THE TRYPTOPHAN INHIBITED 3-DEOXY-D-*ARABINO* HEPTULOSONATE 7-PHOSPHATE SYNTHASE FROM *NEUROSPORA CRASSA*

by

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To my parents.

STATEMENT

The results described in this thesis were obtained by myself under the supervision of Dr. C. H. Doy and Dr. D. M. Halsall in the Department of Genetics of the Research School of Biological Sciences at the Australian National University from December 1971 to April 1975 during the tenure of an Australian National University Research Scholarship.

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PREFACE

Figures, tables and equations are identified by two numbers. The first number indicates the chapter, and the second indicates the number of the figure, table or equation within the chapter.

The abbreviations used are:

PEP	phosphoenolpyruvate
E4P	D-erythrose 4-phosphate
Pi	inorganic phosphate
DAHP	3-deoxy-D-*arabino* heptulosonate 7-phosphate
Phe	L-phenylalanine
Tyr	L-tyrosine
Trp	L-tryptophan
aro	aromatic (nomenclature for the common aromatic pathway, formerly abbreviated as arom in *Neurospora crassa*)
NAD, $NADH^+$	oxidised and reduced nicotinamide adenine dinucleotide
NADP, $NADPH^+$	oxidised and reduced nicotinamide adenine dinucleotide phosphate
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
Tris	*tris*-(hydroxymethyl)-aminoethane
Rm	relative mobility with respect to the dye bromophenol blue
A	absorbance
S	Svedberg units
K_m	Michaelis constant
K_i	Inhibitor constant

mM	millimolar
μM	micromolar
nm	nanometre
w/v	weight/volume

Molecular weights are expressed in dalton units. In general, abbreviations used are those recommended by the editors of Biochimica et Biophysica Acta.

ABSTRACT

In Neurospora crassa, DAHP synthase activity consists of three classes of isoenzymes each of which is feedback inhibited by one of the aromatic amino acids, phenylalanine, tyrosine or tryptophan.

This thesis describes the purification and properties of the tryptophan inhibited DAHP synthase.

DAHP synthase (Trp) has been purified 390 fold in terms of specific activity and 17 000 fold in terms of protein recovery. Purification results in partial loss of activity. A sulphydryl reagent is required for stability and recovery of activity. A divalent metal ion may be important for activity and inhibition. Co^{2+} can restore total activity inhibited by EDTA and in the presence of Zn^{2+}, enzyme activity is reduced but the enzyme is less sensitive to inhibition by Trp.

Indirect evidence shows that the enzyme can exist in several conformations and a specific conformation is required for inhibition by Trp. There also appears to be some interaction between the DAHP synthase (Trp) and DAHP synthase (Phe) activities.

Polyacrylamide gel electrophoresis of the purified enzyme shows two bands, one active and Trp-inhibitable (slow) and one inactive (fast). The active slow band has a molecular weight of about 235 000 and is probably made up of four units of a 59 000 component. The inactive fast band has a molecular weight of about 210 000 and samples can be obtained that contain only a 48 000 component on dodecyl sulphate gel or for some samples, show, in addition to the 48 000 component, a minor contribution from a 59 000 component. On storage, the enzyme loses its activity and the active slow band disappears concomitant with an increase in intensity at the

position of the inactive fast band.

Since aro-8 codes for DAHP synthase (Trp) activity, the 59 000 component must be a product of this locus. The genetic origin of the 48 000 component is not known. As shown by tryptic peptide mapping, the 48 000 component is not a degradation product of the 59 000 component.

The purified enzyme has a weight-average sedimentation coefficient of 9.6 S at a concentration of 1 mg protein per ml and the S value decreases with decrease in protein concentration. Results from sedimentation equilibrium experiments show that the enzyme preparation consists of more than one molecular form and these forms may be undergoing dissociation and association during centrifugation.

The methods of zonal and frontal analyses on gel filtration columns were used to determine the molecular weight of DAHP synthase (Trp) activity. There is a difference in the molecular weight determined by the zonal (240 000) and frontal (165 000) methods which can be explained possibly on the basis of interaction of the lower molecular weight components with the larger active component, resulting in distortion of the elution profile from the frontal column.

Four of the protein peaks as indicated on the elution profile from the hydroxylapatite column and all three protein peaks from the G200 column may contain component that belongs to DAHP synthase (Trp).

The substrates PEP and E4P and the product Pi can protect the purified enzyme against heat denaturation whereas Trp has no effect.

The kinetics of the reaction catalysed by DAHP synthase (Trp) shows that the reaction probably proceeds via a two-site ping-pong bi bi mechanism, with activation by PEP, the first

substrate, and inhibition by E4P, the second substrate. At low substrates concentrations, K_{PEP} is 0.1 mM and K_{E4P} is 0.13 mM.

Product inhibition by Pi is linear noncompetitive with respect to PEP (K_{islope} = 22 mM and $K_{iintercept}$ = 54 mM), and S-linear competitive with respect to E4P (K_{islope} = 25 mM).

The enzyme has an activity optimum at pH 7.3 and a Trp inhibition optimum at pH 6.4. Inhibition by Trp is non-competitive with respect to PEP and S-parabolic competitive with respect to E4P. The $Trp_{0.5}$ is 4 μM. A possible molecular model for the DAHP synthase (Trp) reaction is considered.

Whereas negative cooperativity is not consistent with the symmetry model, it provides strong evidence in favour of a sequential (induced-fit) model for this enzyme.

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CHAPTER 1

DAHP SYNTHASES AND AROMATIC BIOSYNTHESIS IN NEUROSPORA CRASSA

INTRODUCTION

Metabolic processes are basically networks of chemical reactions each of which is controlled by an enzyme. Enzymes are subject to control mechanisms. The net result is a balanced internal environment that can adjust to changes. The two most extensively studied regulatory mechanisms are (1) repression and induction which control the amount of enzymes synthesised and (2) allosteric regulation of enzyme activity.

Repression and induction are readily observed in prokaryotes but are less common in eukaryotes. Perhaps as the degree of functional differentiation increases and becomes more specialised, as growth becomes slower and the environment becomes constant, repression and induction become either unnecessary or inefficient. It may be better to induce or repress the genome in a more permanent manner and control enzyme concentration by degradation and activity by allosteric regulation.

Allosteric regulation was recognised since the discovery of feedback control by end product inhibition in biosynthetic pathways by Umbarger (1956) and Yates and Pardee (1956). In an unbranched bio-

synthetic pathway, the end product, but not the intermediate metabolites, acts as an inhibitor of the first reaction in the sequence. The intermediate enzymatic reactions are not affected by the end product (Monod *et al.*, 1963). In branched biosynthetic pathways, regulatory mechanisms are more complicated and varied.

The biosynthetic pathway that leads to the synthesis of the aromatic amino acids and other vitamins is one example of a branched pathway (Fig. 1.1). The first step in this pathway is catalysed by a regulatory enzyme system, 3-deoxy-D-*arabino* heptulosonate 7-phosphate synthase (DAHP synthase) E.C.4.1.2.15. This chapter will discuss details of this system in *Neurospora crassa* and other background information (including unpublished results from this laboratory) up to the beginning of 1972 when this work began. Similar systems in other organisms are extensively reviewed by Doy (1968b) and Pittard and Gibson (1970).

I. BIOSYNTHESIS OF AROMATIC AMINO ACIDS AND VITAMINS IN NEUROSPORA CRASSA

The first step in the aromatic biosynthetic pathway is a reaction of phosphoenolpyruvate (PEP) with erythrose 4-phosphate (E4P) catalysed by DAHP synthase. The product of this reaction 3-deoxy-D-*arabino* heptulosonate 7-phosphate (DAHP) undergoes six enzymatic steps to chorismic acid which is the branch point compound for the synthesis of all the end products (Gibson, 1964). This portion of the biosynthetic sequence is common to all the end products and is called the common aromatic or shikimate pathway.

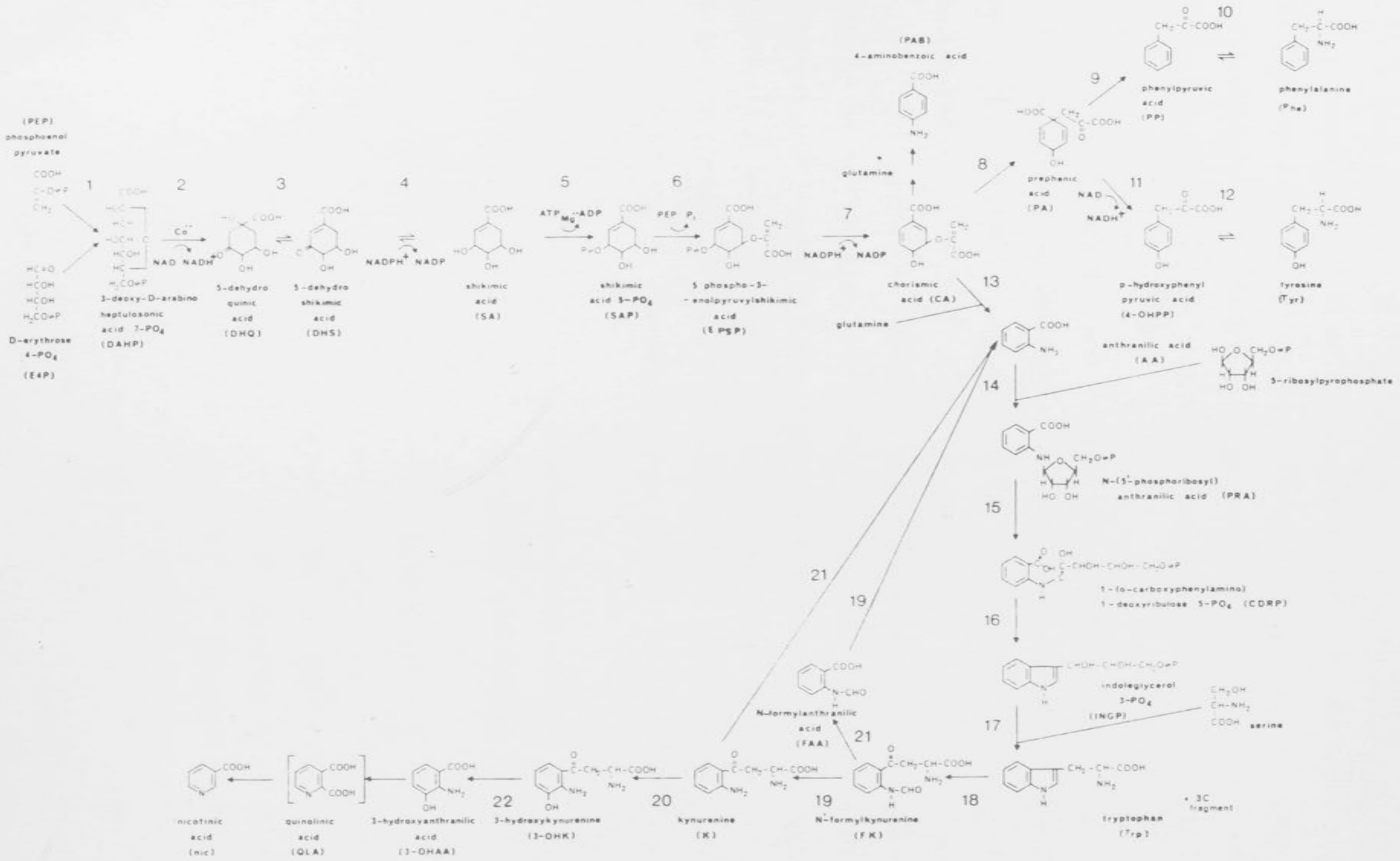

(PEP)
phosphoenol pyruvate
D-erythrose 4-PO4 (E4P)
1
2
Co++
NAD NADH
3-deoxy-D-arabino heptulosonic acid 7-PO4 (DAHP)
3
5-dehydro quinic acid (DHQ)
5-dehydro shikimic acid (DHS)
4
NADPH+ NADP
shikimic acid (SA)
5
ATP ADP Mg++
shikimic acid 5-PO4 (SAP)
6
PEP Pi
5 phospho-3- - enolpyruvylshikimic acid (EPSP)
7
NADPH NADP
chorismic acid (CA)
glutamine
(PAB)
4-aminobenzoic acid
glutamine
8
prephenic acid (PA)
9
phenylpyruvic acid (PP)
10
phenylalanine (Phe)
11
NAD NADH
12
p-hydroxyphenyl pyruvic acid (4-OHPP)
tyrosine (Tyr)
13
glutamine
anthranilic acid (AA)
5-ribosylpyrophosphate
14
N-(5'-phosphoribosyl) anthranilic acid (PRA)
15
1-(o-carboxyphenylamino) 1-deoxyribulose 5-PO4 (CDRP)
16
indoleglycerol 3-PO4 (INGP)
serine
17
tryptophan (Trp)
+ 3C fragment
18
N'-formylkynurenine (FK)
19
kynurenine (K)
20
3-hydroxykynurenine (3-OHK)
22
3-hydroxyanthranilic acid (3-OHAA)
quinolinic acid (QLA)
nicotinic acid (nic)
21
N-formylanthranilic acid (FAA)
21
19

Fig. 1.1 The pathway of aromatic biosynthesis in *Neurospora crassa*.

Commonly used names for the enzyme functions are:

1 3-deoxy-D-*arabino* heptulosonate 7-phosphate synthase
2 5-dehydroquinate synthase
3 5-dehydroquinase I
4- 5-dehydroshikimate reductase
5 shikimate kinase
6 3-enolpyruvylshikimate 5-phosphate synthase
7 chorismate synthase
8 chorismate mutase
9 prephenate dehydratase
10,12 amino-transferase
11 prephenate dehydrogenase
13 anthranilate synthase
14 anthranilate 5-phosphoribosyl-1-pyrophosphate phosphoribosyl transferase
15 N-(5'-phosphoribosyl) anthranilate isomerase
16 indoleglycerol 3-phosphate synthase
17 tryptophan synthase
18 tryptophan pyrrolase
19 kynurenine formamidase
20 kynurenine 3-hydroxylase
21 kynureninase
22 hydroxykynureninase

In Neurospora crassa four end products are known, namely, phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and 4-aminobenzoate (PAB) which is an intermediate in the synthesis of folic acid. Nicotinic acid in Neurospora is synthesized from tryptophan (Partridge, Bonner and Yanofsky, 1952) by way of formylkynurenine, kynurenine (Yanofsky and Bonner, 1950, 1951), 3-hydroxyanthranilic acid, and quinolinic acid (Yanofsky, 1955). Excess tryptophan is degraded to anthranilate and excreted into the medium (Matchett and DeMoss, 1963).

(a) Common Aromatic Pathway

Steps two through six ending at 3-enolpyruvylshikimate 5-phosphate (EPSP) are catalysed by a multifunctional enzyme aggregate, which is coded for by a set of closely linked genes, the aro gene cluster (Gross and Fein, 1960; Giles, Case, Partridge and Ahmed, 1967a). This aro gene cluster on linkage group II (Fig. 1.2) bears some resemblance to a prokaryote's operon but it is not a definite operon since no operator region has been identified. The cluster is transcribed in a polarised fashion via a single messenger RNA. Nonsense mutations at one end of the region lead to the loss of all five activities (Case and Giles, 1968). Fig 1.3 shows the gene-enzyme relationships in the common aromatic pathway.

The aro multi-enzyme complex in N. crassa has been purified (Burgoyne et al., 1969; Gaertner and DeMoss, 1970). The molecular weight of the wild-type aggregate was found to be approximately 230 000 which was dissociable into a half molecular weight component by 6M guanidine hydrochloride or dodecyl sulphate. More recent work

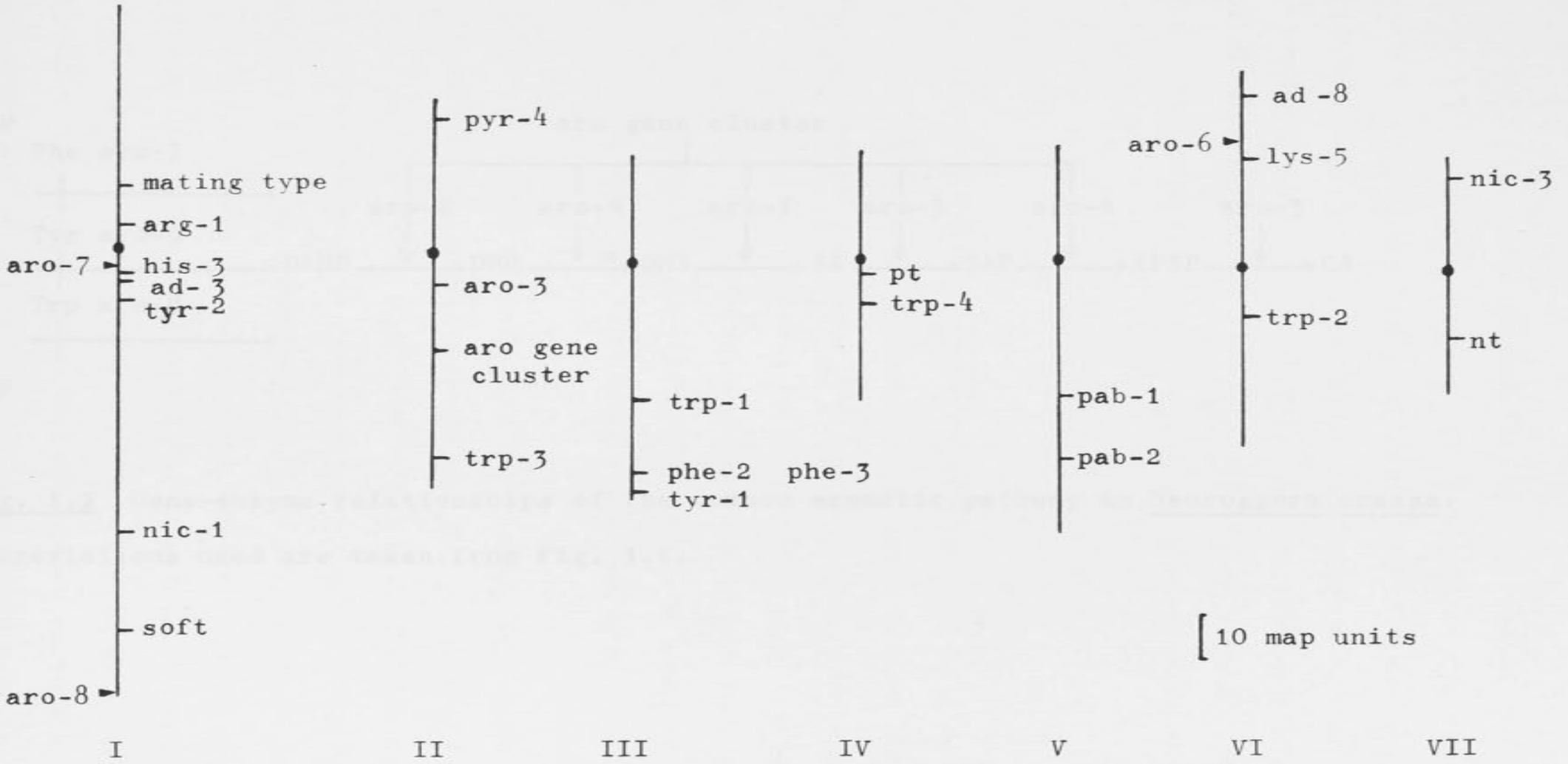


Fig. 1.2 Linkage groups of Neurospora crassa showing the location of the three structural genes for the DAHP synthase isoenzymes (aro-6, aro-7, aro-8) (Halsall and Doy 1969) and their relationship to other structural genes concerned with aromatic biosynthesis (aro = common path, trp = tryptophan, tyr = tyrosine, phe = phenylalanine, pt = phenylalanine tyrosine, pab = p-aminobenzoate). Linkage groups are shown with the left arm uppermost.

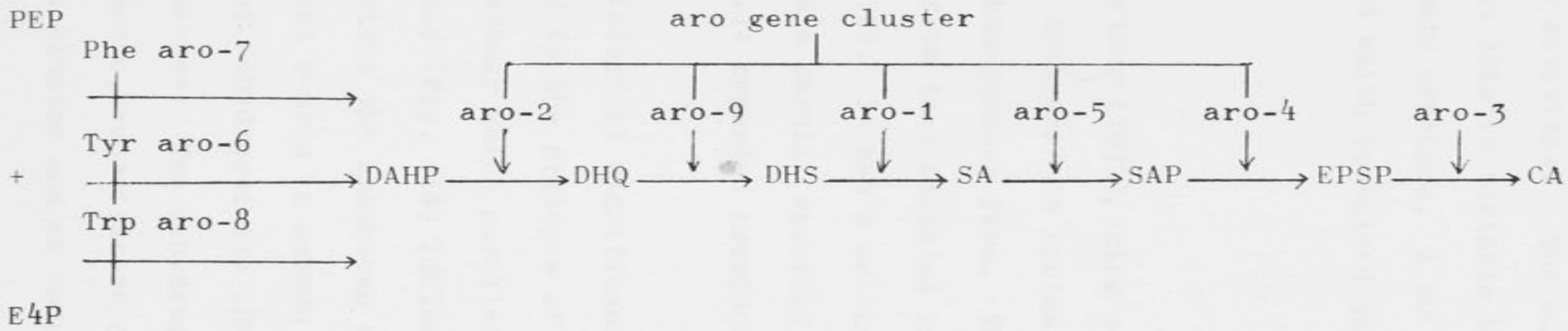


Fig. 1.3 Gene-enzyme relationships of the common aromatic pathway in Neurospora crassa. Abbreviations used are taken from Fig. 1.1.

(Jacobson et al., 1972) showed that the aggregate could be dissociated into 80 000 and 20 000 components. Both the 80 000 and the 20 000 components contain 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase activities. The 80 000 aggregate also contains a third function but this is variable being either shikimate kinase or 5-dehydroquinate synthase. A 60 000 molecular weight component could be recovered which contained only 3-enolpyruvylshikimate 5-phosphate synthase.

Gaertner (1972), using a procedure originally designed to isolate the anthranilate synthase complex, isolated the aromatic complex in homogeneous form. This aro complex differs in physical properties from that isolated by Burgoyne et al., (1969) and Jacobson et al., (1972). It has a molecular weight of 290 000, a sedimentation of 10.6S and a partial specific volume of 0.72 compared with 230 000, 11.4S and 0.74 reported previously (Burgoyne et al., 1969).

A biological significance for the aro aggregate in N. crassa is suggested by the presence of an additional inducible aromatic catabolic pathway which parallels in one step the aromatic biosynthetic pathway (Fig. 1.4) (Giles et al., 1967b). This catabolic pathway involves the breakdown of quinic acid (QA) to protocatechuic acid (PCA) and shares in common with the biosynthetic pathway the conversion of dehydroquinate (DHQ) to dehydroshikimate (DHS) catalysed by dehydroquinase. Two dehydroquinases were isolated in N. crassa, the biosynthetic, constitutive dehydroquinase encoded in aro-9 of the aro gene cluster and an inducible dehydroquinase encoded in the

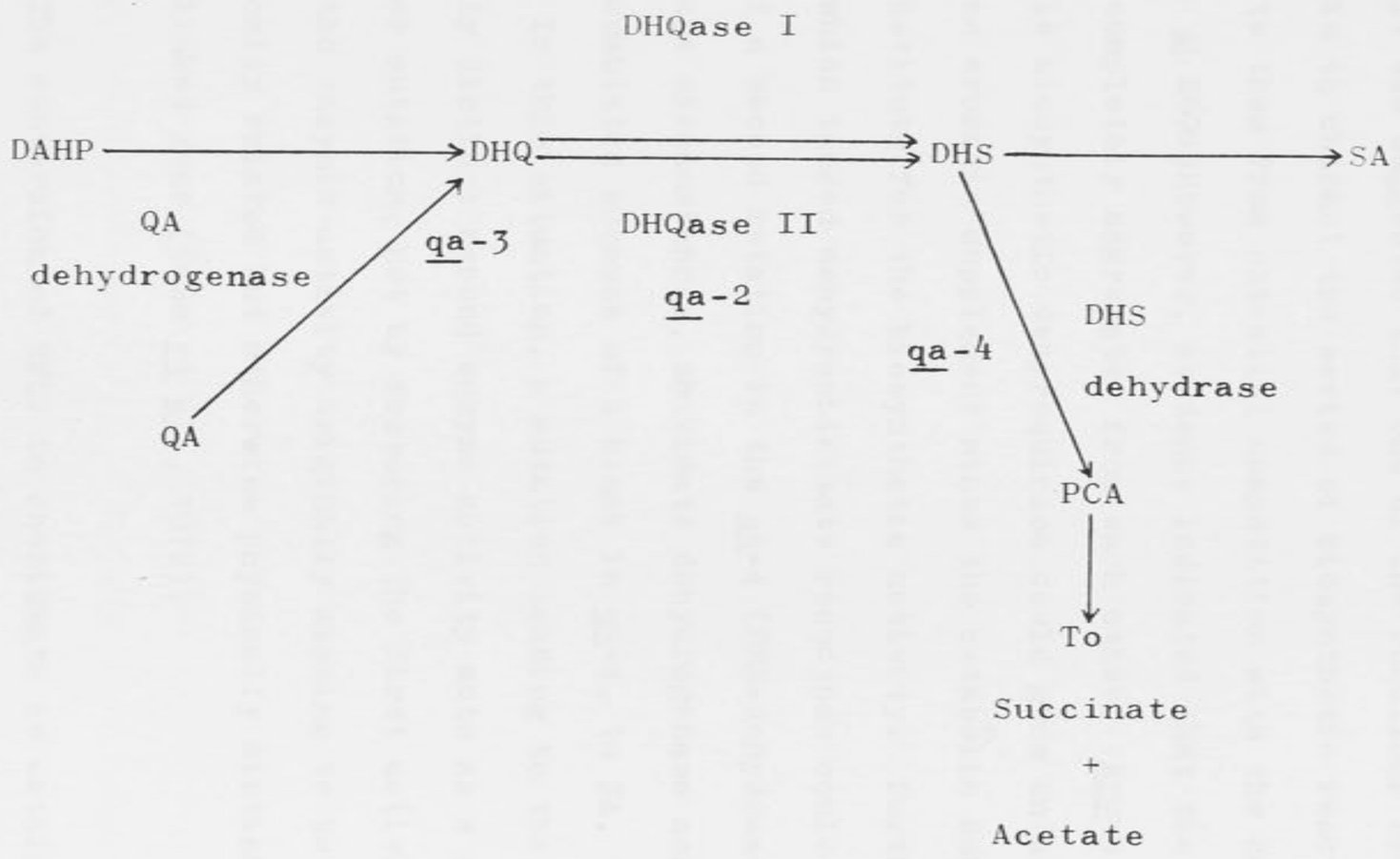


Fig. 1.4 Gene-enzyme relationships of the catabolic quinate pathway and its relationship with the common aromatic pathway.

QA, quinic acid; PCA, protocatachuic acid. Other abbreviations used are taken from Fig. 1.1.

qa-2 structural gene of a second cluster - the qa cluster, containing four genes, three structural and one regulatory, in linkage group VII (Rines, 1968; Chaleff, 1971; Valone, Case and Giles, 1971).

It was suggested that one of the functions of the aro gene cluster is to channel the series of biosynthetic reactions and thus to isolate them from potential competition with the catabolic pathway (Giles, et al. 1967). However, evidence indicated that the two pathways are not completely segregated from each other. Aro-9 mutants lacking detectable biosynthetic dehyroquinase could grow on a minimal medium lacking an aromatic supplement since the catabolic dehydroquinase could substitute for the biosynthetic activity. Furthermore, aro-1 mutants which lacked dehydroshikimate reductase could grow in minimal medium if a second mutation in the qa-4 (DHS-dehydrase) was present. Under these circumstances, shikimate dehydrogenase converted DHS, which accumulated because of a block in qa-4, to SA, thus permitting growth. In this situation, a mutation leading to the loss of a biochemically distinct second enzyme activity acts as a special type of suppressor mutation, not by restoring the first activity, but by causing the enzymic activity originally missing to be replaced by a catalytically related but otherwise physically distinct enzyme encoded in an unlinked gene (Case et al., 1972).

