0.

(2) Though the above treatment is not applicable to competitive inhibition for single substrate systems, it can be adapted for use with systems involving fully competitive inhibition towards one substrate only in a two substrate system. As the inhibition of GSH-aryl transferase by the phthalein-type dyes falls into this category (2b) it is pertinent to consider how this can be done.

For case 2 (b) $i = 1 - \frac{vi}{v}$

where $\alpha = \infty$, $1 < \beta < \infty$, $\gamma = 0$. $i = \dfrac{\frac{K'a}{a} \cdot (\frac{I}{K_i}) \cdot (\frac{I}{\beta} + \frac{Kb}{b})}{\frac{I}{b} + \frac{K'a}{a} \left((1 + \frac{I}{\beta K_i}) + \frac{Kb}{b} (1 + \frac{I}{K_i}) \right)}$

$$= \frac{1}{1 + \frac{\beta K_i}{I_f}} = i, \text{ iff } a \ll K_a; \quad b \gg K_b$$

$$\therefore \quad I_f = \frac{\beta K_i}{1-i} \cdot i .$$

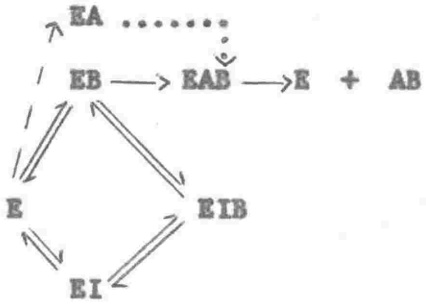
Now $$I_t = \frac{(1 + \frac{b}{\beta Kb}) \cdot \frac{I}{K_i}}{1 + \frac{b}{Kb} + \frac{a}{K_a} \cdot (1 + \frac{b}{K'b}) + \frac{I}{K_i} (1 + \frac{b}{\beta Kb})}$$

$$= \frac{1}{1 + \frac{\beta K_i}{I}} = i, \text{ iff } a \ll K_a; \quad b \gg K_b$$

Hence, $$I_f = \beta K_i \cdot \frac{i}{1-i} + i. \text{ Et.}$$

This equation is identical with that for the non-competitive case except that, in an $\frac{I_t}{I}$ vs $\frac{1}{1-i}$ plot the gradient is βK_i.

The requirements, $a \ll K_a$, $b \gg K_b$, can be explained qualitatively. Firstly, if the enzyme is saturated with respect to b, then the change in affinity due to the presence of inhibitor will not enter into the kinetics. Secondly, if a is very small, EA and EAB become correspondingly small and the pathway involving a becomes negligible.



The system approximates to non-competitive inhibition for a single-substrate enzyme. The EB complex can be regarded as being the enzyme-substrate complex for such a system, the rate of breakdown being directly proportional to a, and EAB being an intermediate product.
Where $\beta = 1$, as in the inhibition studies presented here, it will not be necessary to work at concentrations of $b \gg (K_b, K'b)$.

The same considerations apply to partial non-competitive inhibition. If a is very low and b very high then the system below is again an approximation to the simple non-competitive case.
Here the equation is:

$$I_t = \frac{\beta K_i}{1-i} + i.\,Et.$$

and in the reverse case, where $a \gg (K_a, K'a)$ and $b \ll (K_b, K'b)$,

$$I_t = \frac{\alpha K_i}{1-i} + i.\,Et.$$

Appendix B - Variation of Kinetics with pH.

The various states of ionization of the enzymic species in a two substrate system are shown in fig. B(1). For simplicity, substrate ionization is not shown. The assumptions involved are those of overall equilibrium, and that the singly-protonated HEAB species is the only one capable of breaking down into products. Substrate constants shown are true constants, invariant with pH, representing the equilibrium between only those ionic species shown.