The conversion of EPSP to chorismate is catalysed by chorismate synthase which exists in two forms (Gaertner and DeMoss, 1970).

(b) Post Chorismate Pathway

In the phenylalanine-tyrosine specific portion of the pathway,

only one chrosimate mutase has been demonstrated and this enzyme does not form a stable, functional aggregate with either prephenate dehydratase or prephenate dehydrogenase (Baker, 1968). Chorismate mutase is feedback inhibited by phenylalanine or tyrosine and the activity of either inhibitor is antagonised by tryptophan which acts as an activator (Baker, 1966). Prephenate dehydrogenase, the first enzyme in the tyrosine specific pathway, is inhibited by tyrosine and activated by phenylalanine (Catcheside, 1969). Prephenate dehydratase, the first enzyme in the phenylalanine specific pathway, is inhibited by phenylalanine and unaffected by tyrosine. Anthranilate synthase, the first enzyme in the tryptophan specific pathway, is inhibited by tryptophan. The combined effect of activation of chorismate mutase and prephenate dehydrogenase is stimulation of the biosynthesis of tyrosine. This increase in tyrosine synthesis is thought to be important in supply of the large quantities of tyrosine required during sexual differentiation when tyrosine is converted to precursors of melanin which is required for maturation of protoperithecia, perithecia and ascospores (Catcheside, 1969).

There are four unlinked genes specifying the enzymes which catalyse the five steps in the conversion of chorismate to tryptophan (Fig. 1.5) (Ahmad and Catcheside, 1960; DeMoss and Wegman, 1965; DeMoss, 1965). The polypeptide specific to anthranilate synthase (product of Trp 2) is associated with N-(5'-phosphoribosyl) anthranilate (PRA) isomerase and Indole-3-glycerolphosphate (InGP) synthase (products of Trp 1) to form a complex with a sedimentation coefficient of 10.3S and a molecular weight of 240 000 (Gaertner and DeMoss, 1969). The complex can be dissocated into 7.4S and 4.4S units by 4-hydroxymercuribenzoate.

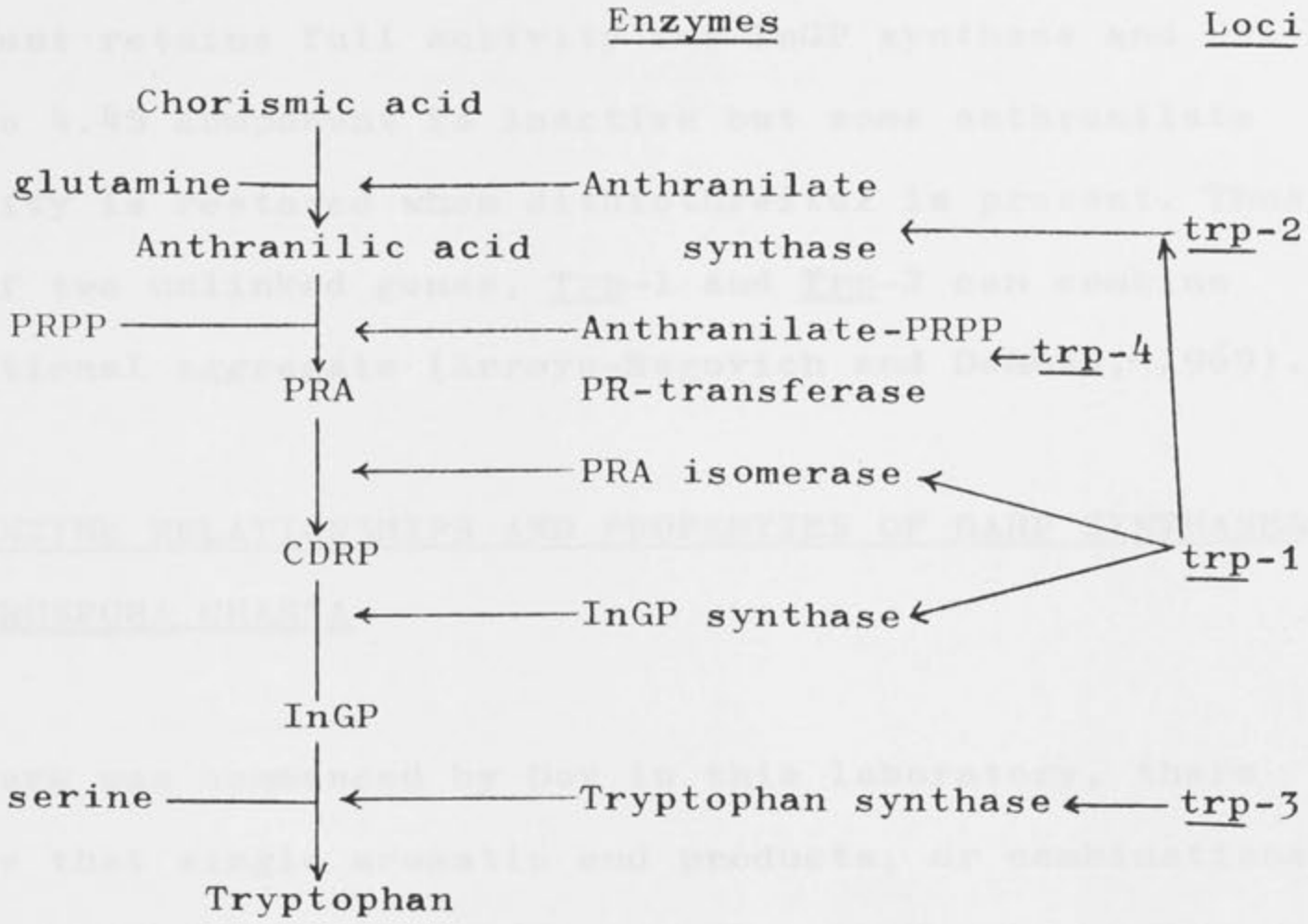


Fig. 1.5 Gene-enzyme relationships of the tryptophan biosynthetic pathway of Neurospora crassa.

PRPP, 5-phosphoribosyl 1-pyrophosphate; PRA, N-(5'-phosphoribosyl) anthranilic acid; CDRP, 1-(O-carboxyphenylamino) 1-deoxyribulose 5-phosphate; InGP, indoleglycerol 3-phosphate; PR, phosphoribosyl.

The 7.4S fragment retains full activity for InGP synthase and PRA isomerase. The 4.4S component is inactive but some anthranilate synthase activity is restored when dithiothreitol is present. Thus, the products of two unlinked genes, Trp-1 and Trp-2 can combine to form a functional aggregate (Arroyo-Begovich and DeMoss, 1969).

II. GENE-ENZYME RELATIONSHIPS AND PROPERTIES OF DAHP SYNTHASES IN NEUROSPORA CRASSA

When work was commenced by Doy in this laboratory, there was no evidence that single aromatic end products, or combinations of them, normally inhibited the common pathway in N. crassa in such a way as to prevent the synthesis of other end products (Doy, 1968b). It was thought that the aromatic end products might inhibit the first enzyme, DAHP synthase, in the biosynthetic sequence. This has been demonstrated. Tyrosine and phenylalanine were shown to inhibit about 82% of the DAHP synthase activity in crude extracts of wild-type N. crassa 74a (Doy, 1966). In this early work, inhibition by tryptophan could not be demonstrated. Later work (Doy, 1967) showed that tryptophan inhibited strongly that portion of the activity not inhibited by phenylalanine and tyrosine. It was also shown that tryptophan inhibited a portion of activity not inhibited by phenylalanine plus tyrosine in Escherichia coli W (Doy, 1967).

In later work with wild-type N. crassa, a typical dialysed crude extract is inhibited about 44% by either tyrosine or phenylalanine and about 10% by tryptophan (Doy, 1968b).

However, this distribution showed wide variation (Halsall, 1969; Hoffmann, 1971). At subsaturating levels, a mixture of phenylalanine and tyrosine inhibits synergistically (Doy, 1968a).

Up to the beginning of 1968 (Doy, 1968a), evidence for the existence of isoenzymes of DAHP synthases sensitive to different inhibitors was accumulating but was not unequivocal. Studies with crude extracts showed an activity plateau between pH 6.2 and pH 8.0 and an activity optimum at 37°C.

The strongest evidence for the existence of three isoenzymes of DAHP synthase came from molecular sieving experiments. When crude extracts were sieved on agarose columns in the presence of PEP, dithiothreitol (DTT) and Mg^{2+}, three kinds of activity were separated, each inhibited by one of the three aromatic amino acids (Doy, 1968b).

Co^{2+} stimulates crude extracts. Activity is strongly inhibited by EDTA. The Tyr- and Phe- sensitive portions are inhibited whereas that of Trp-sensitive activity remains. Addition of Co^{2+} reverses the inhibition and stimulates activity to a higher level with a concomitant increase in Trp-sensitive activity (Doy, unpublished results). Mn^{2+} reverses EDTA but does not stimulate original activity (Doy, 1968b).

In molecular sieving experiments on agarose columns, in the absence of Co^{2+}, the activity recovered approximates to the sum of the separately inhibited activities. In the presence of Co^{2+}, about twice the overall activity is recovered. A specific form of DAHP

synthase (Trp) is unaffected. Fractions which previously were specifically inhibited by Tyr and Phe are inhibited by Trp and the sum of inhibitions by all three allosteric ligands is greatly in excess of the total activity. Thus Co^{2+} has stimulated activity and created cross-inhibition (Doy, unpublished results). Changes of this kind were easily detected but varied beyond the control of the experimenter.

4-Chloromercuribenzoate completely inhibits activity, indicating the importance of thiol groups (Doy, 1968b). Dialysed crude extracts when diluted, lose 80-90% activity within a few minutes. Undiluted extracts (12-17 mg/ml) are stable at room temperature and at 37^{0}C for one hour but are unstable at 45^{0}C. Phosphoenolpyruvate gives considerable protection against the deactivation but E4P does not. The tryptophan sensitive portion and a non-inhibited portion survive all treatments. Phosphoenol-pyruvate protects the tyrosine sensitive portion selectively whereas the phenylalanine sensitive portion is protected by phenyl-alanine (Doy, 1968b). These results show that the portions sensitive to different negative modifiers have different properties. During deactivation, there is no gain of non-inhibited activity which means that either the active sites alone are lost, or that both active and inhibitor sites are lost together. These results suggest that separate isoenzymes sensitive to different inhibitors exist. Furthermore, mutations affecting DAHP synthase were unknown, suggesting that more than one mutation was required for all activity to be lost (Doy and Halsall, 1968).

In 1967, the search for mutation affecting DAHP synthase began (Halsall, 1969). By the middle of 1968, the first mutant lacking Tyr-inhibitable DAHP synthase activity (DAHP synthase (Tyr))

was obtained and the mutation was named aro-6. The aro nomenclature is used in Neurospora to identify the genes which code for the enzymes of the common aromatic pathway leading to chorismate. Later, mutants lacking phenylalanine-inhibitable activity (DAHP synthase (Phe), mutation in aro-7) and mutants lacking Trp-inhibitable activity (DAHP synthase (Trp), mutation in aro-8) were isolated. The three genes, aro-6, aro-7 and aro-8 are widely separated in the genome. Aro-6 is located on linkage group VI L; aro-7 is on linkage group I R near the centromere and aro-8 is at the most distal end of linkage group I R (Fig. 1.2), (Halsall and Doy, 1969). Recombinants containing all three classes of mutation (aro-6, aro-7 and aro-8) required a complete supplements of aromatic end products for growth and no DAHP synthase was detected.

From strains carrying two activity-negative mutations, mutants resistant to each allosteric ligand were isolated and the locations of the mutations were determined by the frequency of recombination between the activity-negative locus and the resistance locus. No recombinants were found. This indicates that the resistance loci and the activity loci are closely linked and possibly pleiotropic. The resistance mutations were named aro-6^r, for non-inhibitable DAHP synthase (Tyr), aro-7^r, for non-inhibitable DAHP synthase (Phe), and aro-8^r, for non-inhibitable DAHP synthase (Trp).

Forced heterocaryon tests also failed to demonstrate complementation between activity-negative and allosteric inhibition mutations. Mixtures of extracts of activity and allosteric inhibition mutant strains, when subjected to conditions favourable to

association and dissociation of subunit polypeptides, also could not demonstrate enzymic complementation. The absence of recombination and complementation *in vitro* (enzymic) and *in vivo* (forced heterocaryon) indicates that the two classes of mutations associated with a specific isoenzyme are **probably pleiotropic (Halsall and Doy, 1969).**

Some *aro*-6^r mutants were isolated that required PEP for *in vitro* stability of the non-inhibitable enzyme derived from DAHP synthase (*Tyr*). The mutation was revertible and the revertants regained both stable activity and inhibition by tyrosine. Similar pleiotropic mutants for DAHP synthase (*Phe*) have also been isolated (Halsall, 1969; Halsall and Catcheside, 1971). Such pleiotropic mutations are further evidence that *aro*-6, -7 and -8 are the structural genes for DAHP synthase (*Tyr*), DAHP synthase (*Phe*), and DAHP synthase (*Trp*), respectively. It is likely that both activity and inhibition sites are specified on the same polypeptide.

The properties of the isoenzymes and their inter-relationships have also been studied. The sieving properties of the isoenzymes in crude extracts from wild-type and various allosteric and activity mutants, were compared on agarose columns equilibrated with different buffers and allosteric effectors (Doy, 1970). Experiments were done in *Tris*-maleate and phosphate buffers (pH 7.4) and enzyme assays were done in the same buffer as used in sieving. Isoenzymes inhibited by the allosteric ligands, phenylalanine, tyrosine, and tryptophan were demonstrated.

When extracts were sieved in Tris-maleate buffer, recovery of DAHP synthase (Phe) was low and inhibition patterns changed on storage. In the absence of allosteric ligands, DAHP synthase (Tyr) and DAHP synthase (Phe) activities exist as high apparent molecular weight forms. When phenylalanine and tyrosine are present, these isoenzymes move to low molecular weight. DAHP synthase (Trp) activity is not affected in this way and remains predominantly as a high molecular weight form. Elution with tyrosine moved DAHP synthase (Tyr) to lower molecular weight whereas elution with phenylalanine moved DAHP synthase (Phe) to lower molecular weight.

Elution in phosphate buffer results in greater recovery of DAHP synthase (Phe) activity but results for DAHP synthase (Trp) are poor. The proportion of non-inhibited activity is high. Activity profiles are distorted which shows that either cross-inhibition occurs or there are mixtures of enzyme forms. The same shifts in molecular weights for the DAHP synthase (Tyr) and DAHP synthase (Phe) isoenzymes occur in the presence of their respective inhibiting ligands as that in Tris buffer.

When extracts of phenylalanine- and tyrosine-resistant mutants were sieved in the presence of the now non-inhibiting ligands, conversion to the low molecular weight form did not occur. It was concluded that both DAHP synthase (Tyr) and DAHP synthase (Phe) could exist in two forms corresponding to apparent molecular weights 106 000 and 68 000 for DAHP synthase (Tyr) and 99 000 and 57 000 for DAHP synthase (Phe). Dissociation of the two isoenzymes might be part of the normal mechanism of allosteric inhibition. Phenylalanine was also found to stabilise phenylalanine-resistant DAHP synthase (Phe) which

shows that phenylalanine can still bind on to the enzyme. Therefore the changes induced by the binding of ligands are separable into activation and inhibition.

Experiments with crude extracts (Halsall, 1969; Doy, 1970) showed a strong similarity between DAHP synthases (Phe) and (Tyr) but DAHP synthase (Trp) had different properties. In summary, the properties characteristic of DAHP synthase (Trp) in crude extracts are as follows: (1) Greater stability and no obvious requirement for PEP stabilisation; (2) Insensitivity to EDTA; (3) No obvious molecular weight shift in the presence of the allosteric ligand Trp; (4) No clear evidence for interaction with the other isoenzymes. On the other hand, properties similar to those of the other DAHP synthases are: (1) Activity and allosteric inhibition are probably encoded in the one gene; (2) The pH optima for activity and inhibition are similar; (3) It is a sulphydryl enzyme; and (4) Reaction with Trp is co-operative.

Attempts were then made to purify the isoenzymes for a detailed comparison. Work on the isoenzymes was made difficult by the instability of DAHP synthases (Tyr) and (Phe) (Jensen and Nasser, 1968; Gaertner and DeMoss, 1970). In this laboratory, PEP was found to protect DAHP synthase (Tyr) and to some degree DAHP synthase (Phe) (Doy, 1968a). Hoffmann (1971) found that three isoenzymes could be partly separated either by anion-exchange chromatography on diethylaminoethyl (DEAE) cellulose eluted with a non-linear salt gradient or by taking different ammonium sulphate saturation fractions. The latter method was used because of its

higher capacity. The next two steps in the purification make use of the ability of Tyr and Phe to dissociate DAHP synthases (Tyr) and (Phe) and the ability of the isoenzymes to reassociate after the ligands are removed. The two isoenzymes could then be separated on a DEAE-cellulose column by eluting with a linear salt gradient. DAHP synthase (Tyr) could be further purified on a hydroxylapatite column. Activity elutes as a single symmetrical peak and analytical polyacrylamide gel electrophoresis gives two protein bands, only one of which is active. However, on storage, activity doubled and electrophoresis now shows only one protein band corresponding in position to the previously active band. It was concluded that an inactive protein is converted to an active enzyme during storage and suggests that the preparation is pure in the sense that it does not contain proteins which do not have DAHP synthase (Tyr) potential (Hoffmann et al., 1972). On prolonged storage, the purified enzyme slowly loses activity. After several months, the loss of activity can be halted (and in most cases activity increased to the original level) by the addition of another quantity of PEP. However, when all enzyme activity was allowed to decay completely, addition of PEP no longer resulted in its restoration (Hoffmann, 1971).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate of the purified DAHP synthase (Tyr) gives as many as twelve protein bands with molecular weights ranging from 76 000 to 11 000. When the native purified DAHP synthase (Tyr) was sieved on an agarose column, the activity profile of the eluted enzyme is not symmetrical, indicating a mixture of enzyme forms.

Molecular weight at the maximum of the activity profile is about 130 000. If the dodecyl sulphate gel result is correct, the enzymes might be composed of as many as twelve subunits (Hoffmann, 1971).

DAHP synthase (Phe) could be partially purified in the same way. The enzyme is unstable and the activity profiles from hydroxylapatite columns are resolved into multiple peaks. Gel electrophoresis of different portions across the activity profile gives additional bands between those found previously for purified DAHP synthase (Tyr). Dodecyl sulphate gel electrophoresis also gives a slightly different set of protein bands from DAHP synthase (Tyr). When sieved on agarose columns, the partially purified enzyme gives an almost symmetrical peak corresponding to molecular weight of about 128 000. All the evidence demonstrates that DAHP synthases (Tyr) and (Phe) are quite similar and that the two isoenzymes might be composed of different proportions of the two polypeptides coded for by aro-6 and aro-7 (Hoffmann, 1971). However, the gene products of aro-6 and aro-7 have not been identified.

In the isolation of mutants for DAHP synthases in N. crassa, strains carrying aro-6 are unable to grow in the presence of phenylalanine tyrosine and tryptophan unless PAB is added. This effect is not observed for aro-7 or aro-8 mutants. It was deduced that DAHP synthase (Tyr) (the product of aro-6) is incompletely inhibited by tyrosine to ensure a supply of PAB when tyrptophan, phenylalanine and tyrosine are present in excess (Halsall and Doy, 1969).

III. KINETIC ASPECTS OF DAHP SYNTHASES IN NEUROSPORA CRASSA

The kinetics of the DAHP synthases has been studied in both crude extracts (Doy, 1968a) and purified DAHP synthase (Tyr) (Hoffmann, 1971). Double-reciprocal plots of velocity against combination of fixed and varied substrate concentration yield families of parallel lines. For PEP as the varied substrate, these are straight, but for E4P they curve, concave upward. Parallel lines are consistent with the ping-pong mechanism and curvature indicates co-operativity between E4P binding sites. In crude extracts, the Hill coefficient, m, for PEP is 1.7 and 1.8 for E4P. For partially purified DAHP synthase (Tyr) from a DEAE-cellulose column, m = 1.35 for E4P and m = 1.0 for PEP.

With purified DAHP synthase (Tyr), PEP was found to be saturating over the concentration range used and the 1/v versus 1/E4P plot at saturating concentration of PEP is a straight line. Inhibition by phosphate with respect to E4P is competitive and with respect to PEP is consistent with noncompetitive inhibition and therefore the ping-pong mechanism (Hoffmann, 1971).

The details of inhibition of purified DAHP synthase (Tyr) by Tyr are complex but with saturating substrates $[Tyr]_{0.5}$ (I) (concentration of Tyr for half maximal inhibition) is about 3 μM and m = 1.76 (strong co-operation between at least two sites). When E4P was varied with various fixed levels of Tyr, velocity-substrate plots became more sigmoidal and double reciprocal plots more curved, with increasing Tyr concentration. These changes are indicative of

increasing co-operativity between sites binding E4P and are examples of a heteromolecular effect of Tyr on E4P. At high E4P concentrations the family of double reciprocal plots are parallel straight lines showing that Tyr is uncompetitive with E4P (both K_m (apparent) and V_{max} (apparent) are changed). Replots of the reciprocal of V_{max} (apparent) give a graphical estimate of $K_{i(Tyr)} = 3$ μM.

Synergism between Phe and Tyr was observed with crude extracts (Doy, 1968a) but not with purified DAHP synthase (Tyr) (Hoffmann, 1971). Interaction between DAHP synthases (Phe) and (Tyr), or the products of the aro-6 and aro-7 loci is probably the basis for synergism.

Limited kinetic experiments have been done (Hoffmann, 1971) with highly purified DAHP synthase (Phe). In general the results are similar to those with DAHP synthase (Tyr). $[Phe]_{0.5}$ (I) is about 5.5 μM and m = 1.25 (weak co-operation between at least two sites). During late stages of purification, DAHP synthase (Phe) separates into several forms some of which are very sensitive to inhibition by Tyr as well as Phe. This suggests that these are heteropolymers between the products of aro-6 and aro-7.

IV. ORGANISATION OF DAHP SYNTHASES

Evidence for an *in vitro* interaction between the polypeptide products of the aro-6 and aro-7 loci has been obtained (Halsall *et*

al., 1971). A mutation in the aro-6 locus was found to affect the molecular forms of DAHP synthase (Phe) as indicated on agarose columns. In the wild type, the elution profile from an agarose column indicates a mixture of a high (minor) and a low (major) molecular weight forms. In one of the aro-6 and aro-8 double mutants, the high molecular weight form alone was observed. It was suggested that the minor form is a heteropolymer between the products of aro-6 and aro-7 and that the major form is a homopolymer of the aro-7 product (Halsall et al., 1971).

Throughout the purification of DAHP synthase (Tyr), it was found that the properties of the enzyme were changing (Hoffmann, 1971). The most obvious changes being (a) loss of sensitivity to EDTA and Co^{2+} ; (b) only a portion of the purified enzyme will dissociate in the presence of Tyr.

Specific mutations within the aro gene cluster can alter the phenotype of DAHP synthase (Doy, unpublished results). DAHP synthases (Phe) and (Tyr) extracted from aro-2, aro-9, or aro-5 mutants show a change in the apparent weight-average molecular weights when sieved on an agarose column. These genes code for the three reactions following DAHP synthase (Fig. 1.3). These results could be the first indication of direct interaction between DAHP synthase and later functions of the pathway.

V. CONTROL OF ENZYME SYNTHESIS

Repression and Induction

Cohn and Monod (1953) first showed that in bacteria the

production of tryptophan synthetase and methionine synthetase were specifically repressed by exogenous tryptophan and methionine respectively. In arginine biosynthesis, repression by arginine affected the production of the majority, if not all, of the enzymes of the pathway (Vogel, 1957; Gorini and Maas, 1958). This shutting off of all the enzymes of a sequence by its end-product is termed repression. The reverse behaviour whereby certain enzymes are synthesized only in the presence of their substrate is termed induction (Monod and Cohn, 1952). Induction is more commonly found in catabolic pathways whereas repression occurs mainly in biosynthetic pathways.

In N. crassa repression of DAHP synthase to below wild-type level has not been observed. Derepression of DAHP synthase activity to about two- to ten-fold above the basal level is observed with the aro-2 mutant, 81-4A (blocked after DAHP). Derepressed activity is mainly Tyr and Phe inhibitable (Doy and Halsall, 1969). Other experiments with 81-4A grown on a limiting amount of any one of the three aromatic amino acids give extensive derepression and there is no obvious relationship between the limiting end product and derepression of specifically inhibited portion of DAHP synthase (Doy, personal communication). Doy (personal communication) also points out that these experiments are not designed to measure changes in rate of synthesis of DAHP synthase, only changes in specific activity. Such changes could affect activation-deactivation rather than derepression-repression. There is, then, a mechanism for controlling the upper limit of enzyme synthesis so that the level found in wild-type is balanced to pro-

duce adequate levels of all three aromatic amino acids.

Induction is observed in the quinate catabolic pathway. The enzymes concerned in quinate catabolism are induced when strains are grown on quinate as the sole source of carbon or when mutants accumulate dehydroquinate (Giles et al., 1967a, b).

VI. REGULATION OF ENZYME ACTIVITY

If one considers (Savageau, 1972) a sequence of reactions constituting a biosynthetic pathway as shown,

$$X_0 \rightarrow X_1 \rightarrow X_2 \rightarrow X_3 \rightarrow \quad ,$$

each step being catalysed by an enzyme, then in the absence of any control mechanism, an increase in concentration of the independent variable X_0 will lead to an increase in the rate of production of X_1 and to a higher level of X_1. This in turn increases the production of X_2 and leads to a higher level of X_2, etc.

In the new steady state, all the concentrations will have increased and all the reaction rates will be higher but equal.

One can think of imposing a control mechanism, for example feedback inhibition, as shown;

$$X_0 \rightarrow X_1 \rightarrow X_2 \rightarrow X_3 \rightarrow \quad ,$$

(with X_3 feeding back to inhibit the first step)

while everything else remains unchanged. A mutation in the structural gene for the first enzyme in the pathway might create an allosteric site on the protein which binds the end product X_3 and lead to

inhibition of the first reaction. As a result of the inhibition, for a given level of the independent concentration variable X_0, the levels of the dependent concentrations will all be lower in the feedback controlled system compared to the otherwise equivalent uncontrolled system. If X_0 is changed to a higher level, at the new steady state, the change in values of the dependent concentrations will be less in the controlled system. Feedback inhibition, or mechanisms which control activity of enzyme in general, thus act to "buffer" the concentration of the end product against internal perturbation in cellular metabolism that could lead to changes in the precursor concentration X_0 (Savageau, 1972). This is essential for preventing wasteful synthesis of compounds already in abundant supply.

Several different methods have evolved for the regulation of enzyme activity. These are considered below.

(a) Feedback Inhibition

In this type of inhibition, the end product of the pathway inhibits the first enzyme in the biosynthetic sequence. In unbranched biosynthetic sequences, the single loop feedback is the predominant pattern of control. The intermediates do not affect the first reaction (Monod *et al.*, 1963).

In a branched biosynthetic pathway, the mechanism of control is necessarily more complex. In general, five different types of feedback mechanisms have been observed. DAHP synthases from different micro-organisms have been shown to exemplify each one of these

mechanisms.

(i) "Inhibition by single end product"

In this case, there is only one enzyme catalysing the first reaction and this enzyme is inhibited only by one of the end products. An excess of that end product will stop the whole of the biosynthetic pathway and deprive the organism of the other end products. Growth will then be inhibited if the other end products are not supplied exogenously. DAHP synthase from Thiobacillus neapolitanus is inhibited by phenylalanine only (Kelly, 1969) and growth of the organism is inhibited in the presence of excess **Phe** added to the medium. DAHP synthases from Streptomyces and Micromonospora species were found to be inhibited by tryptophan only (Jensen et al., 1967). However, the observation of a single enzyme inhibitable by a single end product may be due to a failure to detect other isoenzymes, or inhibition by the other end products.

(ii) "Cumulative feedback"

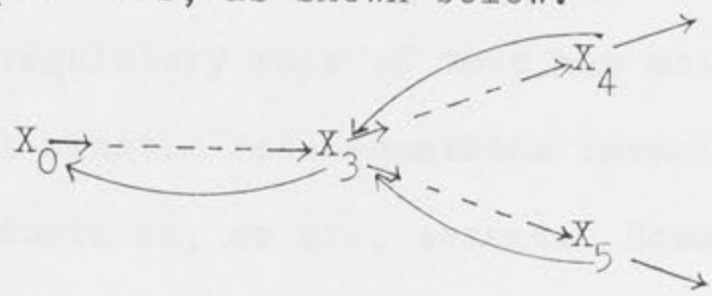
A single enzyme is inhibited by several inhibitors each inhibiting a portion of the activity. The effect of any two inhibitors together is less than the sum of their single inhibitions. Woolfolk and Stadtman (1964) reported that glutamine synthetase from E. coli was inhibited by at least eight inhibitors in a cumulative manner. DAHP synthases from the hydrogen-oxidising chemolithotrophs Hydrogenomonas facilis, H. eutropha and H. pantotropha were controlled by cumulative feedback inhibition by tyrosine and phenylalanine. No measurable inhibition by tryptophan was found in these strains (Jensen et al., 1967).

(iii) "Concerted or multivalent feedback"

The term "concerted feedback" (Datta and Gest, 1964) describes an allosteric pattern in which multiple end products act in synergistic combination to produce feedback inhibition. Individual end products produce little or no inhibition. In Rhodomicrobium vanniellii, DAHP synthase is inhibited only in the presence of all three aromatic amino acids. Depending on the ratio of the substrates PEP and E4P, both phenylalanine and tyrosine potentially, alone or in combination, can stimulate or inhibit activity. Simultaneous presence of all three aromatic amino acids interferes with the binding of E4P so severely that the consequence is a potent inhibition (Jensen and Trentini, 1970).

(iv) "Sequential feedback"

In this type of inhibition, the first reaction is inhibited by an intermediate metabolite whose accumulation in turn is controlled by several end products, as shown below:



DAHP synthase from Bacillus subtilis consists of a single enzyme inhibitable by chorismate or prephenate but not by the aromatic amino acids (Jensen and Nester, 1966a, b). Many of the Bacillus sp. examined also exhibit sequential inhibition of DAHP synthase by prephenate or chorismate. Other species which show this type of inhibition of DAHP synthase include, Staphylococcus sp., Gaffkya tetragena, Flavobacterium sp., Achromobacter parvulus and Alcaligenes

viscolactis (Jensen et al., 1967).

(v) "Isoenzymic feedback"

The same reaction is catalysed by different isoenzymes each of which is inhibited by one of the end products. The existence of different isoenzymes catalysing the same reaction was established by Stadtman et al., (1961) on multiple aspartokinases in the control of lysine, threonine and methionine biosynthesis. DAHP synthases from E.coli (Smith et al., 1962; Brown and Doy, 1963), Salmonella typhimurium (Gollub et al., 1967), N.crassa (Doy, 1968b), Saccharomyces cerevisiae (Lingens et al., 1966), Polytoma obtusum and all of the Enterobacteriaceae that have been examined (Jensen et al., 1967) all consist of isoenzymes inhibitable by different end products.

(b) Regulation by Product Inhibition

Inhibition of an enzyme by its product(s) is a common phenomenon but the regulatory role of this has not been fully explored. Classical kinetic rate equations invariably assume that product or products is, or are, absent. However, in vivo, such an assumption is generally invalid. A normally expected rectangular hyperbolic response of reaction velocity to changes in the substrate concentration may turn out to be quite different in the presence of the reaction product(s). This product effect is especially striking for multi-substrate enzymic reactions. Depending on the type of reaction mechanisms, different products can have different effects on the sensitivity to changes in the

substrates concentration. Product concentration can also alter the co-operativity of substrate binding. For an enzyme system with its substrate poised at an intermediate degree of saturation within the cell, fluctuation in the reaction product level can act much like activators and inhibitors in allosteric systems. An increase in product concentration causes the S(0.5) (substrate concentration at half maximal velocity) to shift toward higher substrate concentration and decreases the degree of substrate saturation. A decrease in product concentration causes the S(0.5) to decrease and thus increase the degree of substrate saturation. No special modifier site on the enzyme is required (Purich and Fromm, 1972).

This effect of product on the degree of substrate saturation is illustrated by the effect of fumarate on aspartase. In E. coli, aspartase catalyses the reversible nonoxidative deamination of aspartate to fumarate and ammonium ion, thus providing fumarate to the citric acid cycle. The presence of fumarate shifts the velocity saturation curve toward higher aspartate concentrations (Purich and Fromm, 1972).

In aromatic biosynthesis, chorismate mutase T from E. coli and A. aerogenes is product inhibited by prephenate whose accumulation in turn is controlled by feedback inhibition of prephenate dehydrogenase by tyrosine (Cotton & Gibson, 1967). In Bacillus subtilis, chorismate mutase-is product inhibited by prephenate, being a part of the pattern of sequential feedback inhibition which regulates the aromatic pathway in this organism (Jensen and Nester, 1966a, b).

(c) Interpathway Regulations.

It is becoming increasingly clear that individual biosynthetic pathways are not isolated. The regulation of biosynthetic enzymes by metabolites which are not produced in that pathway has been termed 'metabolic interlock' by Jensen, (1969). In branching biosynthetic pathways, a metabolite formed in one branch usually influences the activity of an enzyme in one of the other branches. For example, in N. crassa, chorismate mutase is activated by tryptophan (Baker, 1966) and prephenate dehydrogenase is activated by phenylalanine (Catcheside, 1969); in E. coli, threonine deaminase is activated by valine (Changeux, 1963).

In B. subtilis, prephenate dehydratase bears specific allosteric sites for phenylalanine (end product inhibitor), methionine (activator), leucine (activator), tryptophan (inhibitor) and tyrosine (reversal of inhibition by tryptophan) (Rebello and Jensen, 1970). High levels of exogenous tryptophan are able to inhibit growth by inhibiting synthesis of phenylalanine only when intracellular tyrosine level is low. It is possible that this is a mechanism whereby intracellular phenylalanine level is kept low when the intracellular level of tyrosine diminishes. More examples of remote metabolite controlling biosynthetic enzymes in other pathways include inhibition of histidine deaminase by tyrosine in Pseudomonas putida (Hug and Roth, 1968), inhibition of glutamine synthetase by anthranilate (Kapoor et al., 1968) and activation of anthranilate synthase by histidine in B. subtilis (Kane and Jensen, 1970).

(d) Enzymic Reaction Mechanisms in Regulation

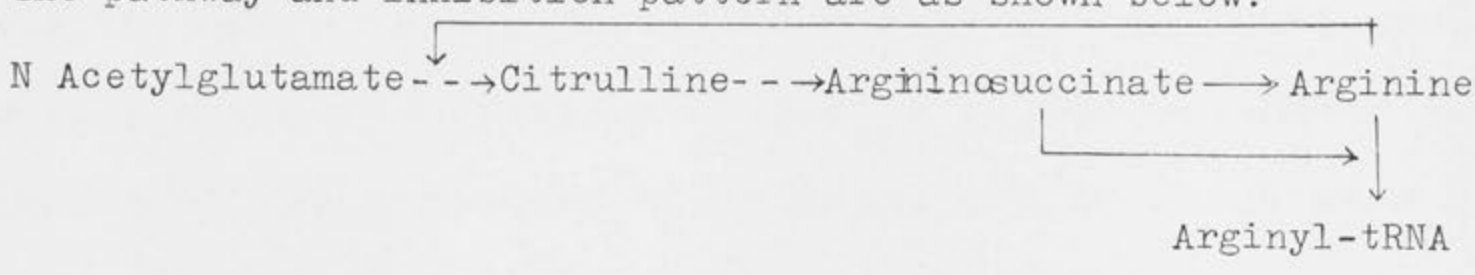
That some enzymes bind their substrates in an ordered manner, whereas other enzymes bind their substrates in a random manner, suggests that the kinetic mechanism of reaction is important in the regulation of enzyme activity. The order of substrate binding and product removal can alter the responses of enzymes to fluctuations in the intracellular substrate and product levels (Purich and Fromm, 1972). Different reaction mechanisms can cause qualitatively and quantitatively different substrate-dependent velocity responses. The effect of products on the reaction velocity has already been discussed. An additional point to note is that depending on the reaction mechanisms, individual substrates may or may not be able to reverse the inhibition by individual products in multi-substrate reaction. In addition, substrate inhibition is also commonly observed and is an important regulatory element. Rabbit muscle lactate dehydrogenase exhibits substrate inhibition by pyruvate at high pyruvate concentration when nicotinamide adenine dinucleotide (NAD) is present. No such inhibition is observed when NAD is absent. In this case, substrate inhibition can be accounted for by the formation of the abortive ternary complex enzyme-NAD-pyruvate at elevated pyruvate and NAD concentrations (Zewe and Fromm, 1965).

Since DAHP synthase catalyses a two-substrate-two-product reaction, one would expect that the reaction mechanism might be important in regulation. DAHP synthase from Rhodomicrobium vanniellii exhibits substrate inhibition by E4P and both phenylalanine and tyrosine are competitive inhibitors with respect to E4P.

At high E4P concentration, phenylalanine or tyrosine decreases the apparent concentration of E4P and thus activates the reaction. The presence of all three aromatic amino acids interferes with E4P binding to produce the observed concerted feedback inhibition (Jensen and Trentini, 1970).

(e) Feedforward Inhibition

The control mechanism by feedforward inhibition was discovered in the arginine biosynthetic pathway of several microorganisms (Nazario, 1967; Sussenbach and Strijkert, 1969; Yem and Williams, 1971). It was found that arginyl-tRNA synthetase was inhibited by argininosuccinate, the immediate precursor of arginine. The pathway and inhibition pattern are as shown below:



The significance of this phenomenon was analysed by Savageau (1971, 1972). For a system in which feedback inhibition does not exist, an increase in citrulline concentration will lead to an increase in concentration of all the metabolites. In the presence of feedforward inhibition, a similar increase in citrulline concentration will lead to an accumulation of arginine, the metabolite immediately before the inhibited step. This by itself confers no advantage to the organism. However, in concert with the feedback inhibition of N-acetylglutamokinase by arginine, a magnification in the arginine concentration will increase the effectiveness of the feedback inhibition. Also, an increase in internal arginine concentration will inhibit the early step, deplete the argininosuccinate and de-inhibit

arginyl-tRNA synthetase leading to increased utilization of arginyl-tRNA. Thus feedforward inhibition appears to have a functionally significant role in improving the effectiveness of the overall regulation in a biosynthetic pathway.

However occurrence of feedforward inhibition is rarely demonstrated and this phenomenon has not been shown to occur in the control of aromatic biosynthesis.

CHAPTER 2

OBJECTIVES

The objectives of this work are to purify the DAHP synthase (Trp), identify the aro-8 gene product and study the subunit constitution, properties and reaction kinetics of the enzyme. The longer term objectives are to compare the three isoenzymes with respect to evolutionary relationships and to elucidate the organisation of DAHP synthase as a whole, including its relationship, if any, with the other enzymes in the aromatic pathway.

The purpose of the purification of an enzyme is to study its properties free of interference from unrelated proteins. One has therefore to decide on the purity of the final product. A commonly accepted criterion of purity is the occurrence of a single protein or a mixture of proteins all of which have enzymatic activity. This is a simple matter if the enzyme consists of a single molecular structure composed of identical subunits of a single polypeptide species. However, an enzyme may consist of several species of polypeptide subunits which may aggregate to form molecules of various molecular weights and conformations each with properties that depend on the manner of assembly of the subunits. In some examples, each species of polypeptide could be essential for enzyme activity but in others some might contribute to the fine tuning of the enzyme to biological function and its assembly into the overall structural organisation of the cell. Thus, depending on the objectives of the investigator, the criterion of purity would vary. At the one extreme of definition, purity will mean the presence of only one species of polypeptide for

chemical analysis, and at the other, the presence of all polypeptide components that normally participate in biological function.

For DAHP synthase (Trp) in N. crassa, even though there is evidence that the activity site and the allosteric site are specified on a single polypeptide (Halsall and Doy, 1969), it is not unequivocally established (Doy, personal communication) that the locus aro-8 (coding for DAHP synthase (Trp)) contains only one cistron. Furthermore, because the product, DAHP, of any one of the DAHP synthase isoenzymes can be metabolised to any one of the aromatic end products, it is possible that in nature, the isoenzymes are organised rather than separated. Thus, in terms of biological purity as opposed to biochemical purity, it is important, during purification, to identifiy the protein (or proteins) coded for by the aro-8 locus and to decide whether other proteins copurify as components of DAHP synthase (Trp).

CHAPTER 3

PURIFICATION AND SOME PROPERTIES OF DAHP SYNTHASE (TRP)

I. INTRODUCTION

Crude cell extracts and whole organisms were used in earlier studies of the DAHP synthases in N. crassa. DAHP synthase (Tyr) has been purified (Hoffmann, 1971). The optimum pH for stability of total DAHP synthase activity in crude extracts was found to be 7.4. The optimum pH for activity was broad, ranging from pH 6.5 to 7.7. Tris-maleate buffer was regarded as unsuitable for use in the purification of the DAHP synthases because of loss of activity after $(NH_4)_2SO_4$ fractionation. Phosphate buffer was used because even though activity was lost during $(NH_4)_2SO_4$ fractionation, it could be restored after desalting. DAHP synthase (Trp) was found to be most abundant in the 0-55% $(NH_4)_2SO_4$ fraction and was stable during fractionation whereas PEP was required for recovery of the Phe- and Tyr- inhibited isoenzymes. It was intended that the purification of DAHP synthase (Trp) could be continued from the 0-55% $(NH_4)_2SO_4$ fraction after the removal of nucleic acid by protamine sulphate precipitation. The 55% $(NH_4)_2SO_4$ precipitate could be stored at -10°C until required.

II. PURIFICATION OF DAHP SYNTHASE (TRP) - A SUMMARY

The purification scheme for DAHP synthase (Trp) is

summarised as follows: all operations were carried out at 4°C.

Step 1: Preparation of crude cell extract.

Mycelia were freeze-dried and then ground immediately to a powder in a coffee mill. 100g of the mycelial powder were homogenized with 1 l. of 0.05M KH_2PO_4 -NaOH buffer pH 7.4 and after one hour cell debris was removed by centrifugation (15 000 x g, 20 min).

Step 2: Removal of nucleic acids.

Protamine sulphate (2.4 g) as a suspension in 100 ml of 0.05M KH_2PO_4 -NaOH buffer pH 7.4 was added to the crude extract. The mixture was stirred for 5 min. and the precipitate removed by centrifugation (15 000 x g, 20 min).

Step 3: $(NH_4)_2SO_4$ fractionation.

Solid $(NH_4)_2SO_4$ was added to the supernatant to 55% satn at 4°C (32 g per 100 ml), the mixture was stirred for 45 min and the precipitate collected by centrifugation (15 000 x g, 20 min). At this stage, the precipitate could be stored at -15°C until required. The precipitate was then dissolved in a minimal volume of 0.02M KH_2PO_4 -NaOH buffer pH 7.4 and dialysed against the same buffer (3 x 2 l) at pH 6.4. After dialysis, any precipitate was removed by centrifugation (30 000 x g, 10 min).

Step 4: Temperature and pH changes.

2-Mercaptoethanoal was added to the supernatant to 0.1% (w/v) and the solution was brought to 39°C in a water-bath and maintained at that temperature for 10 mins. The solution was cooled in ice and adjusted to pH 6.0 with 1 N HCl.

Precipitate was removed by centrifugation (30 000 x g, 5 min), and the supernatant immediately re-adjusted to pH 7.4 by 1 N NaOH.

Step 5: Phenylmethylsulphonyl fluoride (PMSF) treatment.

To the supernatant, a solution of 0.02M PMSF in 2-propanol was added to a final concentration of 1 mM PMSF. The solution was allowed to stand at 4°C for 30 min and the precipitate was removed by centrifugation (30 000 x g, 10 min). The supernatant was dialysed against 0.02M KH_2PO_4 -NaOH buffer pH 7.4 plus 0.1% (w/v) 2-mercaptoethanol.

Step 6: Anion exchange chromatography on DEAE-cellulose.

The dialysed solution was applied to a column of DEAE-cellulose (2.5 cm x 30 cm) equilibrated in the same buffer and the enzyme was eluted with a linear NH_4Cl gradient (0-0.25M, 500 ml per chamber) in 0.02M KH_2PO_4 -NaOH buffer pH 7.4 plus 0.1% (w/v) 2-mercaptoethanol (Fig. 3.1).

Step 7: Hydroxylapatite column.

Active fractions from the previous column were pooled and concentrated to 20 ml by ultrafiltration (Diaflo, Amicon Corp., PM 10 membrane). The concentrated solution was applied on to a hydroxylapatite column (2.5 cm x 25 cm) equilibrated with 0.02M KH_2PO_4 -NaOH buffer pH 7.4 with 0.1% (w/v) 2-mercaptoethanol. The column was eluted with a linear phosphate gradient (0.02 - 0.35M, 500 ml per chamber) plus 0.1% 2-mercaptoethanol (Fig. 3.2).

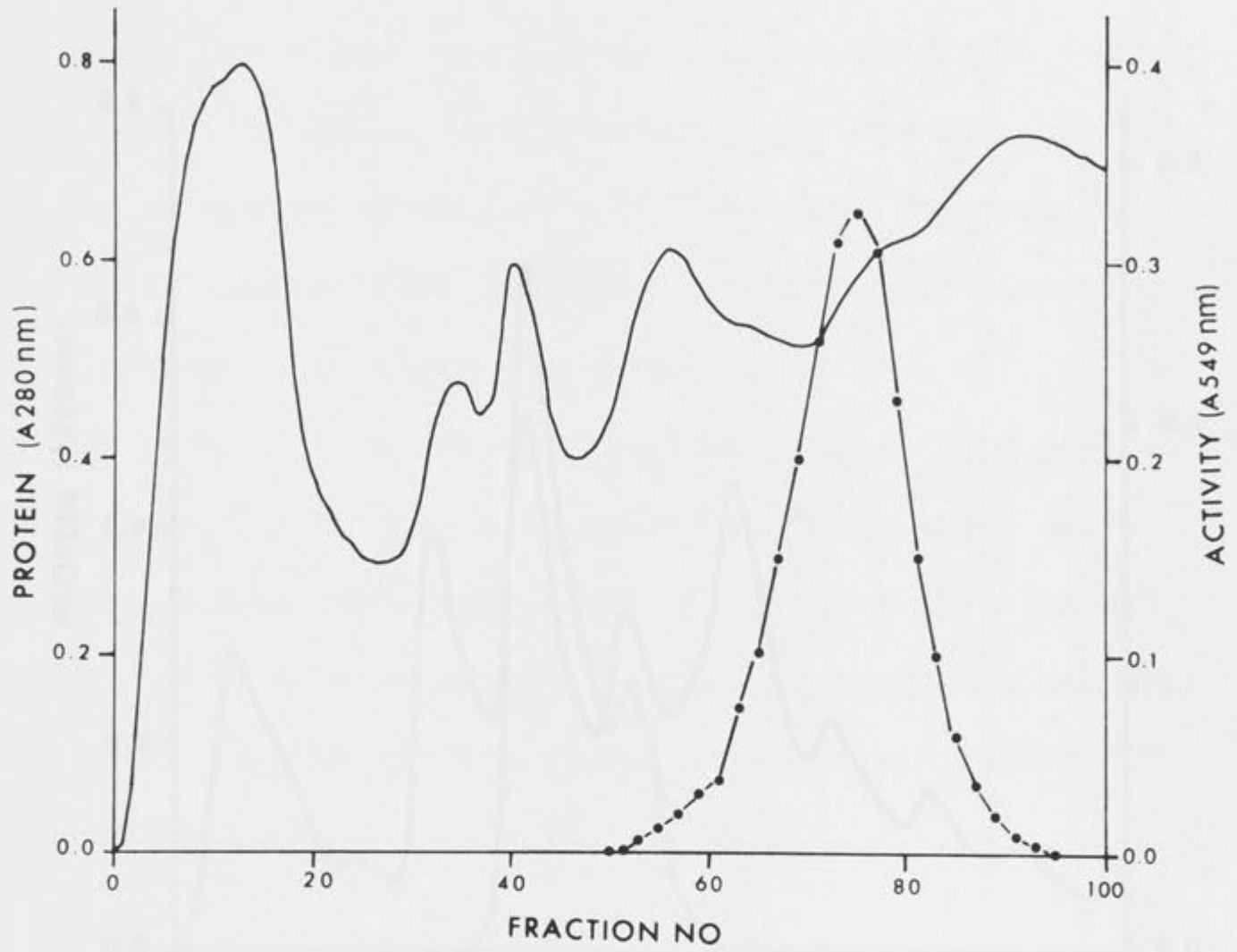


Fig. 3.1 Purification of DAHP synthase (Trp) by chromatography on DEAE-columns (2.5 x 30 cm).

The column was developed by a linear NH_4Cl gradient (0-0.25M, 500 ml/chamber) at 45 ml/h and 5 ml fractions were collected. Peak activity corresponded to 0.1M NH_4Cl.

●——● DAHP synthase activity; —— protein.

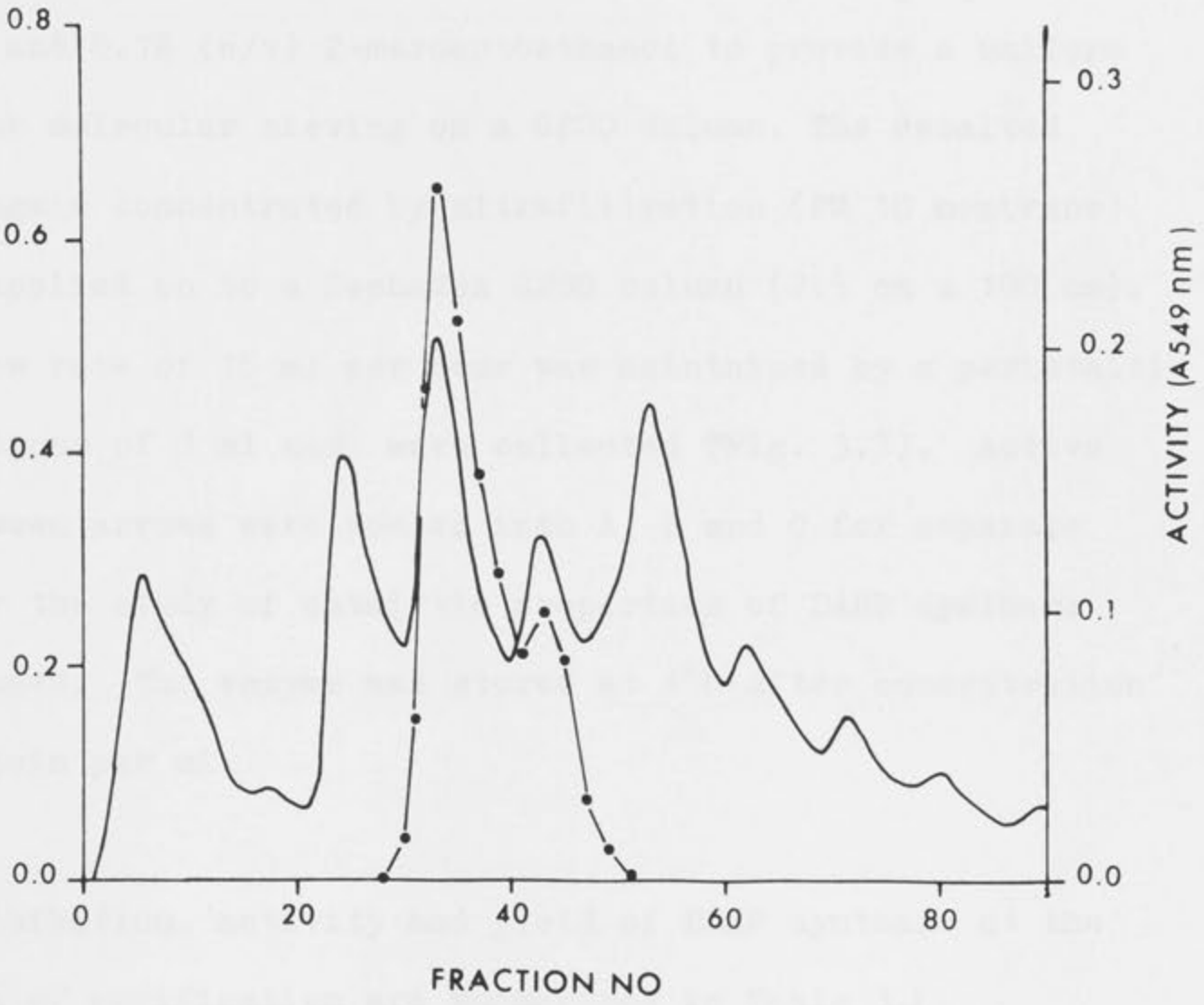


Fig. 3.2 Chromatography of sample collected from a DEAE-cellulose column on a hydroxyl-apatite column (2.5 x 25 cm).

The column was eluted with a linear phosphate gradient (0.02-0.35M, 500 ml/chamber) at 30 ml/h and 4 ml fractions were collected. First peak of activity corresponded to 0.058M phosphate and that for the second peak is 0.067M phosphate. ●——● DAHP synthase activity; ——protein.

Step 8: Sephadex G200.

As the final step, active fractions from the previous column were pooled and concentrated to 10 ml by ultrafiltration (Diaflo, PM 10 membrane). The solution was desalted by passing through a Sephadex G25 column equilibrated with 0.02M KH_2PO_4 -NaOH buffer pH 7.4 and 0.1% (w/v) 2-mercaptoethanol to provide a uniform environment for molecular sieving on a G200 column. The desalted solution was again concentrated by ultrafiltration (PM 10 membrane) to 10 ml and applied on to a Sephadex G200 column (2.5 cm x 100 cm). A constant flow rate of 15 ml per hour was maintained by a peristaltic pump and fractions of 3 ml each were collected (Fig. 3.3). Active fractions between arrows were pooled into A, B and C for separate analysis. For the study of catalytic properties of DAHP synthase (Trp), B was used. The enzyme was stored at 4°C after concentration to 0.1 mg protein per ml.

The inhibition, activity and yield of DAHP synthase at the various stages of purification are summarised in Table 3.1.