Now

$$(E_{Total} - EA_T - EB_T - EAB_T)\ \frac{1}{fhe} = EH$$

where $EA_T = H_2EA + HEA + EA$. etc.

and fhe is the Michaelis pH function for the HE species such that

$$E_{T(free)} = (1 + \frac{H}{Ke_1} + \frac{Ke2}{H}).\ EH = fhe.\ EH$$

$$= (1 + \frac{H}{Ke2} + \frac{H^2}{Ka_1Ke_2}).\ E = fe.\ E$$

$$= (1 + \frac{Ke1}{H} + \frac{Ke_1.Ke_2}{H^2}).\ EH_2 = fh_2e.EH_2$$

Equilibrium requires that

$$\frac{Ka}{Ke_1} = \frac{K^+a}{Kee_1} \quad \text{etc.}$$

Hereafter, for convenience, only the singly ionized species will be considered and the pH function written as fe etc. Equations derived will be equally valid if other forms of the enzyme are active if the appropriate pH functions are substituted in the equation.

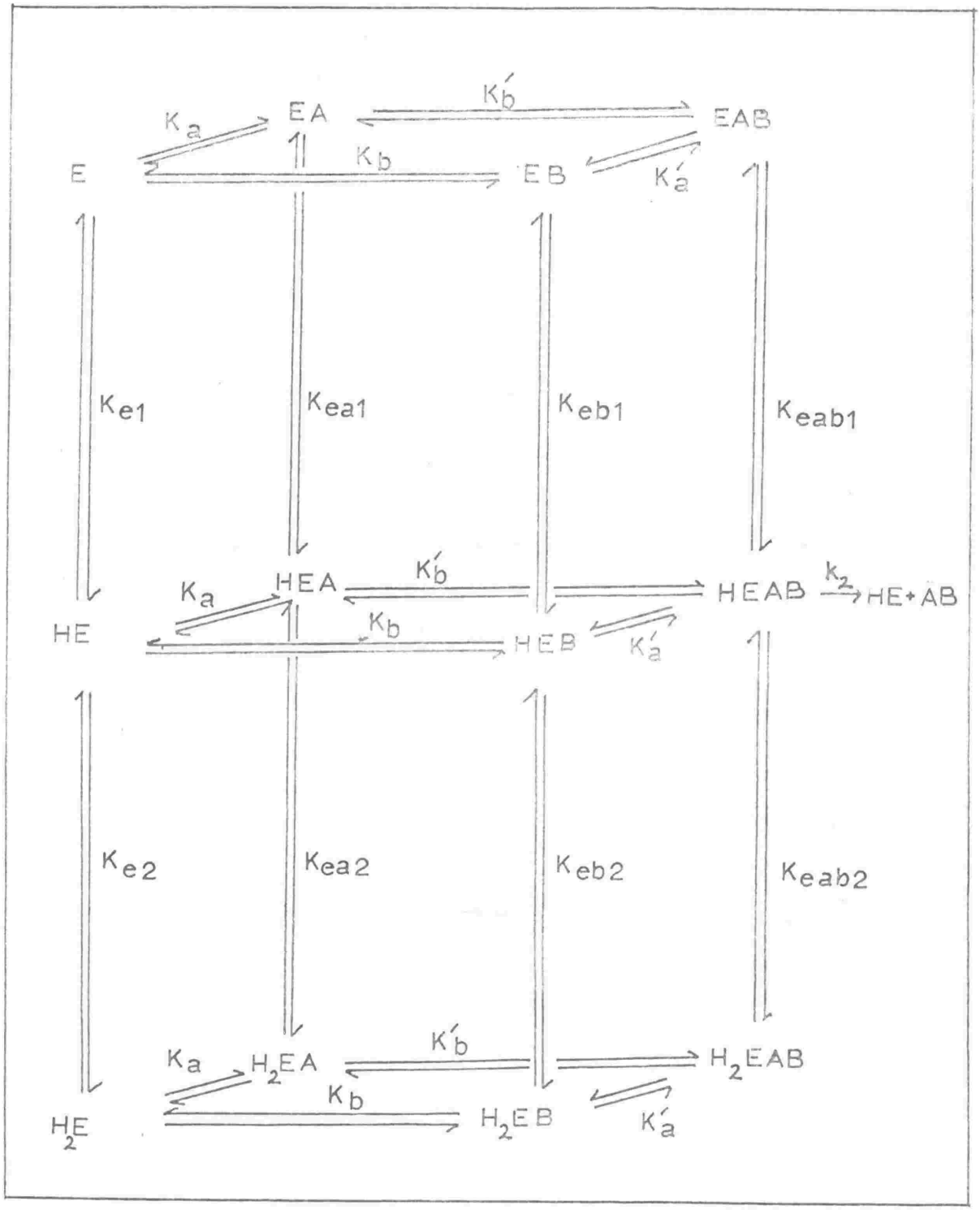


Fig.B.1: Ionic species possible in a two substrate enzyme system.

From the first equation, assuming that

$v = k_{+2}$ [HEAB]

$v_m = k_{+2}$ [E total]

we get $V = \dfrac{\dfrac{V_{m.}\ b}{feab.\ b + K'b\ fea}}{1 + \dfrac{1}{a}\dfrac{KaK'b\ fe + bK'a\ feb}{feab.\ b + K'b\ fea.}}$

Similarly for b.

If one wishes to account for the effects of the substrates A and B, it is merely a matter of substituting $\frac{a}{fa}$, $\frac{b}{fb}$ for a and b in the above equation.

In the ideal experiment, all Ka's and the true V_m should be determined over a range of pH, using the method of Florini & Vestling. This would give:

$K_a(app) = K_a . \frac{fa.\ fe}{fea}$, or $pK'a_{app} = pK'a + pfa + pfe - pfae.$

$K'a(app) = K'a . \frac{fa.feb}{feab}$, or $pK'a_{app} = pK'a + pfa + pfeb - pfeab.$