Phosphoenolpyruvate which is essential for recovery of DAHP synthase (Tyr) and (Phe) but not (Trp) activities is not included in the buffer for the preparation of the crude cell extract because it was intended to study the kinetic properties of the enzyme. The large loss of total activity between steps 2 and 3 is because the Tyr- and Phe-sensitive isoenzymes are discarded. In steps 3 and 4, 40% of the enzymic activity is lost after one day but note that the amount of DAHP synthase (Trp) increases because the total activity becomes almost completely ($\geqslant$90%) tryptophan sensitive. A similar

Fraction Nos.	$A_{549\ nm}/A_{280\ nm}$
9	10.5
12	10.0
14	15.2
16	20.4
17	21.1
18	21.2
20	16.8
22	10.2
25	4.4

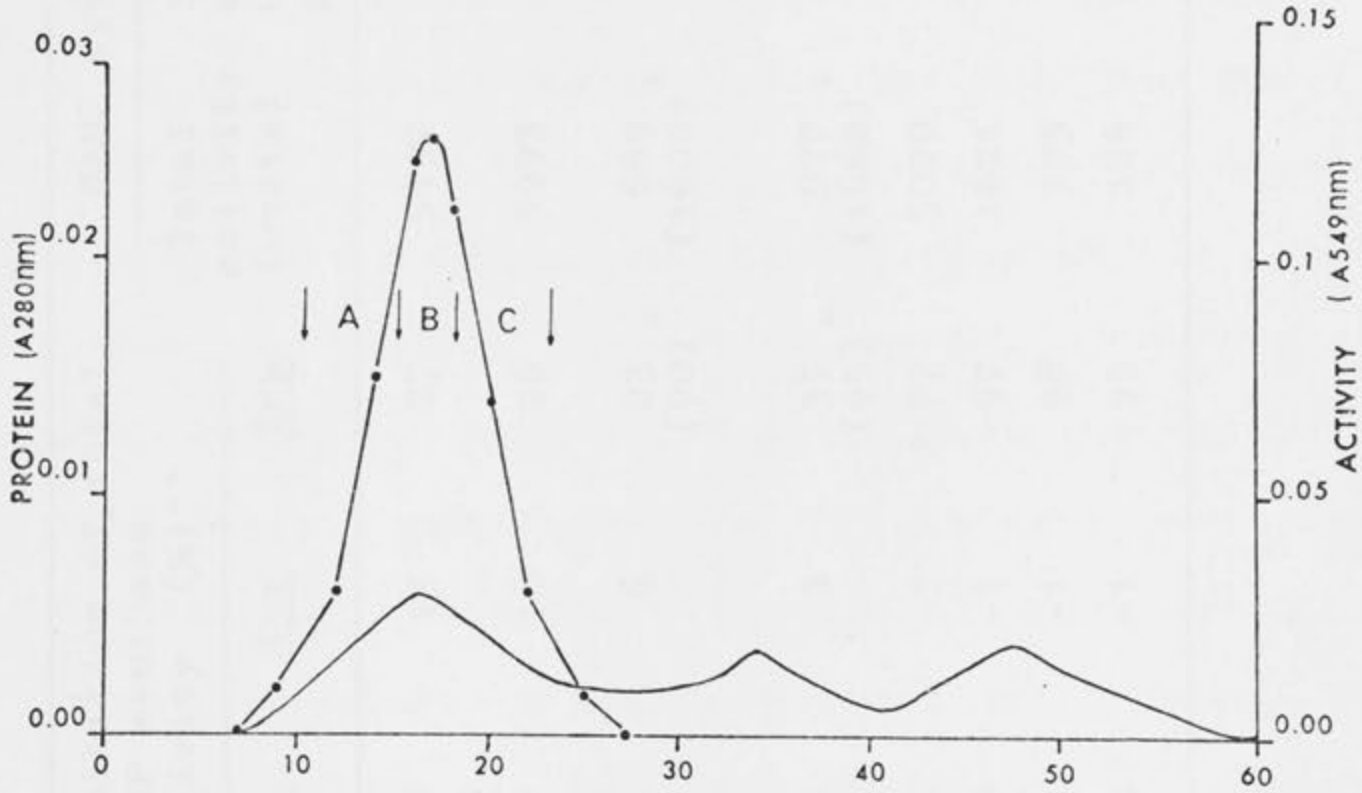


Fig. 3.3 Purification of DAHP synthase (Trp) by sieving on a Sephadex G200 column (2.5 x 100 cm).

The column was eluted at 15 ml/h and 3 ml fractions were collected. Fractions between arrows were pooled separately. A, fractions 10 to 14; B, fractions 15 to 18; C, fractions 19 to 24. ●—● DAHP synthase activity; ——— protein.

TABLE 3.1

Summary of purification of DAHP synthase (Trp)

Purification step	Total protein (mg)	Total DAHP synthase activity (units)	Inhibition of total DAHP synthase activity (%)**			DAHP synthase (Trp)		
			Phe	Tyr	Trp	Total activity (units)	Specific activity units/mg protein	Yield (%)
(1) Crude cell extract	11121	20350	50	13	27	5497	0.49	100
(2) Protamine sulphate	4914	23109	47	16	26	5993	1.22	109
(3) $(NH_4)_2SO_4$ fractionation, 55%	2794	2968 (1780)*	4	6	23 (90)*	669 (1600)*	0.24 (0.57)*	12 (29)*
(4) pH and temperature changes	1287	2612 (1750)*	4	2	37 (95)*	977 (1662)*	0.76 (1.30)*	18 (30)*
(5) PMSF	1007	3977	4	3	77	3070	3.05	56
(6) DEAE-cellulose	55	1667	-10	-1	97	1621	29.50	29
(7) Hydroxylapatite	8.30	402	-3	-1	98	393	47.30	7
(8) Sephadex G200	0.65	124	-2	-1	99	124	191.00	2.2

** Negative inhibition means stimulation

* Values after 24 hours storage

change may also occur at step 5.

III. 0-55% $(NH_4)_2SO_4$ Fraction

After removal of nucleic acid by protamine sulphate, $(NH_4)_2SO_4$ was added to 55% saturation at 4°C. The precipitate was collected by centrifugation and dissolved in and dialysed against 0.02M KH_2PO_4-NaOH buffer pH 7.4. The solution was clear after dialysis. When the $(NH_4)_2SO_4$ fraction was dissolved in and dialysed against 0.02M KH_2PO_4-NaOH buffer pH 6.4, the dialysed solution was turbid. Therefore the $(NH_4)_2SO_4$ precipitate was more soluble at pH 7.4 than at pH 6.4. When the dialysed solution at pH 6.4 was centrifuged, the bulk of the particles remained in suspension. To alter the state of aggregation of the solute and therefore result in precipitation, the pH could be changed or the temperature of the suspension could be altered.

When the solution at pH 6.4 was heated at 39°C for 10 min, precipitation occurred and after centrifugation, the supernatant was clearer than before heating but still slightly turbid. Prolonged heating at 39°C resulted in loss of enzyme activity. Heating at 50°C for 10 min resulted in loss of 90% of the total activity. It was also noticed that the DAHP synthase (Phe) activity which was present in the 0-55% $(NH_4)_2SO_4$ fraction was lost after heating. The activity of DAHP synthase (Trp) remained stable for 10 min and then declined rapidly. When the dialysed solution at pH 7.4 was heated at 39°C, no precipitation occurred.

Enzyme activity after heat treatment remains stable whereas unheated samples lose activity faster. The heating has probably inactivated some proteolytic enzymes present in the solution.

Since the $(NH_4)_2SO_4$ fraction was more soluble at pH 7.4 than at pH 6.4, the pH of the dialysed solution was lowered by dropwise addition of 1N HCl. Precipitation occurred almost immediately. After the pH had been lowered to 5.0 and the suspension centrifuged, the supernatant was clear. However, enzyme activity was unstable at low pH. Earlier studies had shown that DAHP synthase activity in N. crassa is sensitive to 4-chloromercuribenzoate. Therefore, to stabilise activity, a sulphydryl reagent might be important. Dithiothreitol (5×10^{-4}M) was added to the solution before acid treatment and the results are shown in Table 3.2. In the presence of dithiothreitol, the enzyme is more stable at pH 5.0. Recovery of activity is improved but loss of activity is still undesirably large. When the pH of the dialysed $(NH_4)_2SO_4$ fraction was lowered to pH 6.0 instead of 5.0, more activity was recovered. The enzyme is very unstable at pH 6.0 so that immediately after centrifugation to remove the precipitate, the solution was raised to pH 7.4 by 1M NaOH. Even though more protein was precipitated at pH 5.0 than at pH 6.0, the balance between the recovery of activity and the removal of protein favours the latter pH. As long as the time of treatment at pH 6.0 is kept to a minimum, loss of DAHP synthase (Trp) activity is negligible. In any event, a change in pH from 6.4 to 6.0 has removed more than half of the protein without any noticeable loss of activity.

TABLE 3.2

Effect of dithiothreitol on stability at low pH

DTT 5×10^{-4} M	Time of exposure at pH 5.0 (min)	Activity* (arbitrary unit)
-	0	100
+	5	72
-	5	69
+	10	60
-	10	40

*Arbitray unit is used for ease of comparison of results.

100 Arbitrary units = 0.450 $A_{549\ nm}$

If the $(NH_4)_2SO_4$ fraction was dialysed for less than 12 hours, the bulk of the activity was noninhibitable by either one of the three aromatic amino acids, Tyr, Phe and Trp. However, after storage at 4°C for 12 hours 40% of the total activity was lost, but the amount of DAHP synthase (Trp) increased because the total activity became almost completely ($\geqslant$ 90%) tryptophan inhibitable (Table 3.1). If dialysis of the $(NH_4)_2SO_4$ fraction was continued for a prolonged period ($\geqslant$ 12 hours), the activity was almost completely inhibitable by Trp. Enzyme activity was low before the removal of $(NH_4)_2SO_4$. The change in inhibition property shows that there is a change in enzyme forms after removal of $(NH_4)_2SO_4$. It is possible that the enzyme can exist in several conformations and in order that the enzyme is inhibitable by its allosteric effector, Trp, a specific conformation is required. The conformation is drastically changed in the presence of high concentration of $(NH_4)_2SO_4$ so that most of the enzyme activity is lost. However, after and during removal of the $(NH_4)_2SO_4$, the enzyme gradually reassembles itself first into an active conformation and then into an active conformation which is also inhibitable by Trp. The general experience with this enzyme system in N. crassa is that the inhibition can change during storage.

IV. ANION EXCHANGE CHROMATOGRAPHY ON DEAE-CELLULOSE COLUMNS

Since there is still a considerable amount of protein in the sample, molecular sieving on agarose or sephadex columns is not desirable because of technical difficulty when applying concentrated solutions to these columns. A DEAE-cellulose column was therefore tried. The three isoenzymes could be partially separated on a DEAE-cellulose column using a non-linear NaCl

gradient (Hoffmann et al., 1972). The order of elution is, the Trp-, Phe- and Tyr-inhibitable isoenzymes.

In the present example, the column was equilibrated with 0.02M KH_2PO_4-NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol. A linear NH_4Cl gradient (0 to 0.25M, 500 ml per chamber) was used to develop the column. The enzyme eluted at about 0.1M Cl^-. A symmetrical peak is obtained (Fig. 3.1) . The total activity is about 97% inhibitable by 0.1 mM Trp, and stimulated slightly by both Phe and Tyr. If mercaptoethanol was not included in the buffer, recovery of activity was low confirming the importance of sulphydryl group to enzymic activity. If mercaptoethanol was added to the collection tubes instead of to the elution buffer, recovery of activity was again low showing that the -SH bond has to be protected from oxidation for enzyme activity and that the -SH bond is not readily reduced once oxidised.

In crude extracts, EDTA was shown to inhibit strongly that part of the DAHP synthase activity which is Tyr- and Phe-inhibitable. Co^{2+} was found to restore EDTA inhibited activity to a higher level than before EDTA treatment and the Trp-inhibitable activity was increased (Doy, 1968b). The effects of EDTA and divalent metal ions on DAHP synthase (Trp) were tested at this stage of purification. The results (Table 3.3a) show that 41% of the total activity is inhibited by EDTA. Whereas Co^{2+}, Ca^{2+} and Mg^{2+} can stimulate activity, Zn^{2+} inhibits 32% and Mn^{2+} inhibits 15% of the total activity. The result is in contrast with result from crude extracts where DAHP synthase (Trp) is not affected by EDTA.

In one particular example, by mistake, a sample of

TABLE 3.3

Effects of EDTA and divalent metal ions on activity of sample after DEAE-cellulose column.

	Ligands*	% Inhibition**
(a) Sample at pH 7.4 applied to column at pH 7.4.	EDTA	41
	Ca^{2+}	-10.4
	Co^{2+}	- 6.25
	Mg^{2+}	- 3.8
	Mn^{2+}	14.6
	Zn^{2+}	32
(b) Sample at pH 6.4 applied to column at pH 7.4.	EDTA	45.6
	Ca^{2+}	-24.6
	Co^{2+}	-103
	Mg^{2+}	0
	Mn^{2+}	0
	Zn^{2+}	-1021

* Concentration of EDTA = 0.25mM;
concentration of metal ions = 1 mM.

** Negative inhibition means stimulation of activity.

enzyme solution at pH 6.4 after heat treatment was applied to the DEAE-cellulose column equilibrated at pH 7.4 and eluted with the same linear NH_4Cl gradient. The activity appeared earlier than before, at about 0.04M Cl^-. This sample initially was inhibited 18.6% by Phe, 30.4% by Tyr and 80.4% by Trp. After about one month, 80% of the activity was lost. When the effects of EDTA and divalent metal ions were tested then, Zn^{2+} was shown to stimulate activity by as much as 10 fold (Table 3.3b), and activity was inhibited by EDTA and stimulated 100% by Co^{2+}. So, instead of being an inhibitor, Zn^{2+} is a powerful activator. When the inhibition of this enzyme was tested in the presence of Zn^{2+}, the Zn^{2+}-stimulated activity was stimulated a further 10% by Phe, about 7% stimulated by Tyr and inhibited 29% by Trp. Therefore, this Zn^{2+}-stimulated activity is mostly noninhibitable. This result prompted an investigation of the effects of Zn^{2+} on the inhibition properties of enzyme earlier on in the purification procedures. The results are summarised in Table 34. These results show that in the presence of Zn^{2+}, the enzyme becomes less sensitive to inhibition by Trp.

The inhibition of the DAHP synthase (Trp) activity by Zn^{2+} and EDTA and the occurrence of noninhibitable activity in the presence of Zn^{2+} show that a divalent metal ion is important for both activity and inhibition. In crude extracts, this has not been shown to be the case.

It was originally intended to use affinity chromatography after the pH changes, in the purification of DAHP synthase (Trp). An affinity column with Trp binding through the NH_2 group directly to the Sepharose backbone was made according to the method of Cuatrecasas & Anfinsen (1971). The procedure is described in Materials &

TABLE 3.4

Effect of Zn^{2+} on the inhibitory properties of samples at various stages of purification.

Sample	DAHP synthase activity in the absence of Zn^{2+} (arbitrary units)*				DAHP synthase activity in the presence of Zn^{2+} (arbitrary units)*			
	-	Phe	Tyr	Trp	-	Phe	Tyr	Trp
After heat treatment.	100	107.7	112.6	4	65	66.7	72.8	49.6
After pH changes	100	100	104	5.4	90.4	97.4	95.6	72.7
After DEAE-cellulose (dilute)	100	102.6	107.5	3	77.4	72	70	39
After DEAE-cellulose (conc.)	100	96	99	3	84.5	67.6	84.5	45.6
Sample at pH6.4 applied to column at pH 7.4	100	81.4	69.6	19.6	1260	1391	1344	895

* Arbitrary units are used for ease of comparison of results
Concentration of Zn^{2+} in the reaction mixture = 1 mM.

Methods, Chapter 7. When a sample after acid treatment and readjusted to pH 6.4 was applied to the small Sepharose-Trp column, the activity came through unbound.

When this sample from the affinity column was applied to the DEAE-cellulose column, two peaks of activity were resolved (Fig. 3.4). The first peak, fraction 68 (corresponding to 0.08M Cl^-) was inhibited 7.1% by Phe, 14.2% by Tyr and 95% by Trp. In the presence of Zn^{2+}, 34% of the activity was lost and the remaining activity was inhibited 5% by Phe, 10% by Tyr and 39% by Trp. The second peak, fraction 93 (corresponding to 0.12M Cl^-) was inhibited 83% by Phe, 22% by Tyr and 22% by Trp. However, in the presence of Zn^{2+}, 86% of the activity was lost, and the remaining activity was too low to give reliable inhibition data. Therefore, it can be concluded that the first peak consists of Trp-sensitive activity and the second peak consists of mostly Phe-sensitive activity. The two peaks were pooled separately (between arrows, Fig 3.4) and inhibition was analysed. The pooled first peak was stimulated 7% by Phe and 32.6% by Tyr and inhibited 72% by Trp. Zn^{2+} inhibited 6% of the total activity and the remaining activity was inhibited 25% by Phe, 25.5% by Tyr and 67% by Trp. The pooled second peak was inhibited 65.6% by Phe and 38% by Trp and stimulated 71% by Tyr. 85% of the total activity was lost in the presence of Zn^{2+} and the enzyme was very unstable.

It can be concluded that the Phe-sensitive DAHP synthase activity is very sensitive to inhibition by Zn^{2+}.

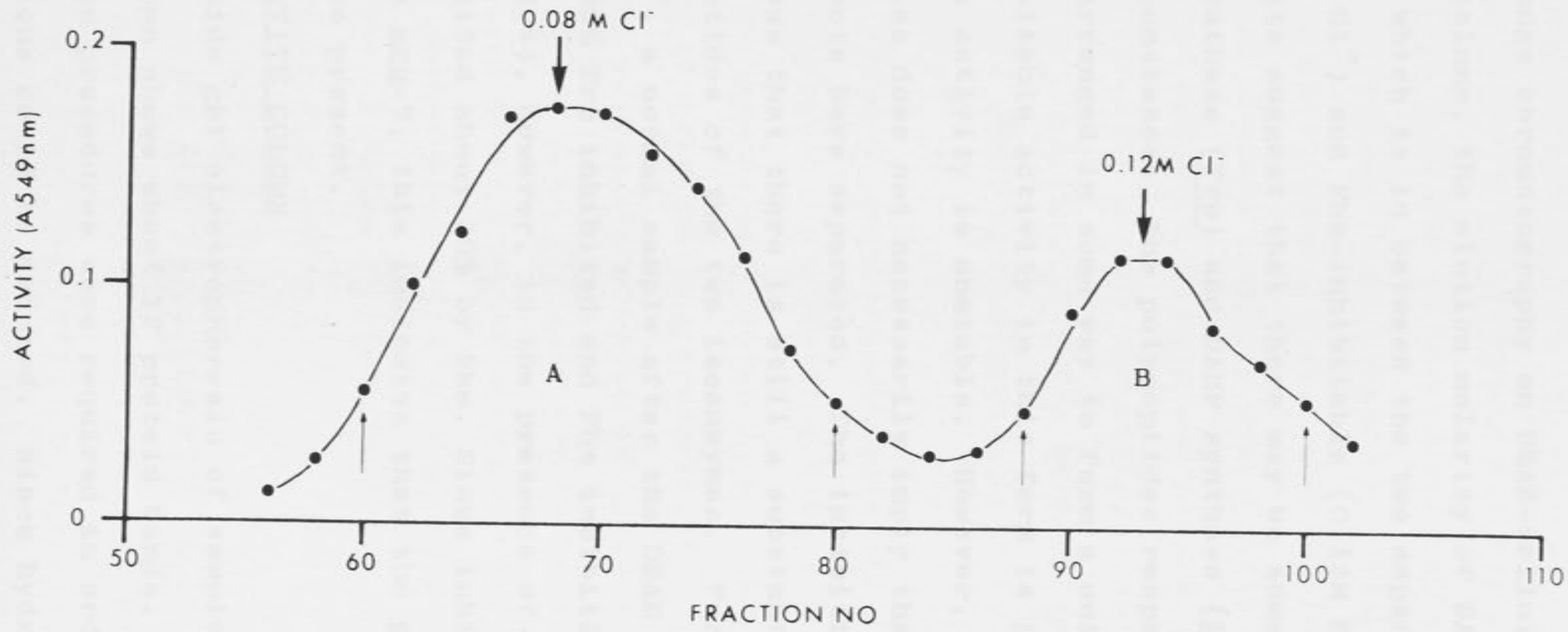


Fig. 3.4 Separation of DAHP synthase activity into two activity peaks on a DEAE-cellulose column (2.5 x 24 cm).

The sample applied was eluted from a Sepharose-Trp affinity column. The column was developed by a linear NH_4Cl gradient (0-0.25M, 500 ml/chamber) at a flow rate of 45 ml/h and 5 ml fractions were collected. Total activity was measured.

What exactly happened on the Sepharose-Trp column is not clear. It seems that the passage through this column makes possible the separation of the Trp- and Phe-inhibitable DAHP synthase activity by anion exchange chromatography on DEAE-cellulose. Without the Sepharose-Trp column, the elution molarity of DAHP synthase (Trp) is 0.1M Cl^-, which is in between the two separated Trp-inhibitable (0.08M Cl^-) and Phe-inhibitable (0.12M Cl^-) activity peaks. These results suggest that there may be some interaction between the DAHP synthase (Trp) and DAHP synthase (Phe) isoenzymes. Under ordinary circumstances, the polypeptides responsible for the isoenzymes may be arranged in some way to form a unit. The failure to detect Phe-inhibitable activity in this form is probably because the Phe-inhibitable activity is unstable. However, the separation of the two activities does not necessarily imply that the two specific gene products have separated. The inhibition of the two separated peaks shows that there is still a substantial interaction between the polypeptides of the two isoenzymes. Furthermore, in the absence of Zn^{2+}, a normal sample after the DEAE cellulose column is almost 100% Trp inhibited and Phe inhibition is insignificant (see Table 3.4). However, in the presence of Zn^{2+}, the same sample can be inhibited about 20% by Phe. Since inhibition by Phe depends entirely on aro-7, this indicates that the product of this locus might still be present.

V. HYDROXYLAPATITE COLUMN

Polyacrylamide gel electrophoresis of samples from the DEAE-cellulose column shows about 12 protein bands. Therefore, further purification procedures were required in order that meaningful conclusions could be deduced. Since hydroxylapatite separates protein by a different mechanism from an ion exchanger, a hyrdoxylapatite column is useful at this stage.

Initially the sample from the DEAE-cellulose column was dialysed against 0.02M KH_2PO_4-NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol before application to the hydroxylapatite column. The column was eluted with a linear phosphate gradient of 0.02M to 0.35M KH_2PO_4-NaOH pH 7.4, 500 ml per chamber with 0.1% (w/v) mercaptoethanol. The gradient was obtained by trial and error. A column length of 20 to 25 cm was used. With a steeper gradient, resolution in terms of protein peaks is poor. With a shallower gradient, the proteins elute as a smear and separation is poor. It is a major characteristic of the hydroxylapatite column that the gradient has to be just right for the column length and the property of the protein under investigation.

A series of protein peaks are obtained (Fig. 3.2). The pattern is very reproducible. The bulk of the coloured material in the sample does not adsorb and elutes as a sharp peak at the front. The activity profile shows a sharp front followed by a shoulder and for some preparations, a second peak appears at the position of the shoulder. The two activity peaks coincide with two consecutive protein peaks and the height of the second activity peak depends on the amount of protein present in the second peak (Fig. 3.5). Sometimes, a third activity peak can be detected after the two major activity peaks.

When the activity peak was re-applied on to the column, even though the protein eluted at the same position, very low activity was recovered. Since without any enzyme activity, one could not be sure whether one was purifying the enzyme or some other protein

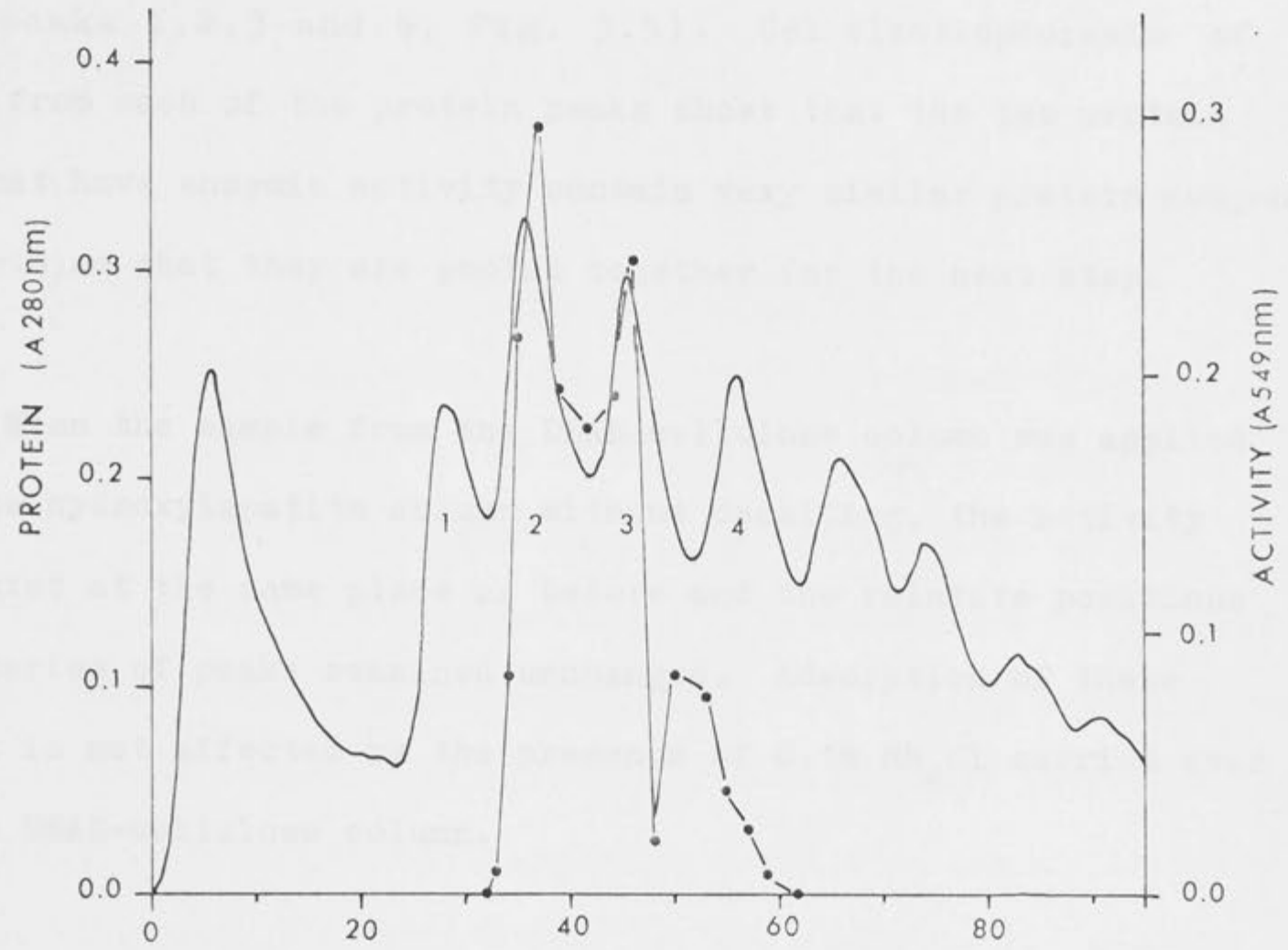


Fig. 3.5 Chromatography on a hydroxylapatite column (2.5 x 25 cm).

Condition for elution was the same as in Fig. 3.2. Note increase in activity corresponding to protein peak 3 concomitant with increase in amount of protein.

●——● DAHP synthase activity; —— protein.

which happened to be there, a second passage through hydroxylapatite was unsuitable at this stage. However, this technique of re-running the sample through the column a couple of times has been used to identify some of the components making up the series of protein peaks (peaks 1,2,3 and 4; Fig. 3.5). Gel electrophoresis of samples from each of the protein peaks shows that the two protein peaks that have enzymic activity contain very similar protein components (see Chapter 4) so that they are pooled together for the next step.

When the sample from the DEAE-cellulose column was applied on to the hydroxylapatite column without desalting, the activity peak eluted at the same place as before and the relative positions of the series of peaks remained unchanged. Adsorption of these proteins is not affected by the presence of 0.1M NH_4Cl carried over from the DEAE-cellulose column.

Hydroxylapatite crystals have two different adsorption sites on their surfaces. These depend on phosphate and calcium, which are responsible for the binding of basic and acidic side groups of proteins, respectively (Bernardi et al.,1972). Binding of acidic protein is through the carboxyl groups on the protein to the calcium on the crystals and elution by a phosphate gradient is thought to be a result of the strong affinity of phosphate for calcium thus displacing the protein from the crystals. The adsorption is therefore not affected by Na^+ or K^+. For basic proteins, binding is through the basic groups to the phosphate on the crystals and elution by a phosphate gradient usually requires high elution molarities. However, these proteins can be eluted by using NaCl or KCl molarity gradients. They can also be eluted by rather weak molarities of $CaCl_2$. Therefore,

the presence of K^+ or Na^+ would interfere with the binding. It seems likely that for DAHP synthase (Trp), the binding is through the carboxyl group to the calcium on the crystal. The elution molarity for the first peak of activity is 0.058M phosphate and that for the second peak of activity is 0.067M phosphate. Therefore, the bindings are weak.

VI. MOLECULAR SIEVINGS ON GEL FILTRATION MEDIA

The apparent molecular weight of DAHP synthase (Trp) in crude extracts was estimated by sieving on agarose columns to be about 150 000 - 165 000 with tailing into 130 000 (Doy, 1968b). When the pooled active fractions from the hydroxylapatite column were applied on to an agarose column (2.5 cm x 100 cm) equilibrated with 0.02M KH_2PO_4 -NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol, the separation as judged by the protein profile is poor. Activity is associated with the early eluted protein (Fig. 3.6). Agarose, with its large pore size, is not suitable for the separation of proteins below molecular weight 200 000. A Sephadex G200 column is more suitable for this enzyme.

The pooled active fractions from the hydroxylapatite column was desalted on a G25 column (2.5 cm x 40 cm) equilibrated with 0.02M KH_2PO_4 -NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol to provide a more constant environment. The desalted sample was concentrated by ultrafiltration and applied on to a Sephadex G200 column (2.5 cm x 100 cm) equilibrated with the same buffer. Mainly three protein peaks could be resolved and activity is associated with the first and fastest eluting peak (Fig. 3.3). The activity

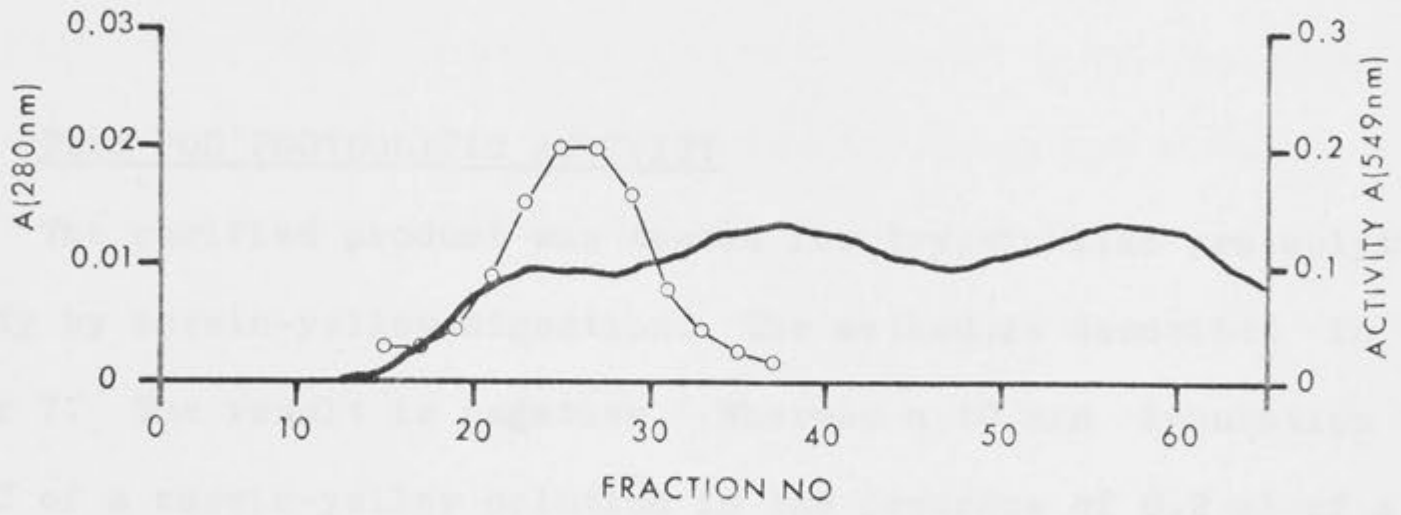


Fig. 3.6 Chromatography of sample eluted from a hydroxylapatite column on an agarose (0.5M) column (2.5 x 100 cm).

The flow rate was maintained at 40 ml/h by a peristaltic pump and 3 ml fractions were collected.

O——O DAHP synthase activity; ——— protein.

peak is symmetrical showing the possibility of a single active species. If the pooled active fractions were reapplied to the same column, recovery of activity was low showing possible breakdown of enzyme on sieving. The elution volume is not changed in the presence of 0.1 mM Trp, which is consistent with results from the crude extracts (Halsall *et al.*, 1971).

VII. TEST FOR PROTEOLYTIC ACTIVITY

The purified product was tested for trypsin-like proteolytic activity by casein-yellow digestion. The method is described in Chapter 7. The result is negative. Whereas a 10 min incubation at 37°C of a casein-yellow solution in the presence of 0.2 ml of a trypsin solution (1mg/ml) resulted in 50% digestion, an incubation for two hours in the presence of 0.5 ml of enzyme solution (0.1 mg/ml) gave no digestion.

However, since polyacrylamide gel electrophoresis results (Chapter 4) show multiple bands it was decided to include a phenylmethylsulphonyl fluoride (PMSF) treatment after the pH changes step.

The PMSF was first dissolved in 2-propanol before addition to the enzyme solution. The solution was allowed to stand at 4°C for 30 min and the precipitate was removed by centrifugation. The supernatant was dialysed against 0.02M KH_2PO_4 -NaOH buffer pH 7.4 containing 0.1% (w/v) mercaptoethanol. When activity was tested after dialysis, it was found that the activity had increased. The reason for the increase in activity is not known but could be a result of the precipitation of some inhibitors by the alcohol. The resulting gel patterns of the final purified enzyme remain unchanged

even when PMSF is included in each step during purification. However, in terms of purification, this step increases the specific activity by two to three fold.

VIII. EFFECT OF EDTA AND DIVALENT METAL ION ON PURIFIED DAHP SYNTHASE (TRP)

The enzyme was dialysed against 0.02M Tris-maleate buffer pH 7.4 and 0.1% (w/v) 2-mercaptoethanol to remove the inorganic phosphate which could precipitate with the metal ions. The results are shown in Table 3.5. 54% of the activity was inhibited when EDTA was added to the enzyme solution to a concentration of 0.25 mM. Of the metal ions tested, only Co^{2+} at 1 mM concentration in the assay mixture will reverse the inhibition by EDTA. In the absence of EDTA, Co^{2+} stimulates activity by about 10%. Inhibition by EDTA can be slightly reversed by Mn^{2+} but not to full activity. Zn^{2+} is an inhibitor of the enzyme. Inhibition is increased in the presence of EDTA.

In the presence of Co^{2+}, inhibition by 0.1 mM Trp is about 90%.

IX. OTHER INHIBITORY PROPERTIES

The ability of various amino acids, and compounds related to Trp, to inhibit the enzyme was tested. Results are summarised in Table 3.6. None of the other amino acids tested has any significant effect on DAHP synthase (Trp). D-Trp stimulates activity slightly and so does indole. The enzyme is not inhibited by tryptamine and anthranilate, whereas 5-methyltryptophan inhibits 16.3%. The structures of indole, tryptamine, anthranilic acid and 5-methyltryptophan are shown below.

TABLE 3.5

Effects of EDTA and divalent metal ions on purified DAHP synthase (Trp) from G200 column.

Metal ions **	% Inhibition *	
	+EDTA	-EDTA
-	54	-
Mg^{2+}	55	- 3.6
Mn^{2+}	33	0
Ca^{2+}	52	- 5.1
Zn^{2+}	62.5	57.4
Co^{2+}	3.6	- 9.2
Cu^{2+}	Interfere with assay method	
Fe^{2+}	Interfere with assay method	

* Negative inhibition means stimulation of activity.

** Concentration = 1 mM

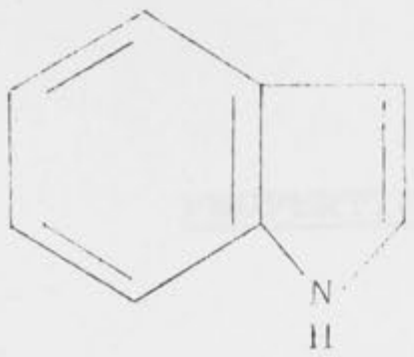
TABLE 3.6

Effects of amino acids and related compounds on activity of purified DAHP synthase (Trp).

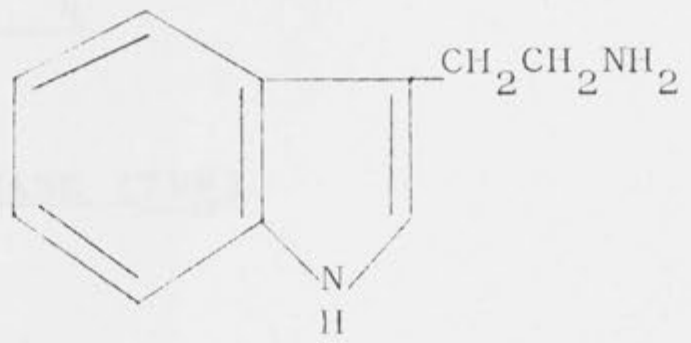
Ligand*	% Inhibition**
L-Trp	99
D-Trp	- 3.6
DL-5-Methyl Trp	16.3
Anthranilate	1.0
Tryptamine	1.2
Indole	- 3.6
L-Ala	4
L-His	1
L-Arg	0
L-Lys	2
L-Asp	3
L-Met	- 3
L-Thr	- 1.7
L-Asn	2.7
L-Ser	- 3.7
L-Val	2.4
L-Glu	2

* Concentration = 0.1mM.

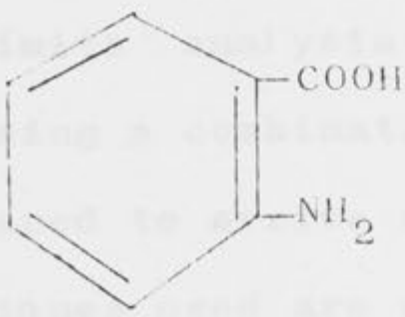
** Negative inhibition means stimulation of activity.



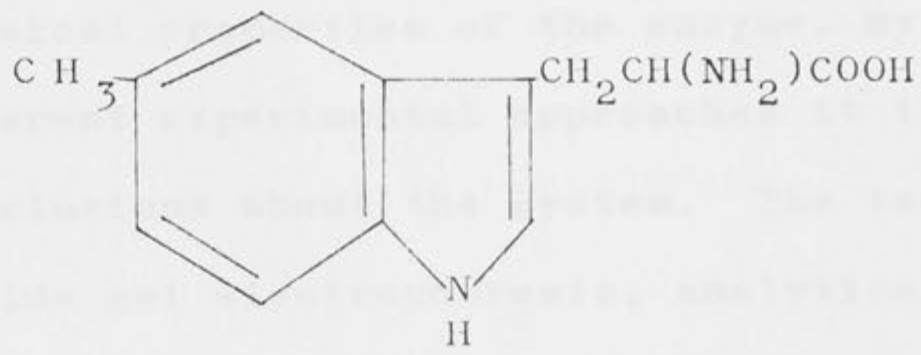
INDOLE



TRYPTAMINE



ANTHRANILIC ACID



5-METHYLTRYPTOPHAN

It can be concluded that the indole group is important for binding to the enzyme, and the carboxyl group and the side arm is important for inhibition. Whether the amino group on the side arm is important for inhibition or not was not tested.

CHAPTER 4

PROPERTIES OF DAHP SYNTHASE (TRP)

I. INTRODUCTION

The low yield from the present purification procedure limits analysis of the physical properties of the enzyme. By using a combination of different experimental approaches it is hoped to arrive at some conclusions about the system. The techniques used are polyacrylamide gel electrophoresis, analytical ultracentrifugation and chromatography on gel filtration columns. Details of the methods are described in Chapter 7.

II. SUBUNIT STRUCTURE OF ENZYME FROM FINAL STEP, SEPHADEX G200 COLUMN (CHAPTER 3)

The activity peak eluted from the Sephadex G200 column was divided into three portions, A, B and C (Chapter 3, Fig. 3.3), pooled and concentrated separately. The three portions, A, B and C representing the leading, peak and trailing activity fractions, respectively, were then analysed separately by polyacrylamide gel electrophoresis.

(a) Peak Activity (Portion B)

Non-denaturing polyacrylamide gel electrophoresis initially shows two bands (band 1, Rm=0.1 and band 3, Rm=0.2, Fig. 4.1a). Occasionally, an intermediate band 2 (Rm=0.15), appears in between bands 1 and 3 (Fig. 4.1b). For the majority of samples, the two-band pattern is typical. However, depending on the preparation, the two bands can be 1 and 3 or 2 and 3, with the former combination the more common.

Dodecyl sulphate gel electrophoresis gives two main bands

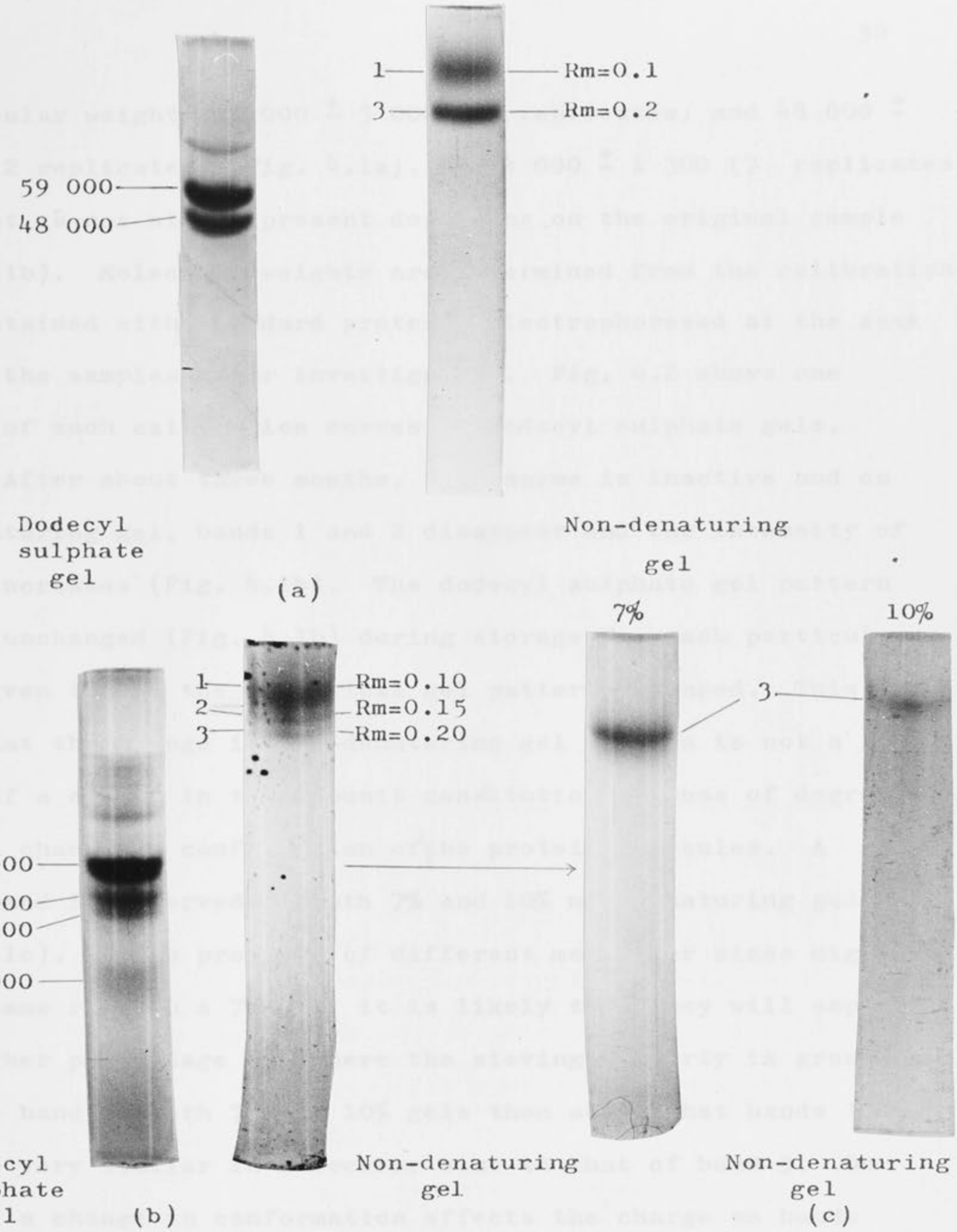


Fig. 4.1 Polyacrylamide gel electrophoresis of peak activity fractions from G200 columns. 7% gels were used for non-denaturing in (a) and (b) and 10% dodecyl sulphate gels were used. Migration is from top to bottom. Approximately 100 µg protein was applied to each gel.

(a) Two bands on non-denaturing gel and two major bands on dodecyl sulphate gel.

(b) Three band pattern on non-denaturing gel and slightly different dodecyl sulphate gel from (a).

(c) Three band pattern changed into one band on both 7% and 10% gels on storage.

of molecular weights 59 000 ± 3 000 (12 replicates) and 48 000 ± 1 700 (12 replicates) (Fig. 4.1a). A 44 000 ± 1 300 (7 replicates) component is not always present depending on the original sample (Fig. 4.1b). Molecular weights are determined from the calibration curve obtained with standard proteins electrophoresed at the same time as the samples under investigation. Fig. 4.2 shows one example of such calibration curves on dodecyl sulphate gels.

After about three months, the enzyme is inactive and on non-denaturing gel, bands 1 and 2 disappear and the intensity of band 3 increases (Fig. 4.1b). The dodecyl sulphate gel pattern remains unchanged (Fig. 4.1b) during storage for each particular sample even though the analytical gel patterns changed. This shows that the change in non-denaturing gel pattern is not a result of a change in the subunit constitution because of degradation but is a change in conformation of the protein molecules. A single band is observed on both 7% and 10% non-denaturing gels (Fig. 4.1c). If two proteins of different molecular sizes migrate at the same rate on a 7% gel, it is likely that they will separate on a higher percentage gel where the sieving property is greater. A single band on both 7% and 10% gels then shows that bands 1 and 2 may be very similar in molecular size to that of band 3. On storage, a change in conformation affects the charge on bands 1 and 2 proteins so that they now migrate at the same rate as band 3. If bands 1,2 and 3 have different molecular sizes, then, it is possible that the single band after storage is a change in conformation that result in association of bands 1, 2 and 3 proteins. This is investigated further in Section IV.

Attempts were made to stain for enzymatic activity on the gel. The usual enzyme assay depends on the formation of chromagen

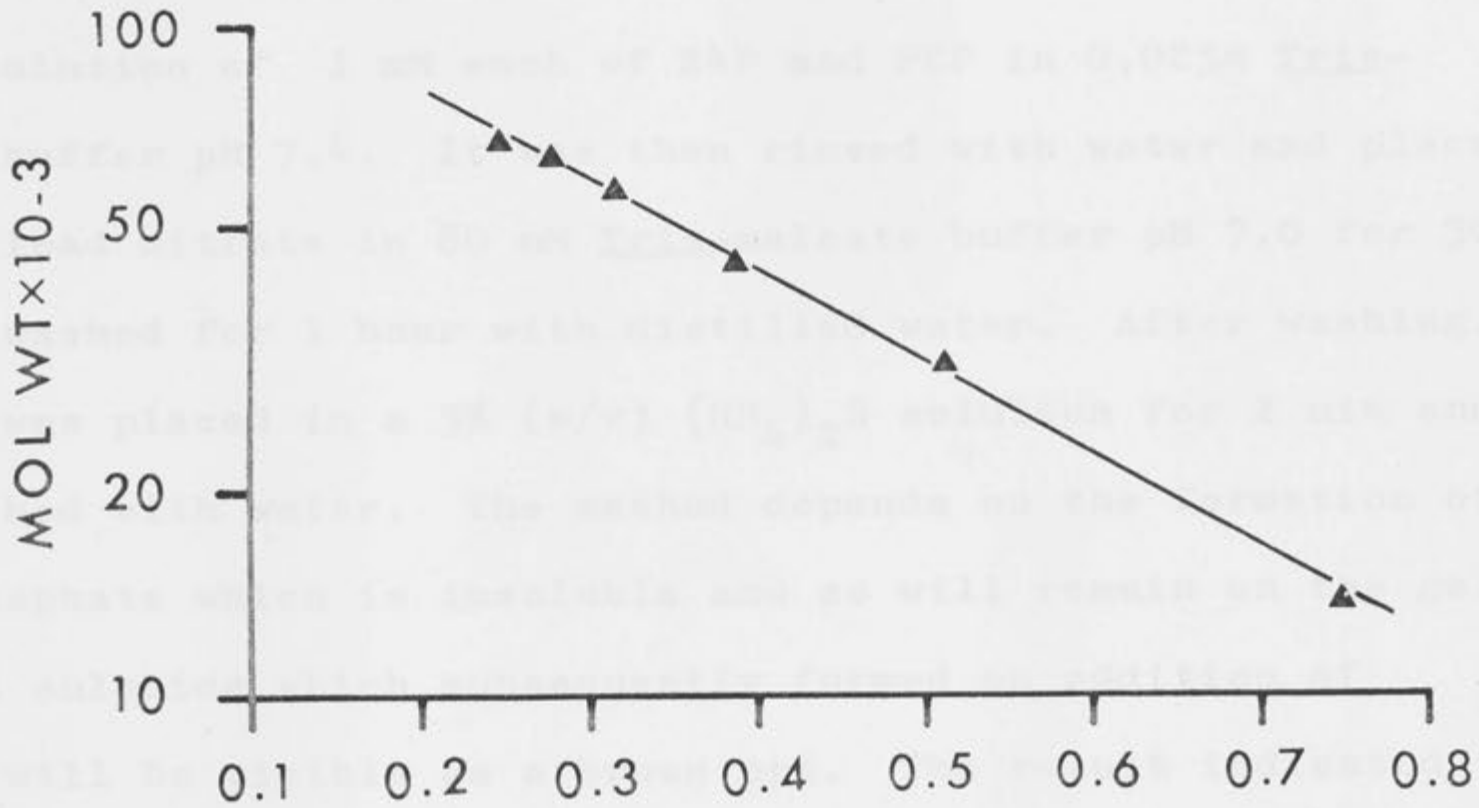


Fig. 4.2 Molecular weight calibration of dodecyl sulphate polyacrylamide gel; a plot of log molecular weight versus relative mobility (Rm).

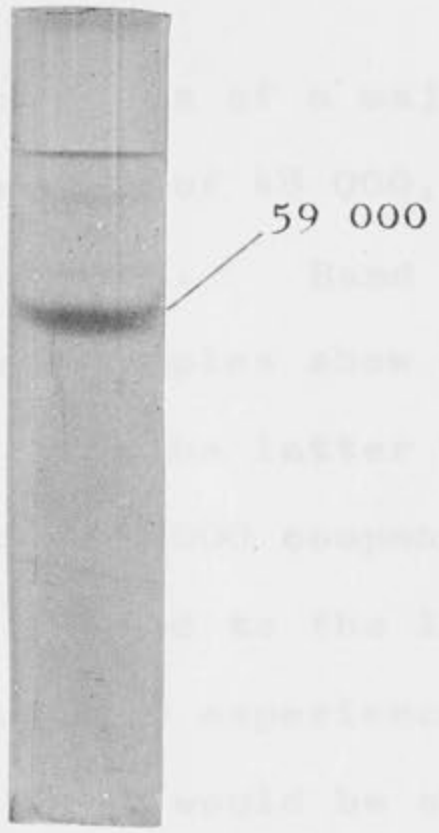
Protein standards used were: bovine serum albumin (68 000); glucose 6-phosphate dehydrogenase (63 000); pyruvate kinase (57 000); ovalbumin (44 000); deoxyribonuclease B (37 000) and ribonuclease A (13 700).

from one of the two reaction products, DAHP (see Chapter 7), and requires heating at 100^{0}C for 8 min. This method is unsuitable for assay on the gel. Since inorganic phosphate is the other reaction product it was intended to stain for inorganic phosphate on the gel (Gabriel, 1971).

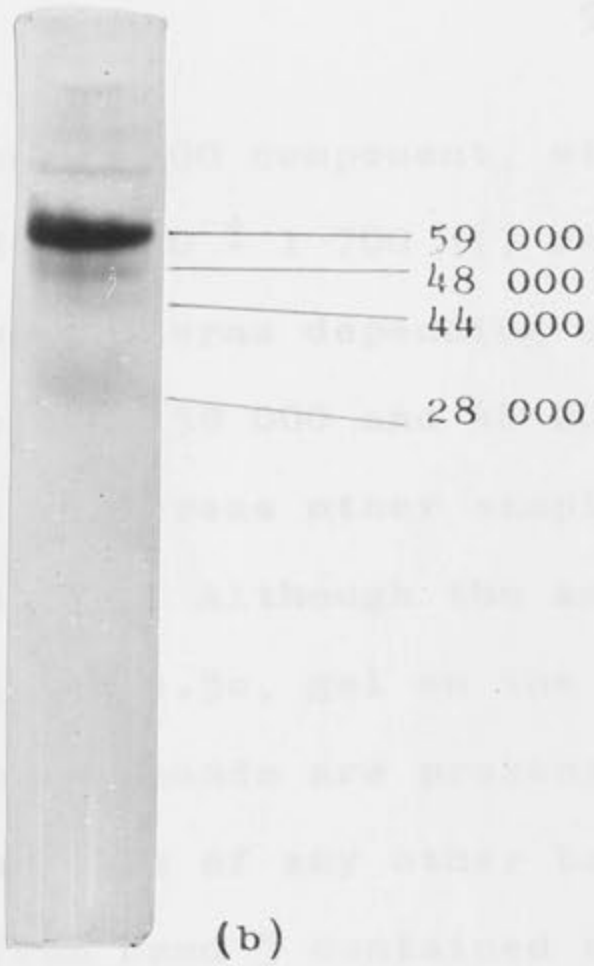
After electrophoresis, the gel was incubated for 30 min with a solution of 1 mM each of E4P and PEP in 0.025M Tris-maleate buffer pH 7.4. It was then rinsed with water and placed in 3 mM lead nitrate in 80 mM Tris-maleate buffer pH 7.0 for 30 min and washed for 1 hour with distilled water. After washing, the gel was placed in a 5% (w/v) $(NH_4)_2S$ solution for 2 min and then washed with water. The method depends on the formation of lead phosphate which is insoluble and so will remain on the gel. The lead sulphide which subsequently formed on addition of $(NH_4)_2S$ will be visible as a brown ppt. The result indicated that brown ppt. could be found in both positions of bands 1 and 3. However, a similar gel without incubation with substrates also showed pptn. at the same positions. This means that the proteins contained bound inorganic phosphate.