$V_{m.}(app) = \frac{V_m}{feab}$, or $\log V_{m_{app}} = \log V_m - \log feab.$

Similarly with K_b and K'b.

This however, would be an extremely tedious method. One can shorten it to two series of experiments by determining K_m and $V_{m_{app}}$. with respect to one substrate at very high and very low concentrations of one substrate. Vo is the velocity at very low concentrations of a.

Thus: $b \gg \{K_b, K'b\}$,

$$V_{ma_{app}} = \frac{V_m}{feab} \quad , \quad Km = K'a \ \frac{feb}{feab} \cdot fa \ , \ Vo = \frac{V_{m.\ a}}{K'a.\ feb.\ fa.} \ .$$

$b \ll \{K_b, K'b\}$,

$$V_{ma_{app}} = \frac{V_{m.\ b}}{K'b\ fea\ fb.} \quad , \quad K_{ma} = K_a \ \frac{fe.\ fa}{fea} \ , \ Vo = \frac{V_{m.\ b.\ a}}{KaK'b\ fa.fb.fe} \ .$$

If an intermediate case is taken where, as in the experiments described earlier, the concentration of the co-substrate is of the order of the substrate constant, we get

$b \doteqdot \{K_b, K'b\}$,

$$V_{ma_{app}} = \frac{V_{m.\ b}}{feab.b + K'b.\ fea.\ fb.} \ ,$$

$$K_{ma} = K'a.\ fa.\ \frac{Kb.fe.fb. + b.feb}{K'b.fea.fb + b.feab} \ ,$$

$$Vo = \frac{V_{m.\ a.\ b}}{fa.\ K'a\ (Kb.\ fe.fb + b.feb)} \ .$$

It is probably this intermediate case that has led to anomalous behaviour of the logarthmic plots obtained for the aryltransferase, i.e. plots of the Km GSH will be affected by ionization of the various complexes able to bind the aromatic substrate. To remove this ambiguity of interpretation, the work should be repeated working at limiting co-substrate concentrations as outlined.