It was thus decided to chop the gel up into slices and assay for activity after eluting with 0.02M phosphate buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol. Tryptophan inhibited activity could be found associated with bands 1 and 2. The recovery of activity is 10% to 19%. Mixing of the proteins eluted from the gel slices did not increase the activity. This could mean that denaturation of the enzyme occurred during electrophoresis on the gel or that the dissociation of the enzyme into two proteins leads to instability.

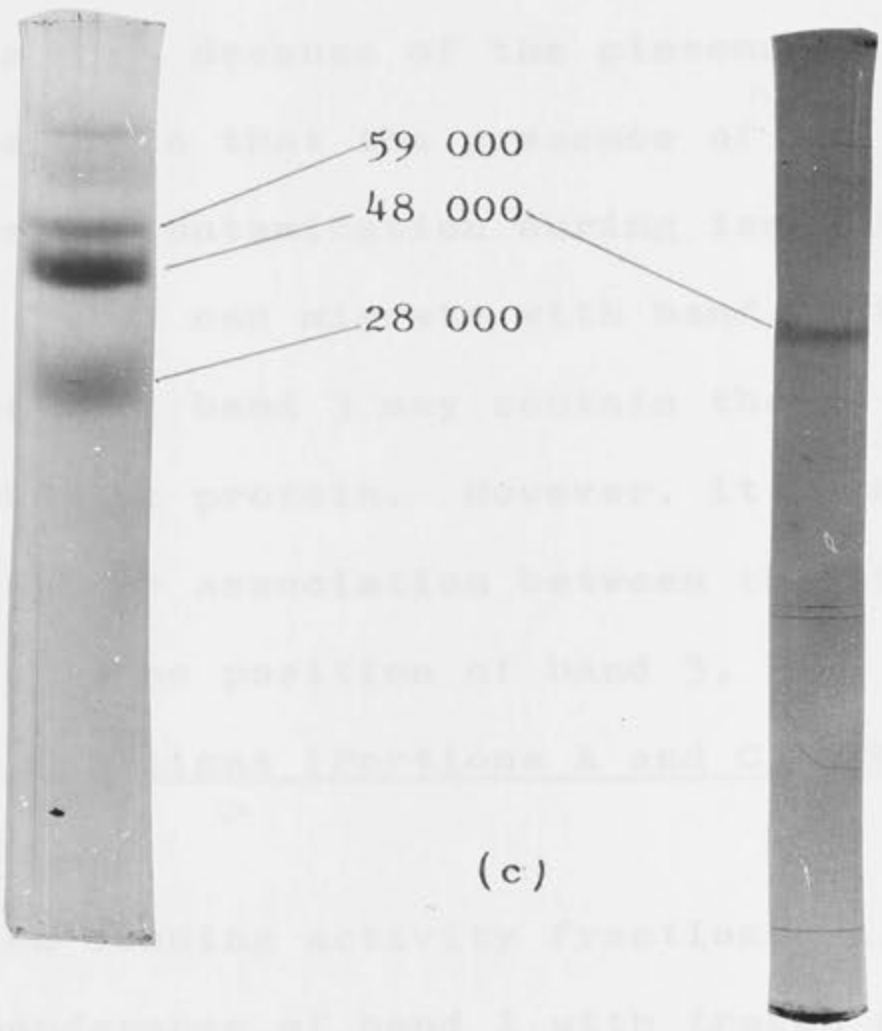
Dodecyl sulphate gel electrophoresis of protein 1 eluted from gel slices shows essentially the 59 000 component (Fig. 4.3a).



(a)



(b)



(c)

Fig. 4.3 Dodecyl sulphate gel (10%) electrophoresis of (a) protein 1; (b) protein 2; (c) protein 3. Samples of approxiately 50 µg protein was applied to each gel except for (c) gel on the right in which about 30 µg protein was applied. For protein 3 (c), preparations could be obtained that contained only the 48 000 component. Molecular weight is obtained by reference to calibration standards which were electrophoresed simultaneously with the sample under investigation.

Band 2 consists of a majority of the 59 000 component, with minor components of 48 000, 44 000 and 28 000 ± 1 700 (7 replicates)(Fig. 4.3b). Band 3 shows two patterns depending on the sample. Some samples show a mixture of the 59 000 and 48 000 components with the latter predominating whereas other samples show only the 48 000 component (Fig. 4.3c). Although the amount of protein applied to the latter gel (Fig. 4.3c, gel on the right) is less, it is my experience that if other bands are present, some faint bands would be observed. No trace of any other bands was observed showing that the sample from band 3 contained only the 48 000 component. Because of the closeness of the bands 1, 2 and 3, it is possible that the presence of the 59 000 component in band 3 is due to contamination during isolation or as discussed earlier, bands 1 and 2 can migrate with band 3 at some stage during storage so that band 3 may contain the 59 000 component that belongs to band 1 protein. However, it remains possible that there may be a genuine association between the 59 000 and the 48 000 molecules at the position of band 3.

(b) Off-peak Fractions (Portions A and C) (Chapter 3, Fig. 3.3)

The pooled leading activity fractions, A, on non-denaturing gels show a preponderance of band 1 with traces of bands 2 and 3 (Fig. 4.4a). With some samples, a trace amount of a faster migrating component can be observed (band 4, Rm=0.25) (Fig. 4.4b). The dodecyl sulphate gel pattern (Fig. 4.4a) shows a major component of molecular weight 59 000.

The pooled trailing activity fractions, C, on analytical gel show a preponderance of band 3 which from these fractions, characteristically stains more diffusely on the edge nearer to the origin (Fig. 4.4c). A few faster migrating minor bands,

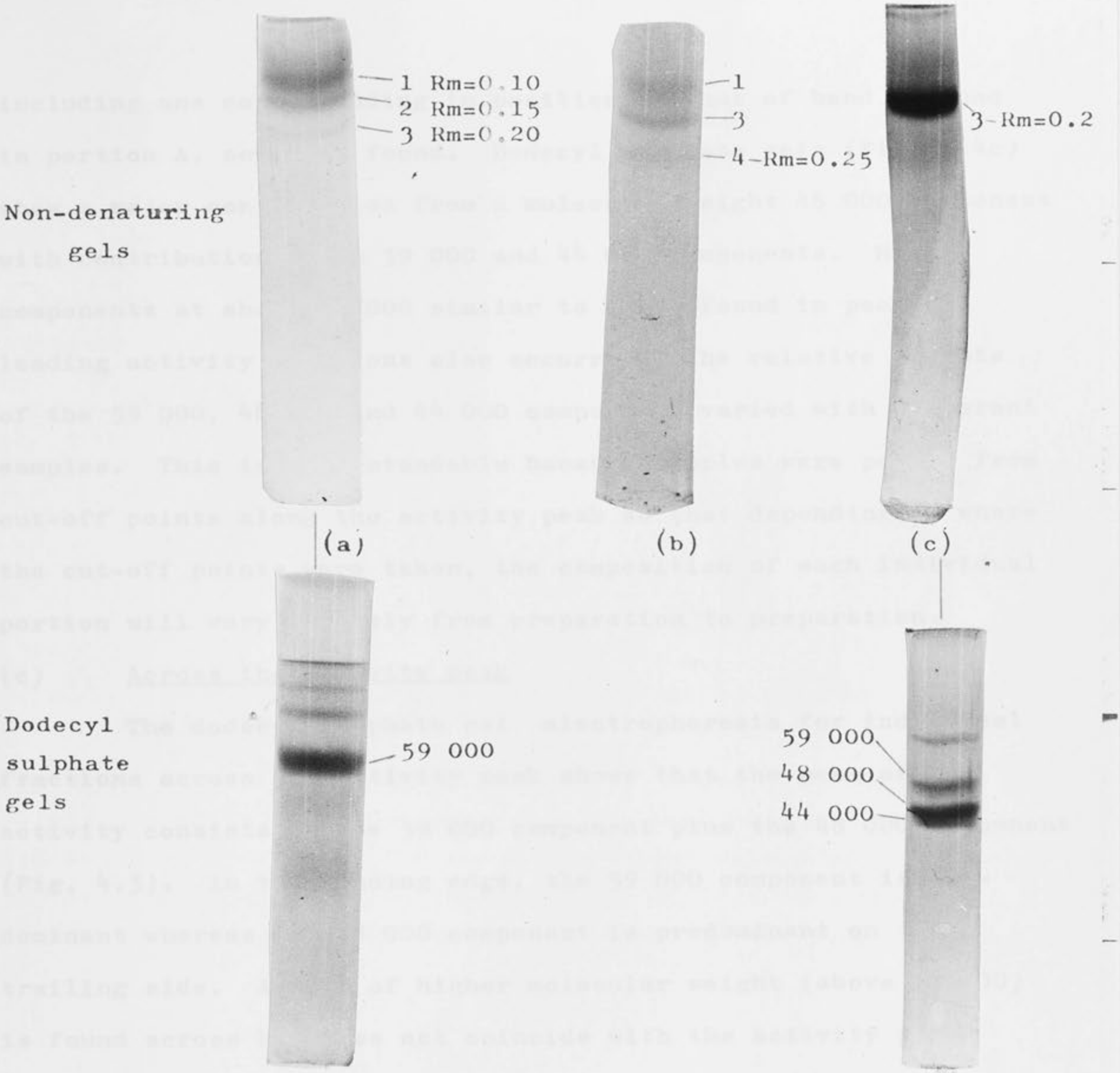


Fig. 4.4 Polyacrylamide gel electrophoresis of samples from leading activity and trailing activity fractions (portions A and C on Fig. 3.3). Approximately 100 µg protein was applied.

(a) Leading activity fractions (A) on 7% non-denaturing gels and 10% dodecyl sulphate gels.

(b) Leading activity fraction (A) sometimes showed an extra band.

(c) Trailing activity fraction (C) on 7% non-denaturing gel and 10% dodecyl sulphate gel.

including one corresponding in position to that of band 4 found in portion A, could be found. Dodecyl sulphate gels (Fig. 4.4c) show a major contribution from a molecular weight 48 000 component with contributions from 59 000 and 44 000 components. Minor components at about 28 000 similar to those found in peak and leading activity fractions also occurred. The relative amounts of the 59 000, 48 000 and 44 000 components varied with different samples. This is understandable because samples were pooled from cut-off points along the activity peak so that depending on where the cut-off points were taken, the composition of each individual portion will vary slightly from preparation to preparation.

(c) Across the activity peak

The dodecyl sulphate gel electrophoresis for individual fractions across the activity peak shows that the peak of the activity consists of the 59 000 component plus the 48 000 component (Fig. 4.5). In the leading edge, the 59 000 component is predominant whereas the 48 000 component is predominant on the trailing side. A band of higher molecular weight (above 80 000) is found across but does not coincide with the activity peak.

The $A_{549\ nm}/A_{280\ nm}$ ratio across the activity peak (Fig. 3.3) is approximately constant at the peak of the activity and declines on both leading and trailing edges of the activity profile. Since protein 1 has enzyme activity and is made up of the 59 000 component, it is possible that the specific activity of protein 1 increases with increase in concentration of protein 1 as a result of stabilisation of molecular forms or some other co-operative effect. It is also possible that maximum activity depends on a certain ratio of 59 000 to 48 000 components. This interpretation implies that the 48 000 component is connected with DAHP synthase (Trp) activity. The effect of protein concentration on activity is not non-specific because the activity is increased

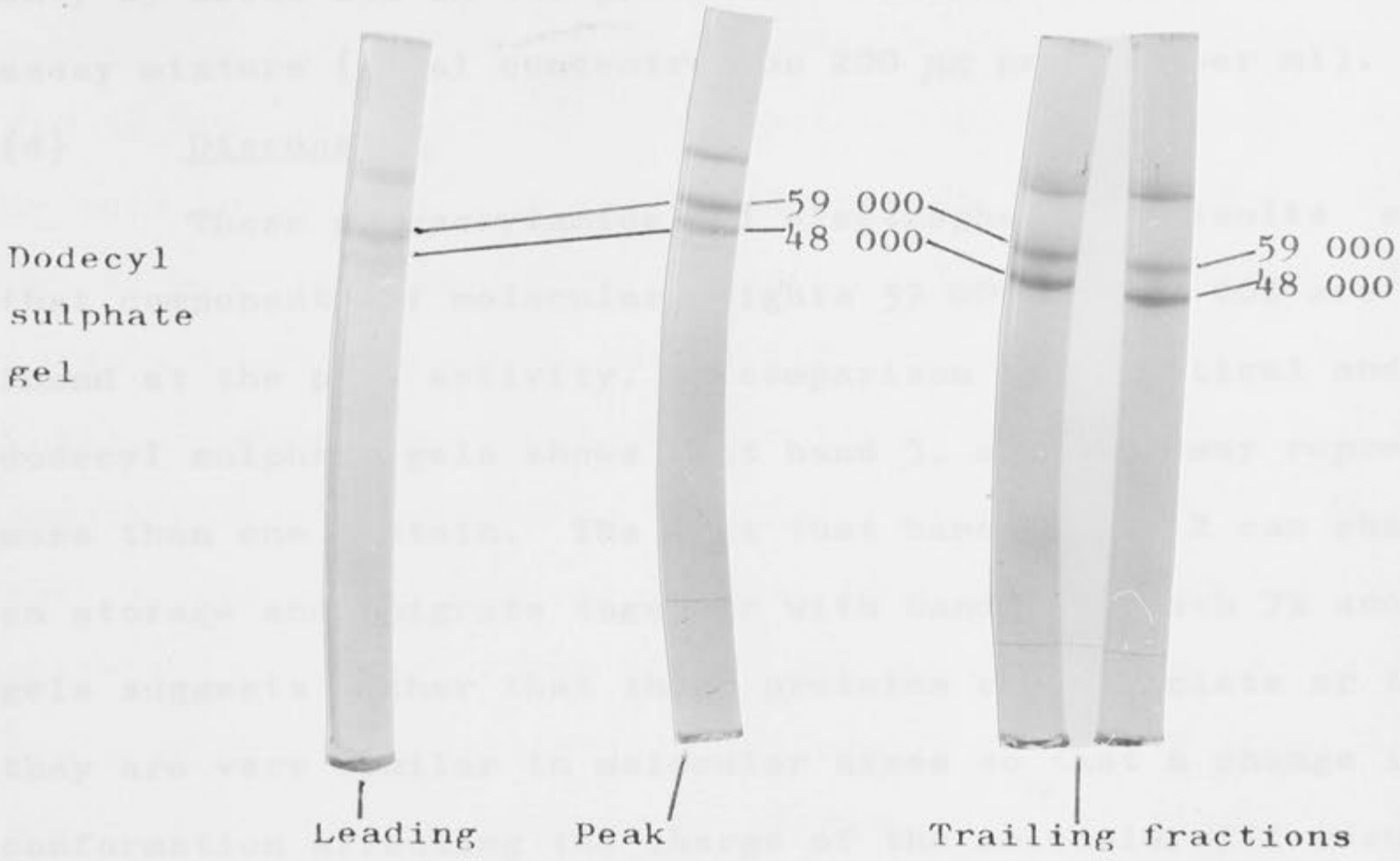


Fig. 4.5 Dodecyl sulphate gels (10%) of individual fractions across the activity peak eluted from the G200 column (Fig. 3.3) Migration is from top to bottom. Equivalent volume of sample was applied to each gel so that each gel will reflect the amount of protein in each fraction relative to the other fractions.

only by about 15% in the presence of bovine serum albumin in the assay mixture (final concentration 200 µg protein per ml).

(d) Discussion

These polyacrylamide gel electrophoresis results show that components of molecular weights 59 000 and 48 000 are found at the peak activity. A comparison of analytical and dodecyl sulphate gels shows that band 3, at times, may represent more than one protein. The fact that bands 1 and 2 can change on storage and migrate together with band 3 on both 7% and 10% gels suggests either that these proteins can associate or that they are very similar in molecular sizes so that a change in conformation affecting the charge of the molecules will result in a similar rate of migration on the gel.

The change in enzyme forms as detected on gels is not unique to DAHP synthase (Trp). DAHP synthase (Tyr) when purified initially showed two bands and only the fast migrating band was active (Hoffmann et al., 1972). On storage, the enzyme slowly activated and subsequently the inactive slow band disappeared concomitantly with an increase in the active fast band. However, when the active fast band was isolated by preparative gel electrophoresis, analytical gels showed a number of bands, some of which were not observed before. Molecular sieving of the purified DAHP synthase (Tyr) on an agarose column also showed a nonsymmetrical activity peak with a number of shoulders indicating a mixture of enzyme forms (Hoffmann, 1971). The activity peak for DAHP synthase (Trp) eluted from the Sephadex G200 column is, however, smooth and symmetrical (Fig. 3.3).

In terms of DAHP synthase (Trp) activity, protein 1 contains both the activity and the allosteric sites and is made

up of the 59 000 component. Since aro-8 is the genetic locus coding for DAHP synthase (Trp) activity, it presumably codes for the 59 000 component. As it is not certain how many cistrons are in this locus (see Chapter 2), the genetic origins of the 48 000 and 44 000 components remain unknown. These proteins represent those with the strongest affinities for each other or with similar physical properties under the condition of purification. The simplest interpretation is that the 48 000 and the 44 000 components are contaminants in the sense that they have no DAHP synthase (Trp) activity. However, since the 48 000 component can be found at the peak of the activity in significant proportion (Fig. 4.5) and since protein 1 can change and migrate with protein 3, it is possible that the 48 000 component can become associated with the 59 000 component which is responsible for DAHP synthase (Trp) activity. Another possibility is that the 48 000 and the 44 000 components are degradation products of the 59 000 component. This possibility is especially true for the 44 000 component which is not always present. This was investigated further and the results are discussed in Section III.

The presence of the 28 000 component which is about half the size of the 59 000 component seems to suggest that the 59 000 protein may not be the ultimate subunit. If the 59 000 component does exist as an aggregate with inter- and/or intra-chain cross-links, the results of the determination of subunit molecular weights by the method of dodecyl sulphate gel electrophoresis will have to be taken with caution. Reynolds and Tanford (1970) postulated that in the presence of excess dodecyl sulphate, the binding of dodecyl sulphate to each protein molecule transforms the protein into a uniform rod-like protein-dodecyl sulphate complex whose length varies directly with the molecular weight of the protein.

However, it is becoming increasingly clear that the electrophoretic mobility of some proteins in dodecyl sulphate polyacrylamide gel is not determined by molecular weight alone but rather by a complex interplay of molecular weight, net charge, differential dodecyl sulphate binding, size, shape and structural compactness of the protein molecules (Tung and Knight, 1972; Griffith, 1972). It was also found that this method does not yield accurate molecular weights for proteins with polypeptides held together by interchain peptide bonds (McDonagh et al., 1972). Two subunits being held together by interchain cross-links could not have an effective surface area equal to that for two free subunits so that the estimation of molecular weight will not be accurate (McDonagh et al., 1972). The validity of the method thus depends on complete unfolding of the polypeptides such that the binding ratio is constant for all proteins. This ideal condition may not be achieved for complex aggregates, especially for multi-enzyme complexes. In this work, proteins were reduced by mercaptoethanol but the thiol groups were not blocked, for example, by iodoacetamide. If intra- and inter-chain peptide as well as disulphide bonds are involved, the separation of subunits may not be complete. However, it is possible that polypeptides smaller than the 59 000, 48 000 and 44 000 components are degradation products of the heavier components since they are not always present.

III. PEPTIDE MAPPING OF PURIFIED BAND 1 AND BAND 3 PROTEINS

The previous section has shown that protein 1 is made up from the 59 000 component and band 3 can be obtained that contains only the 48 000 component. The possibility that the 48 000 polypeptide is a degradation product of the 59 000 polypeptide is tested by peptide mapping of the two proteins.

Since there is no change in the dodecyl sulphate gel

pattern when band 1 changed and migrated with band 3 on storage (Section II), the change on non-denaturing gel is not a result of a change in subunit constitution and if the 48 000 molecule is a degradation product of the 59 000 molecule, the degradation must have occurred before the last step in the purification procedures and not during storage.

Proteins 1 and 3 were purified by polyacrylamide gel electrophoresis followed by eluting the proteins from the gel slices which corresponded in positions to the two proteins. For band 3, only those samples which contained the 48 000 component alone were pooled and used. Attempts to elute the proteins from the bottom of the gel was unsuccessful because the bands ceased to migrate on prolonged electrophoresis.

Proteins were reduced and alkylated by the method of Crestfield _et al_. (1963). Methods of digestion with trypsin and dansylation of peptides by dansyl chloride are described in Chapter 7. The dansylated peptides were separated and visualised by thin-layer chromatography on polyamide sheet. The control with trypsin alone was similarly dansylated and chromatographed to determine which spots on the chromatogram were derived from trypsin. The results of these analyses are shown in Fig. 4.6a for protein 1 and Fig. 4.6b for protein 3. Spots due to trypsin alone are omitted from the diagrams.

If the 48 000 molecule is degraded from the 59 000 molecules, one would expect the peptide maps of the two proteins to show about 80% similarity. The results show (Fig. 4.6a,b) only about 30% similarity between the two proteins which means that the 48 000 molecule is not a degradation product of the 59 000 molecule. However, it is possible that the two molecules are related. If the 48 000 molecule is a product of either _aro_-6 or _aro_-7, DAHP synthases (_Tyr_) and (_Phe_) respectively, then it is not surprising that the

Fig. 4.6a Tryptic peptide map on thin-layer polyamide sheet for protein 1 isolated from non-denaturing gels by eluting from gel slices. Protein was digested with trypsin and dansylated with dansyl chloride.

Solvent for first dimension:

water-90% formic acid (200:3, v/v)

Solvent for second dimension:

benzene-acetic acid (9:1, v/v)

The solvent was allowed to run 16 cm in the first dimension and 14 cm in the second dimension.

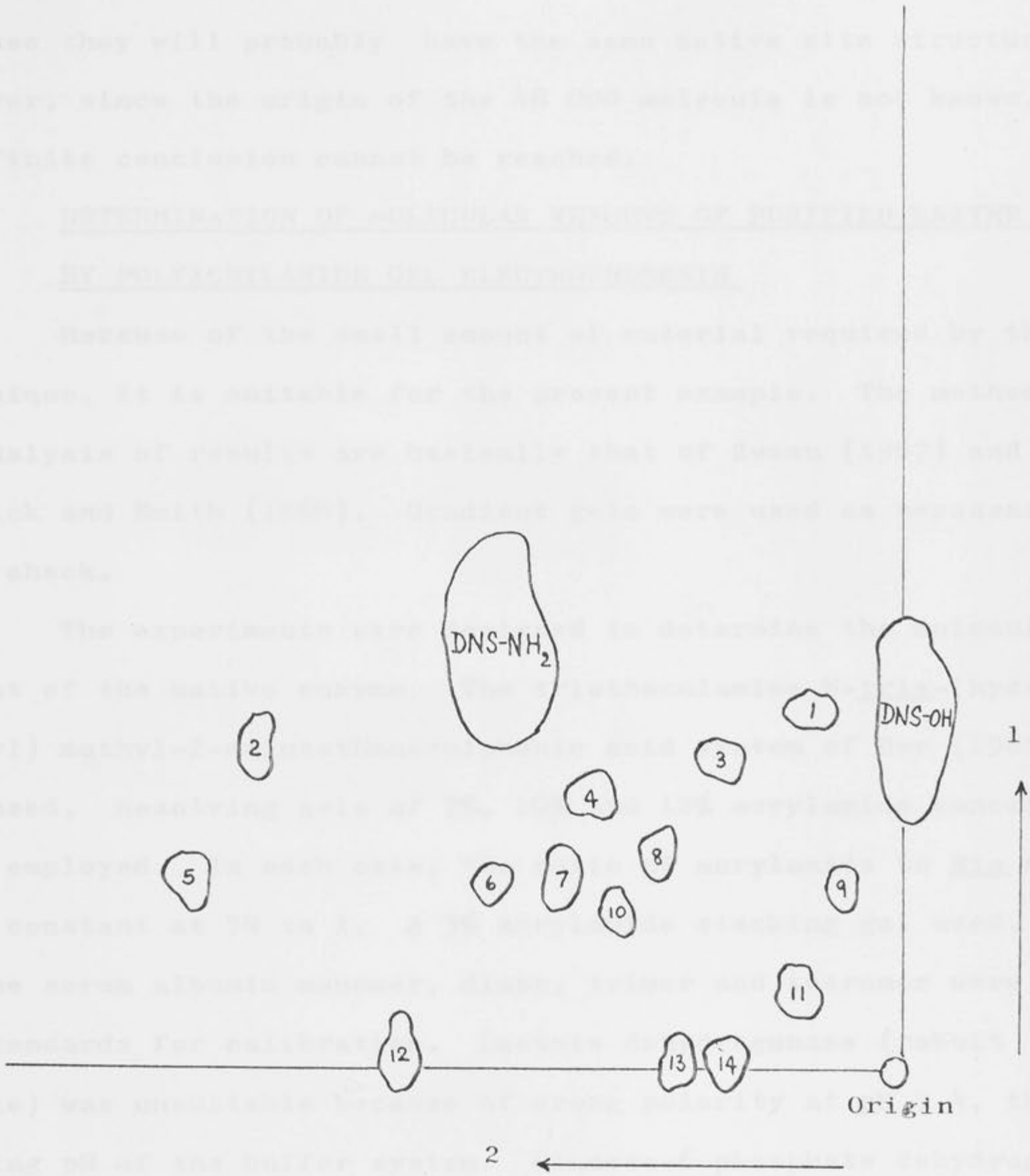


Fig. 4.6b Tryptic peptide map on thin-layer polyamide sheet for protein 3 (which contained only the 48 000 component) isolated from non-denaturing gels by eluting from gel slices. Protein was digested with trypsin and dansylated with dansyl chloride.

Solvent for first dimension:
water-90% formic acid (200:3, v/v)
Solvent for second dimension:
benzene-acetic acid (9:1, v/v)

The solvent was allowed to run 16 cm in the first dimension and 13 cm in the second dimension.

48 000 and the 59 000 molecules have some degree of similarity because they will probably have the same active site structure. However, since the origin of the 48 000 molecule is not known, a definite conclusion cannot be reached.

IV. DETERMINATION OF MOLECULAR WEIGHTS OF PURIFIED ENZYME BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Because of the small amount of material required by this technique, it is suitable for the present example. The methods of analysis of results are basically that of Zwaan (1967) and Hedrick and Smith (1968). Gradient gels were used as necessary as a check.

The experiments were designed to determine the molecular weight of the native enzyme. The triethanolamine N-tris-(hydroxymethyl) methyl-2-aminoethanesulphonic acid system of Orr (1969) was used. Resolving gels of 7%, 10% and 12% acrylamide concentration were employed. In each case, the ratio of acrylamide to Bis was kept constant at 70 to 1. A 3% acrylamide stacking gel used. Bovine serum albumin monomer, dimer, trimer and tetramer were used as standards for calibration. Lactate dehydrogenase (rabbit muscle) was unsuitable because of wrong polarity at pH 7.4, the running pH of the buffer system. Glucose-6-phosphate dehydrogenase (yeast) was tried but the result gave a lower molecular weight than reported. It was subsequently found out that glucose-6-phosphate dehydrogenase tends to dissociate in the absence of NADP which was not added to the buffer (Kirkman and Hendrickson, 1962).

The results of these electrophoretic runs are shown for DAHP synthase (Trp) (Fig. 4.7a) and the albumin polymers (Fig. 4.7b).

In the method of Zwaan (1967), the log of the molecular

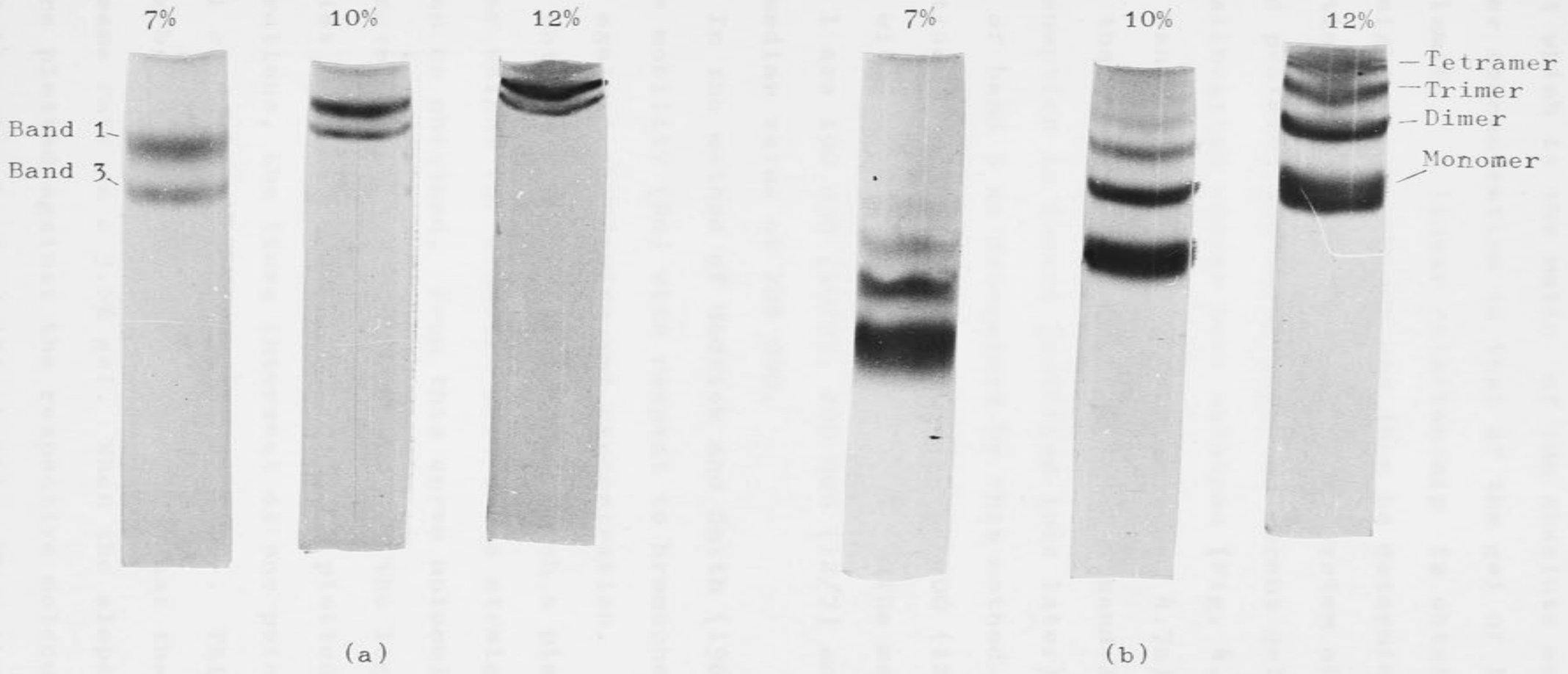


Fig. 4.7 (a) Electrophoresis of portion B (Fig. 3.3) on three different percentages gels. (b) Electrophoresis of serum albumin on three different gel concentrations showing polymer system. Migration is from top to bottom.

The triethanolamine tris-(hydroxymethyl) methyl-2-aminoethanesulphonic acid system of Orr (1969) with a running pH of 7.6 was used. Approximately 100 ug protein was applied to each gel.

weight of the standard protein is plotted against the retardation quotient which is the ratio of the absolute mobility in the gel of higher concentration to that of the gel of lower concentration of acrylamide. A linear relationship is obtained and the molecular weight of an unknown can then be determined from the calibration curve. Using the polymer system of serum albumin as standard proteins and using three different gel concentrations three calibration curves were obtained (Fig. 4.8). Since there are two bands, 1 and 3, on the gels (Fig. 4.7a), it is first assumed that band 3 migrates faster than band 1 in all three gels. This assumption is indeed justified (see later). The molecular weights of band 3 as determined by this method are, for the three calibration curves, 156 000 (10/7), 160 000 (12/7) and 136 000 (12/10) with a median value of 156 000. The molecular weights of band 1 are 190 000 (10/7), 208 000 (12/7) and 204 000 (12/10) with a median value of 204 000.

In the method of Hedrick and Smith (1968), the log of relative mobility (Rm) with respect to bromophenol blue is plotted against acrylamide gel concentration. A linear relationship is obtained. When the slope of such a plot is plotted against molecular weight for standard protein, a straight line calibration curve can be obtained. From this curve molecular weights of unknown proteins can be estimated. When the log of relative mobilities for the albumin polymers are plotted against gel concentrations, the lines intersect at one point corresponding to a gel concentration of 3.6% (Fig. 4.9). This proves that the four bands are indeed polymers and that they will all migrate at the same rate on a 3.6% gel. When the slopes from these plots are plotted against the respective molecular weights, a straight line is obtained (Fig.4.10). When the log of relative

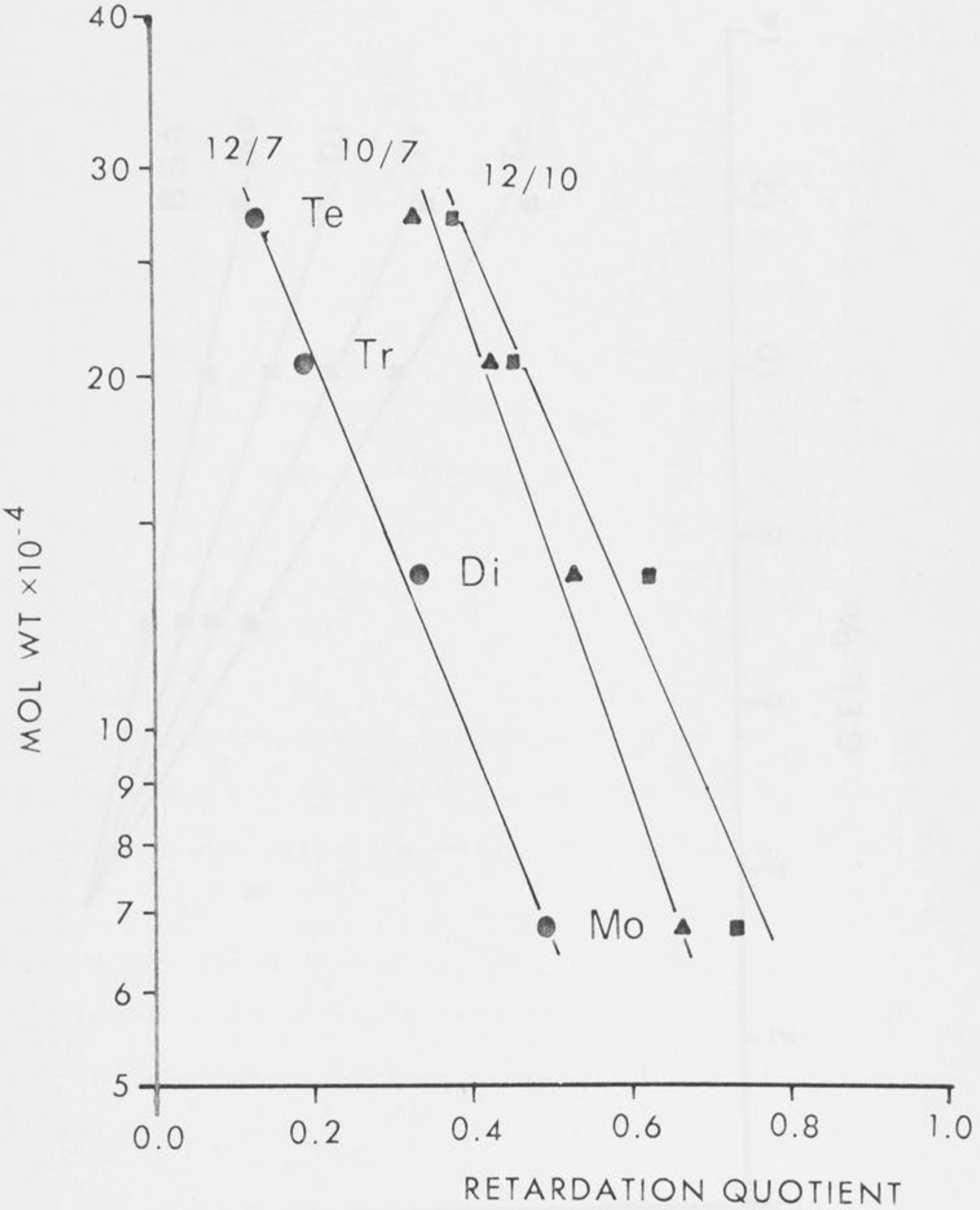
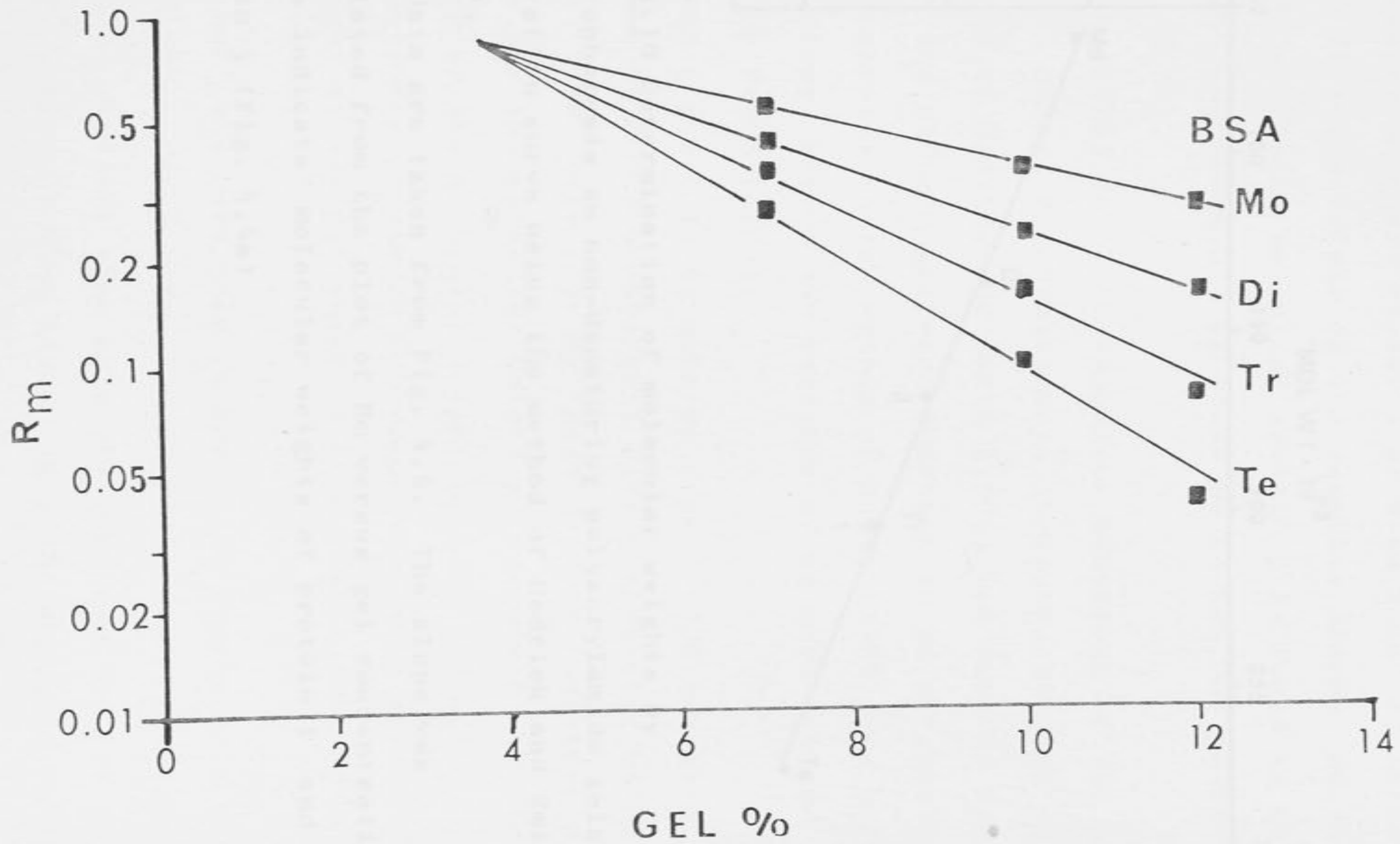


Fig. 4.8 Determination of molecular weights by electrophoresis on non-denaturing polyacrylamide gels. Calibration curves using the method of Zwaan (1967).

Serum albumin polymers used: Mo, monomer (68 000); Di, dimer (136 000); Tr, trimer (204 000); Te, tetramer (272 000). Retardation quotient is the ratio of absolute mobility in the gel of higher concentration to that in the gel of lower concn. These gel ratios are indicated for each calibration curve.

Fig. 4.9 The behaviour of serum albumin polymers on different concentrations gels. Abbreviations used are the same as in Fig. 4.5.



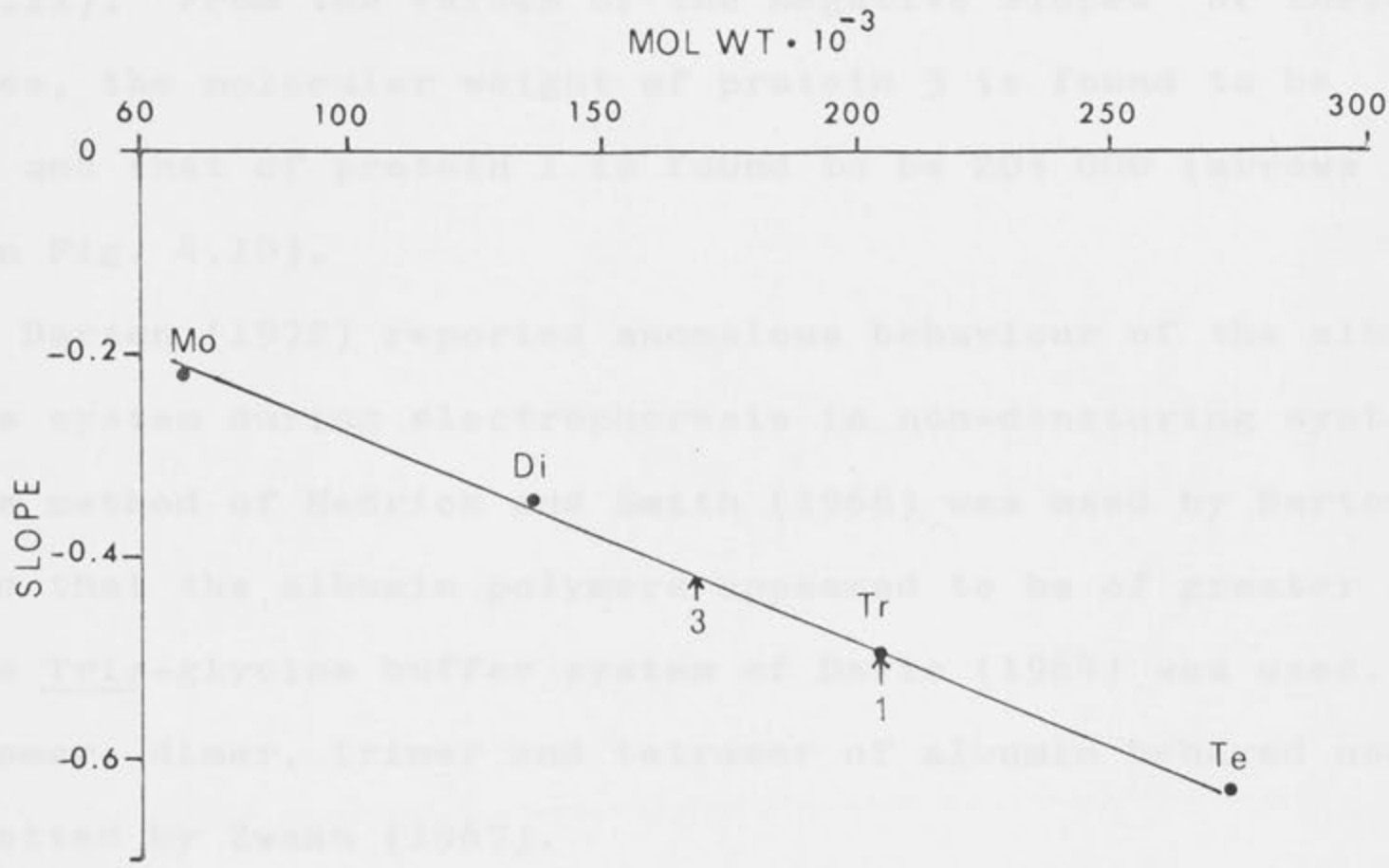


Fig. 4.10 Determination of molecular weights by electrophoresis on non-denaturing polyacrylamide gels. Calibration curve using the method of Hedrick and Smith (1968).

Data are taken from Fig. 4.6. The slope was calculated from the plot of Rm versus gel concentration. Arrows indicate molecular weights of protein 1 and protein 3 (Fig. 4.4a)

mobilities of the two bands of the enzyme are plotted against acrylamide gel concentrations, two straight lines are obtained, each passing through three points corresponding to the assumption that band 3 always migrates faster than band 1 in all three gels (Fig. 4.11). From the values of the negative slopes of these two lines, the molecular weight of protein 3 is found to be 168 000 and that of protein 1 is found to be 204 000 (arrows 3 and 1 on Fig. 4.10).

Barton (1972) reported anomalous behaviour of the albumin polymers system during electrophoresis in non-denaturing system. When the method of Hedrick and Smith (1968) was used by Barton, he found that the albumin polymers appeared to be of greater size when the Tris-glycine buffer system of Davis (1964) was used. The monomer, dimer, trimer and tetramer of albumin behaved normally when plotted by Zwaan (1967).

Protein 1 (Rm=0.1) is made up from 59 000 subunits. A tetramer of the 59 000 subunits will give a molecular weight of about 236 000. The estimation by these methods therefore gives a lower value for the molecular weight of protein 1. Similarly the estimation of the molecular weight of band 3 (Rm=0.2) may be too low. A tetramer of the 48 000 components will give a molecular weight of 192 000 which is higher than the estimated value.

Gradient gels were used to check the approximate molecular weights of proteins 1 and 3. Samples were electrophresed for six hours on gradient slab gels and were stained for protein with coomassie brilliant blue R250. When the absolute mobilities were plotted against the log of the molecular weights of ovalbumin and the serum albumin polymers, an approximate straight line relationship was obtained (Fig. 4.12). From the absolute mobility

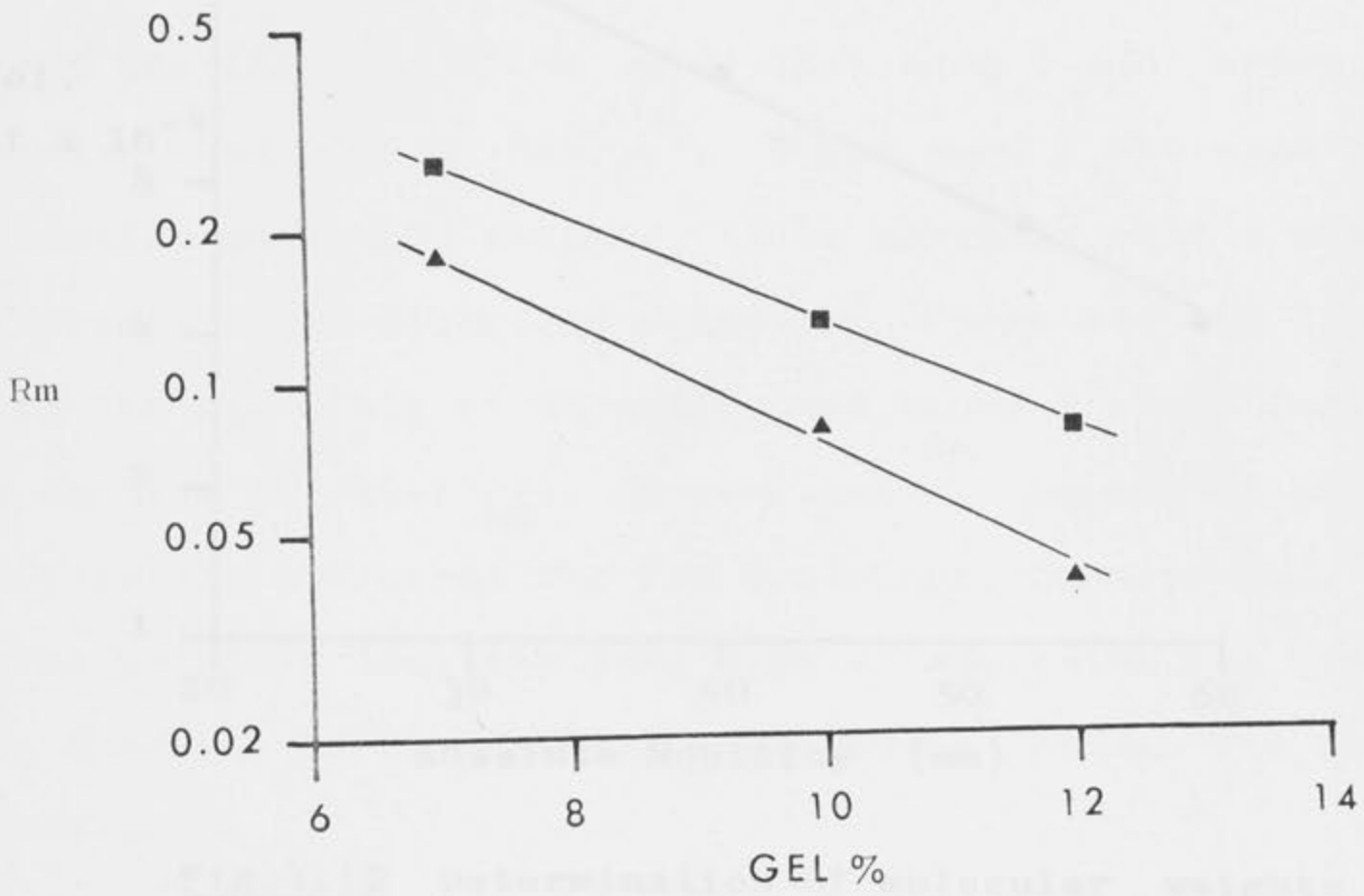


Fig. 4.11 The behaviour of bands 1 and 3 on gels of different acrylamide concentrations. (■) band 3; (▲) band 1

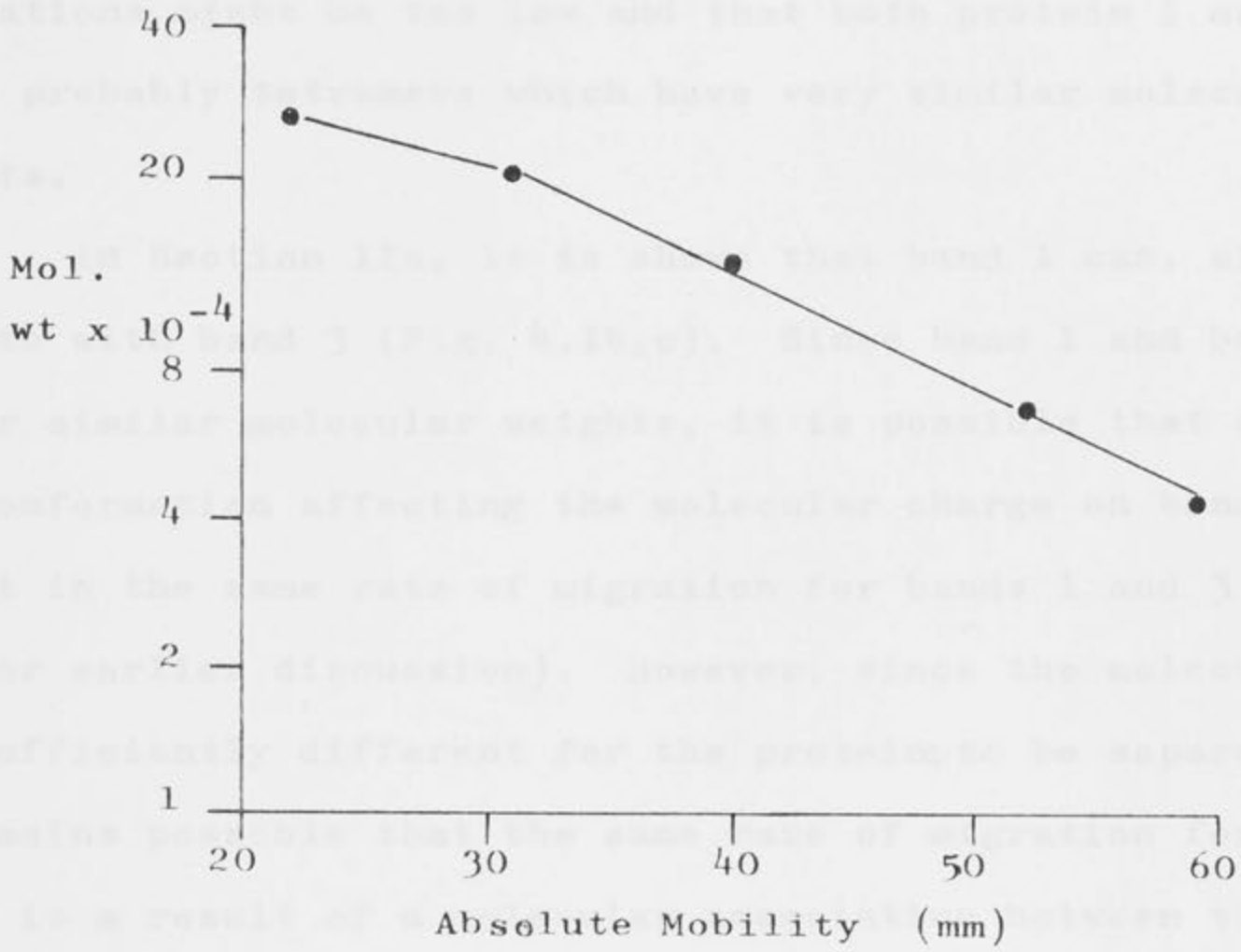


Fig.4.12 Determination of molecular weights by gradient gel electrophoresis. Plot of log of molecular weight versus absolute mobility. The graph is linear approximately within the range 230 000 to 44 000.

Standards used : ovalbumin 44 000; bovine serum albumin monomer 68 000, dimer 136 000, trimer 204 000, tetramer 272 000.

of protein 1, the molecular weight was estimated to be about 235 000. Protein 3 has a molecular weight of about 210 000 daltons. These molecular weight values show that the previous estimations might be too low and that both protein 1 and protein 3 are probably tetramers which have very similar molecular weights.

In Section IIa, it is shown that band 1 can, after storage, migrate with band 3 (Fig. 4.1b,c). Since band 1 and band 3 have rather similar molecular weights, it is possible that a change in conformation affecting the molecular charge on band 1 will result in the same rate of migration for bands 1 and 3 (see Section IIa for earlier discussion). However, since the molecular weights are sufficiently different for the proteins to be separable on gels, it remains possible that the same rate of migration for bands 1 and 3 is a result of a molecular association between the proteins in the two bands.