Variation of Inhibition with pH

The general equation for the variation of v_i over a pH range is as below:

$$v_i = \frac{V_m.\ a\ (1 + \frac{\gamma}{\alpha\beta} \cdot \frac{I}{K_i})}{a\ (feab + \frac{I}{\alpha\beta K_i} \cdot feiab) + K'a\ (feb + \frac{I}{\alpha K_i} \cdot feib)} \Bigg/ 1 + \frac{1}{b}\left\{\frac{KaK'b\ (fe. + \frac{I}{K_i} \cdot fei) + aK'b\ (fea + \frac{I}{\alpha K_i} \cdot feia)}{a\ (feab + \frac{I}{\alpha\beta K_i} \cdot feiab) + K'a\ (feb + \frac{I}{\beta K_i} \cdot feib)}\right\}$$

This rather fearsome equation is, in fact, because of the high degree of symmetry, iittle more complex than that where pH changes are ignored. For clarity, pH functions for A,B, and I have been omitted. This can be rectified by substituting $\frac{a}{fa}$, $\frac{b}{fb}$ and $\frac{I}{fi}$ for a, b and I respectively.

To obtain the expression for the experimentally determined apparent K_i, for any particular type of inhibition, one has only to substitute for K_a, $K'a$, K_b, $K'b$, K_i, α, and β in the appropriate expression obtained from appendix A, as detailed below:

$$K_a.\ app. = \frac{Ka.fa.fe}{fea} \qquad K'a\ app = \frac{K'a\ fa.\ feb}{feab}$$

$$Kb.\ app. = Kb.\ \frac{fe.fb}{feb} \qquad K'b\ app = K'b\ \frac{fea.fb}{feab}$$

$$K_i\ app. = K_i\ \frac{fe.fi}{fei} \qquad \alpha_{app} = \alpha\ \frac{fei}{fe}\frac{fea}{feia}$$

$$\beta\ app = \beta\ \frac{feb}{fe}\ \frac{fei}{feib}$$

To relate this to the experimental work, in the type of inhibition studied (competitive with respect to GSH only) the apparent $K_{i_{GSH}}$

determined by either of the two plotting methods is

$$K_{i_{G(app)}} = K_{i\,(app)}\beta . \frac{(Kd.app + d)}{(\beta Kd.app + d)}$$

This becomes

$$K'i_{G(app)} = \beta . K_i fi . \frac{(Kd.\ fe.\ fd. + d.fed.)}{(\beta Kd.fei.fd + d.feid)}$$

This is, to a first approximation, capable of being interpreted by the use of Dixon's rules (Dixon 1953). At high and low d, where $K_i = K_i\beta . \frac{fi.fed}{feid}$ and $K_i . \frac{fi.fe.}{fei}$, Dixon's rules can be applied with no ambiguity.

If, however, the K_i is determined with respect to the aromatic substrate $K_{i_D(app)} = K_{i_{(app.)}}(1 + \frac{g}{Kg(app)})$ as determined from the intercept of the $\frac{1}{v}$ vs I type plot. This becomes $K_{i_{D(app)}} = K_i . \frac{fi}{fei} . (fe + \frac{g.feg}{Kg.fg})$.

This expression is obviously going to be influenced by irrelevant ionization, and Dixon's rules will not be applicable.

It is relevant here to mention the theoretical work of J.L. Webb (1963) in this field. Webb appears to have been the first to recognise that calculation of K_i's from experimental data might require the use of algebraic functions involving quite complex combinations of pH functions, so that the use of Dixon's rules would result in an incorrect interpretation. He showed that, for several different simple enzyme systems, depending on the type of plotting methods used, several different apparent K_i's could be obtained (see Table B).

However, Webb's work is misleading and inconsistent. For the

$\frac{1}{v}$ vs $\frac{1}{s}$, and $\frac{1}{v}$ vs I plots, K_i's have been calculated from the gradient using the <u>true</u> K_s. This could cause confusion as the common practice seems to be to calculate K_i's from the experimentally determined K_m at that pH. Further, in calculating K_i's from the third type of plot (I $(\frac{1-i}{i})$ vs S), Webb implicitly uses the apparent K_s, obtaining a series of expressions which are thus inconsistent with the previous two series.

Expressions for the apparent K_i, calculated using the apparent K_s, obtained from the three plotting methods, are also shown in table 2. It will be seen that if the K_i's are obtained only from the gradients in $\frac{1}{v}$ vs $\frac{1}{s}$ type plots, then Dixon's rules can be applied to all the cases examined.

This digression is relevant to the interpretation of some of the experimental results presented here. In the $pK_{m\ GSH}$, log V_m, and log V_o vs pH curves for the grass grub enzyme, the two-fold inflection at pH 9.3 could be due to either a double ionization of GSH, or the enzyme, or to a single ionization of each. The pK_i vs pH plot also has this inflection and as the Dixon treatment assumes that $pK_{i_{app}} = pK_i + pfi + pfe - pfei$, one concludes that an ionization of GSH will not affect the plot, and that the inflection is due to a double ionization of the enzyme only.

Webb's expression for a K_i determined in the same manner for competitive inhibition is

$$K_{i(app)} = \frac{I.\ K_i}{fs.I + (fe.fs-1)\ K_i}$$

In this case, it would be impossible to state which of the three alternative situations existed, as the expression contains both fe and fs. The demonstration that these experimental data can be interpreted by Dixon's rules removes the apparent ambiguity.

Table B.

Experimentally derived K_i's

K_i determined from	$K_m = K_s$		$K_m = K_s.fe.fs$		
Type of plot	A	D	A	D	F
Ionizing species					
I I only	$fi.K_i$	$fi.K_i$	$fi.K_i$	$fi.K_i$	$fi.K_i$
II S only	$\frac{I \quad K_i}{fs. \; I + (fs-1) \; K_i}$	$\frac{K_i}{fs}$	K_i	K_i	K_i
III I and S	$\frac{Ifi.K_i}{fs. \; I + (fs-1) \; K_i}$	$\frac{fi}{fs} K_i$	$fi.K_i$	$fi.K_i$	$fi.K_i$
IV E and S 1 Competition on active form HE	$\frac{I.K_i}{fs. \; I + (fhe.fs-1) \; K_i}$	$\frac{K_i}{fs}$	$fhe.K_i$	$fhe. \; K_i$	$fhe.K_i$
2 Competition on active form E	$\frac{I. \quad K_i}{fs. \; I + (fe. \; fs-1) \; K_i}$	$\frac{K_i}{fs}$	feK_i	$fe.K_i$	$fe.K_i$
3 Non-competitive an active form HE	$\frac{I.K_i}{fs. \; I + (fhe.fs-1) \; K_i}$	$\frac{(S + Ks).K_i}{S + fs.Ks}$	$\frac{feh.K_i}{K_i}$ (3a)	$\frac{S + Ks.feh \; fs}{S + Ks.fs} . K_i$	K_i
4 Non. comp. an active form E	$\frac{I. \quad K_i}{fs. \quad I+(fe.fs-1) \; K_i}$	$\frac{(S + Ks).K_i}{S + fs.Ks}$	$\frac{fe.K_i}{K_i}$ (4a)	$\frac{S + Ks.fe.fs}{S + Ks. \; fs} . K_i$	K_i
5 Inhibition on inactive HE	$\frac{I. \; K_i}{fs. \; I. \frac{Ka}{H} + (fs.fhe-1) \; K_i}$	$\frac{H}{Ka.K_i}$	$fe.K_i$	$fe.K_i$	$fe.K_i$
6 Inhibition on inactive E	$\frac{I. \quad K_i}{fs. \; I.\frac{H}{Ka} + (fs.fhe-1) \; K_i}$	$\frac{Ka. \; K_i}{H}$	$fhe. \; K_i$	$f_{he}.K_i$	$f_{he}.K_i$

K_i's estimated from slope in plots A + D; intercept in Plot F, and from the apparent V_m in A, 3 a' and (4 a.).

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