V. ANALYTICAL ULTRACENTRIFUGATION OF PURIFIED ENZYME

A complete analysis of a protein by the analytical ultracentrifuge requires the use of samples of a wide range of concentration. The small amount of material obtained limits analysis by this method. However, it is hoped to correlate the results of the chromatography on various kinds of columns and the polyacrylamide gel electrophoresis with that of the ultracentrifuge and in this way, more completely analyse the system.

(a) Sedimentation velocity

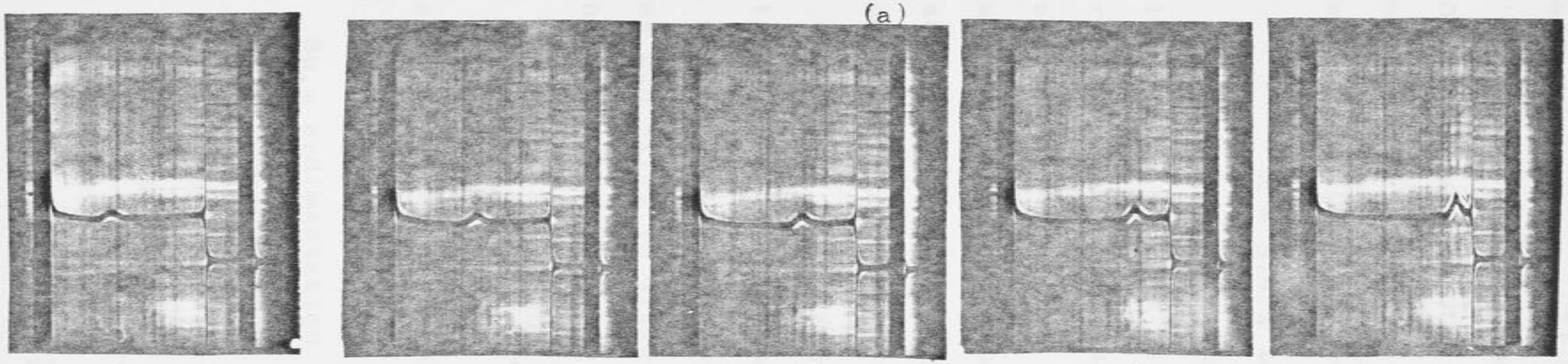
The pooled activity peak fractions, B, from the G200 column (Step 8; Fig. 3.3) show a single, symmetrical peak in the schlieren pattern with an $S_{20,w}$ of 9.6 S (concn. 1mg protein/ml) corresponding to a molecular weight of approximately 230 000. The patterns obtained using a single sector cell (Fig. 4.13a)

Fig. 4.13 (a) Schlieren pattern of DAHP synthase (Trp) in a single sector cell.
Speed = 59 630 rpm. Phase angle = 60°. Temperature = 20°C.

(b) Schlieren pattern of DAHP synthase (Trp) in a double sector cell.
Speed = 59 700 rpm. Phase angle = 50°. Temperature of run = 20°C.

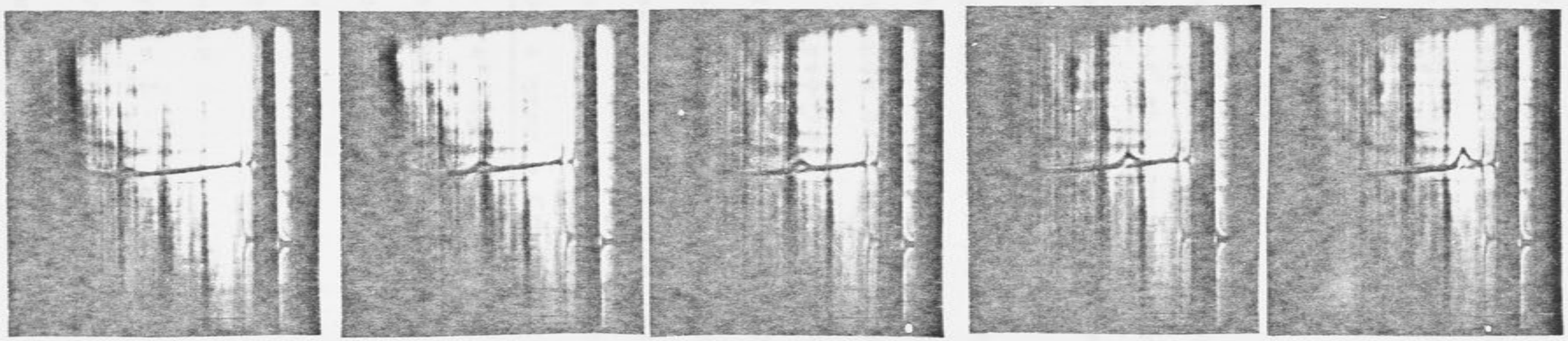
Sedimentation is from right to left. Concentration = 1 mg protein / ml.

(a)



45 min 35 min 25 min 15 min 5 min

(b)



56 min 40 min 32 min 24 min 8 min

and a double sector cell (Fig. 4.13b) are identical. A single peak was observed when the sample was spun at 60 000 r.p.m., 40 000 r.p.m. and 30 000 r.p.m.. The approximate molecular weight from the $S_{20,w}$ value was calculated from the equation of Paetkau and Lardy (1967),

$$M = 5.4 \times 10^3 \times (S_{20,w})^{1.66}$$

The sedimentation of the enzyme at lower protein concentration can be studied by the use of the photoelectric scanner. The results (Table 4.1) show that the $S_{20,w}$ value decreases as the concentration of the protein is decreased. The lowest value in Table 4.1 for absorbancy ($A_{280\ nm}$) is the limiting value where reliable measurements of the rate of sedimentation could be taken. The positive concentration dependence of the $S_{20,w}$ value indicates that the enzyme is undergoing a dissociation and association reaction, that is, the enzyme dissociates into smaller aggregates when its concentration is lowered. These results show that the enzyme can exist in different degrees of aggregation. The $S_{20,w}$ is therefore a weight-average quantity represented by the equation

$$S_w = \frac{CmSm + Cp_1Sp_1 + \text{----} + Cp_nSp_n}{Cm + Cp_1 + \text{----} + Cp_n}$$

where Sw = weight-average sedimentation coefficient

Sm = sedimentation coefficient of monomer

Sp_1 = sedimentation coefficient of polymer 1

Sp_n = sedimentation coefficient of polymer n

Cm = concentration of monomer

Cp_1 = concentration of polymer 1

and Cp_n = concentration of polymer n.

Theoretically, the $S_{20,w}$ of the monomer can be obtained by plotting the $S_{20,w}$ against the concentration and extrapolate to zero concentration. This has not been done for this enzyme

TABLE 4.1

Variation of $S_{20,w}$ with protein concentration

A_{280nm}	$S_{20,w}$
0.283	8.92
0.170	8.87
0.122	8.60
0.083	8.20

Samples were spun at 60 000 r.p.m. at 20^{0}C. Concentration of protein was measured in terms of $A_{549\ nm}$ by the Photoelectric scanner in the analytical ultracentrifuge, Model E, by Beckman.

because it is already established by dodecyl sulphate gels electrophoresis that there are more than one subunit present. What is more, the complex may not dissociate completely to its subunits under ordinary condition within the measurable absorbancy range of the instrument. Attempts to analyse the sample in the presence of 8M guanidine hydrochloride or 1% dodecyl sulphate were unsuccessful due to the complication in the interpretation of sedimentation data for denatured protein.

Using proteins 1 and 3 purified from gel slices, very similar sedimentation coefficients were obtained for the two proteins within the range 9.3 S to 8.2 S depending on protein concentrations. The two proteins, therefore, may not be separable by ultracentrifugation. The S values also confirm the earlier estimation of molecular weights on the gels of about 230 000 and therefore both band 1 and band 3 may be tetramers.

(b) Sedimentation equilibrium

Since the results of analytical polyacrylamide gels of portion B from the G200 column (Fig. 3.3) show that more than one component is present, the sedimentation equilibrium is expected to be complicated and analysis is difficult. When the initial overspeeding technique was used, the sample was spun at 20 000 r.p.m. for 5 hours and then the speed was slowed down to 10 000 r.p.m. for 12 hours. No noticeable gradient was formed and most of the material that had sedimented at 20 000 r.p.m. moved back to the top of the column. The absorbance ($A_{280\ nm}$) at the meniscus was lower than the previous initial reading each time after relaxation of speed.

The fact that on relaxation of speed, the proteins moved back to the meniscus may indicate a concentration dependent dissocoation-association. When a high molecular weight complex

moves back into a region of low concentration and dissociates, the equilibrium favours the low molecular weight form and more material is pulled towards the meniscus with more dissociation. The slower speed is not sufficient to maintain a gradient with the smaller molecule. Since part of the material has sedimented, the concentration of the lighter form is thus lower than the initial reading at the start of the experiment. The other explanation is that there are two kinds of molecules in the sample, one is large and one is small. At the higher speed, both molecules will sediment whereas at the lower speed, the smaller molecule will not sediment. However, other results presented in previous sections show that both protein 1 and protein 3 are large molecules. This result is inconsistent with the second explanation.

The same phenomenon was observed with an initial speed of 15 000 r.p.m. and relaxed to 12 000 r.p.m. after 7 hours. When a fresh sample was used and centrifuged for 24 hours at 15 000 r.p.m., a gradient was obtained throughout the solution. The plot of log of concentration against distance squared is a curve (Fig. 4.14) and the molecular weight is calculated from the slope at any point. The curvature means that more than one molecular form is present and the molecular weight at the bottom of the cell is 300 000 corresponding therefore to the highest weight-average molecular weight present.

In summary, the results of these ultracentrifuge studies are not very helpful in molecular weight determinations, but are consistent with the complexity observed on the gels.

VI. DETERMINATION OF MOLECULAR WEIGHTS BY GEL FILTRATION ON SEPHADEX G200 COLUMNS

The methods for the estimation of molecular weights by gel filtration are described in Chapter 7, Section VIII. The

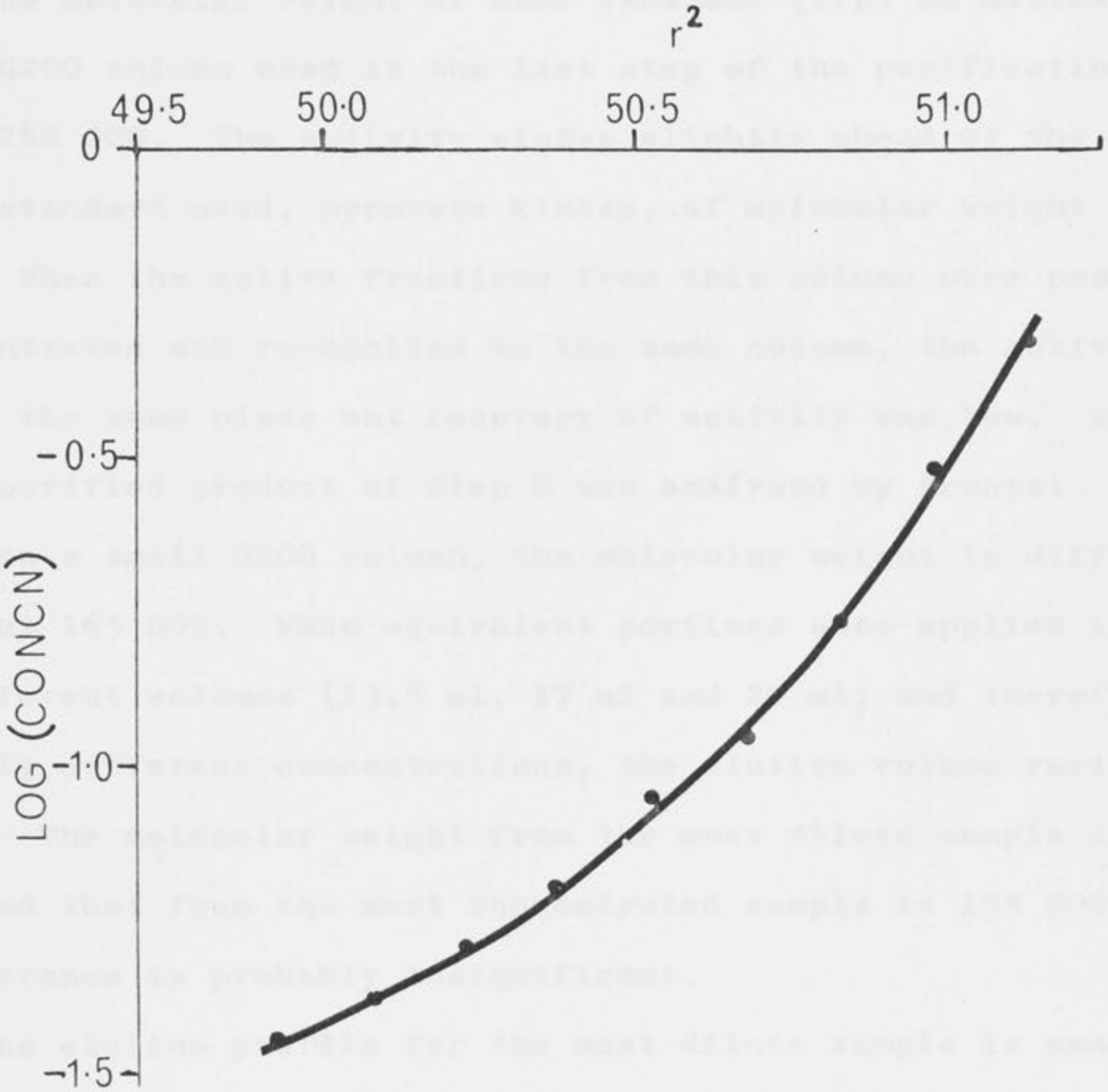


Fig. 4.14. Determination of molecular weight of DAHP synthase (Trp) by sedimentation equilibrium in the ultracentrifuge. Plot of log of concentration ($A_{280\ nm}$) against the square of the distance from the centre of rotation, r^2, cm^2. Samples were spun at 15 000 rpm for 24 hours at 20°C in a double sector cell and concentration was monitored by the photoelectric scanner.

plot of log of molecular weight versus the $(V_e - V_o)/(V_b - V_o)$ ratio is the same for both the zonal and the frontal methods (Fig. 4.15), and the value of the ratio for each of the four standard proteins obtained by either method is the same.

The molecular weight of DAHP synthase (Trp) as estimated from the G200 column used in the last step of the purification is about 240 000. The activity elutes slightly ahead of the heaviest standard used, pyruvate kinase, of molecular weight 237 000. When the active fractions from this column were pooled and concentrated and re-applied to the same column, the activity eluted at the same place but recovery of activity was low. However, when the purified product of Step 8 was analysed by frontal analysis on a small G200 column, the molecular weight is different, being about 165 000. When equivalent portions were applied in three different volumes (13.5 ml, 17 ml and 20 ml) and therefore as slightly different concentrations, the elution volume varied slightly. The molecular weight from the most dilute sample is 165 000 and that from the most concentrated sample is 155 000. This difference is probably insignificant.

The elution profile for the most dilute sample is smooth and nearly enantiographic (Fig. 4.16), whereas that for the most concentrated sample is smooth but nonenantiographic (Fig. 4.17). Enantiography is tested by the plot of the quantity (Ca+Ct-Co) versus V, where Ca and Ct are the enzyme activity corresponding to an effluent volume V in the advancing and trailing boundaries respectively. Co is the initial activity which corresponds to the activity at the plateau region (Ronalds and Winzor, 1969). For the most dilute sample, (Ca+Ct-Co) is approximately zero for all values of V showing that the profile is enantiographic. A similar plot for the most concentrated sample gives both positive and negative values of the ordinate parameter (Fig. 4.18) showing

Fig. 4.15 Calibration curve for determination of molecular weight of DAHP synthase (Trp) by molecular sievings on G200 columns.

Standard proteins used were: Pyruvate kinase (235 000); lactate dehydrogenase (150 000); bovine serum albumin (68 000) and ovalbumin (44 000). Columns were eluted at 20°C. The line was the same for zonal and frontal analyses.

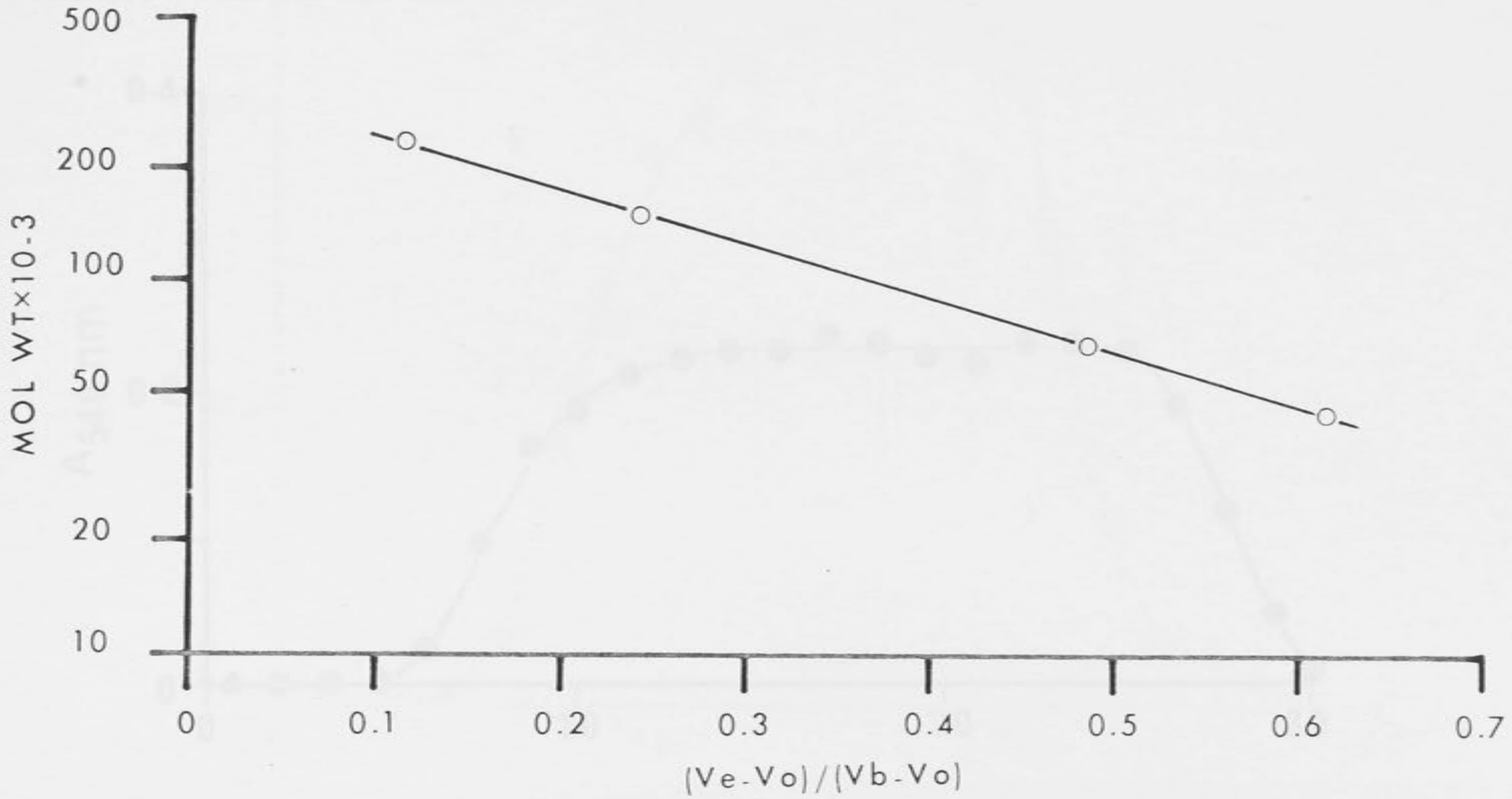

500
200
100
50
20
10
MOL WT×10-3
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
(Ve-Vo)/(Vb-Vo)

Fig. 4.16 Elution profile of DAHP synthase (Trp) from the small frontal column (13.5 ml bed volume). The column was eluted at 20°C and a sample volume of 20 ml was applied.

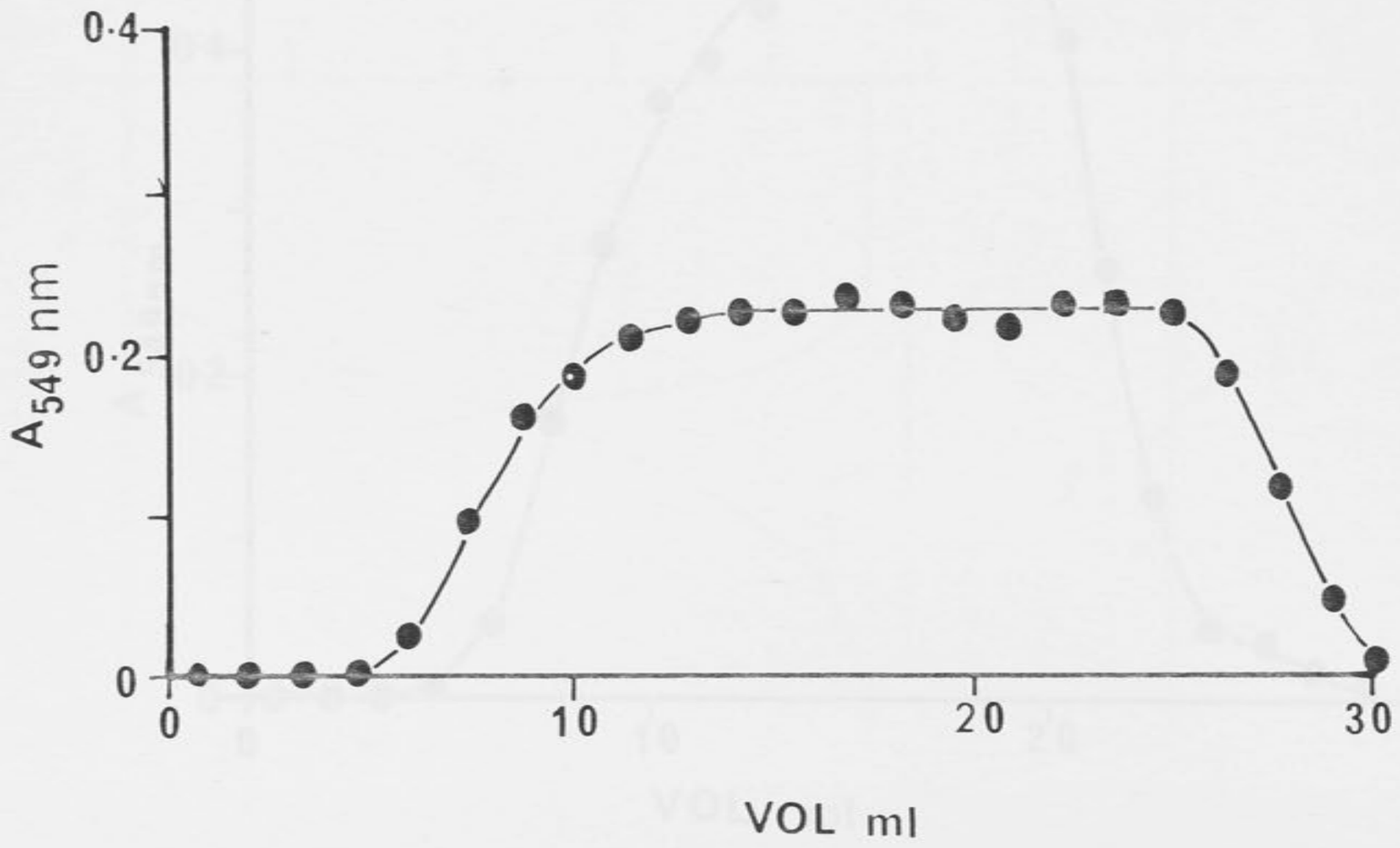


Fig. 4.17 Elution profile of DAHP synthase (Trp) from the small frontal column (13.5 ml bed volume). The column was eluted at 20°C and a sample volume of 13.5 ml (0.05 mg/ml) was applied.

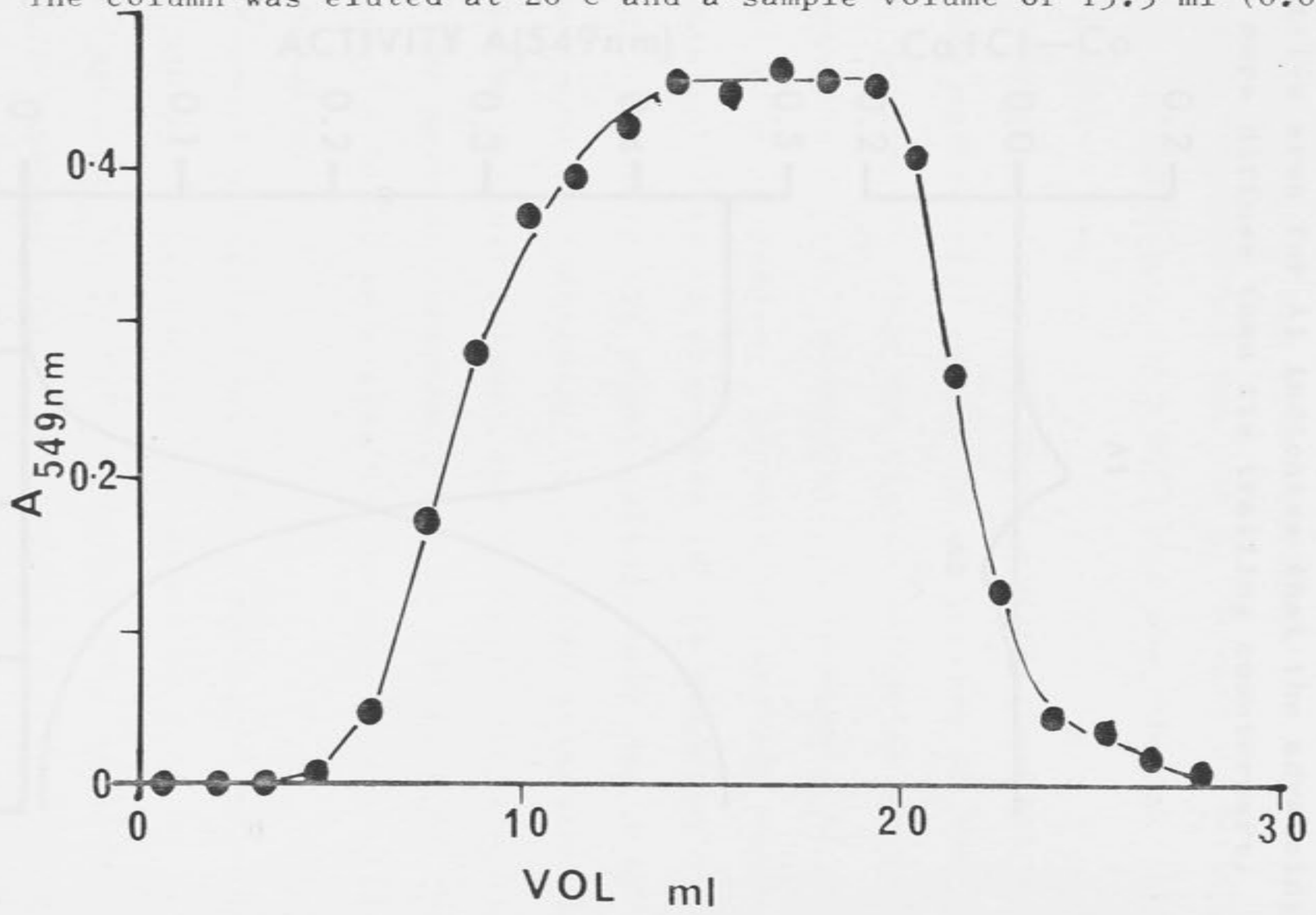


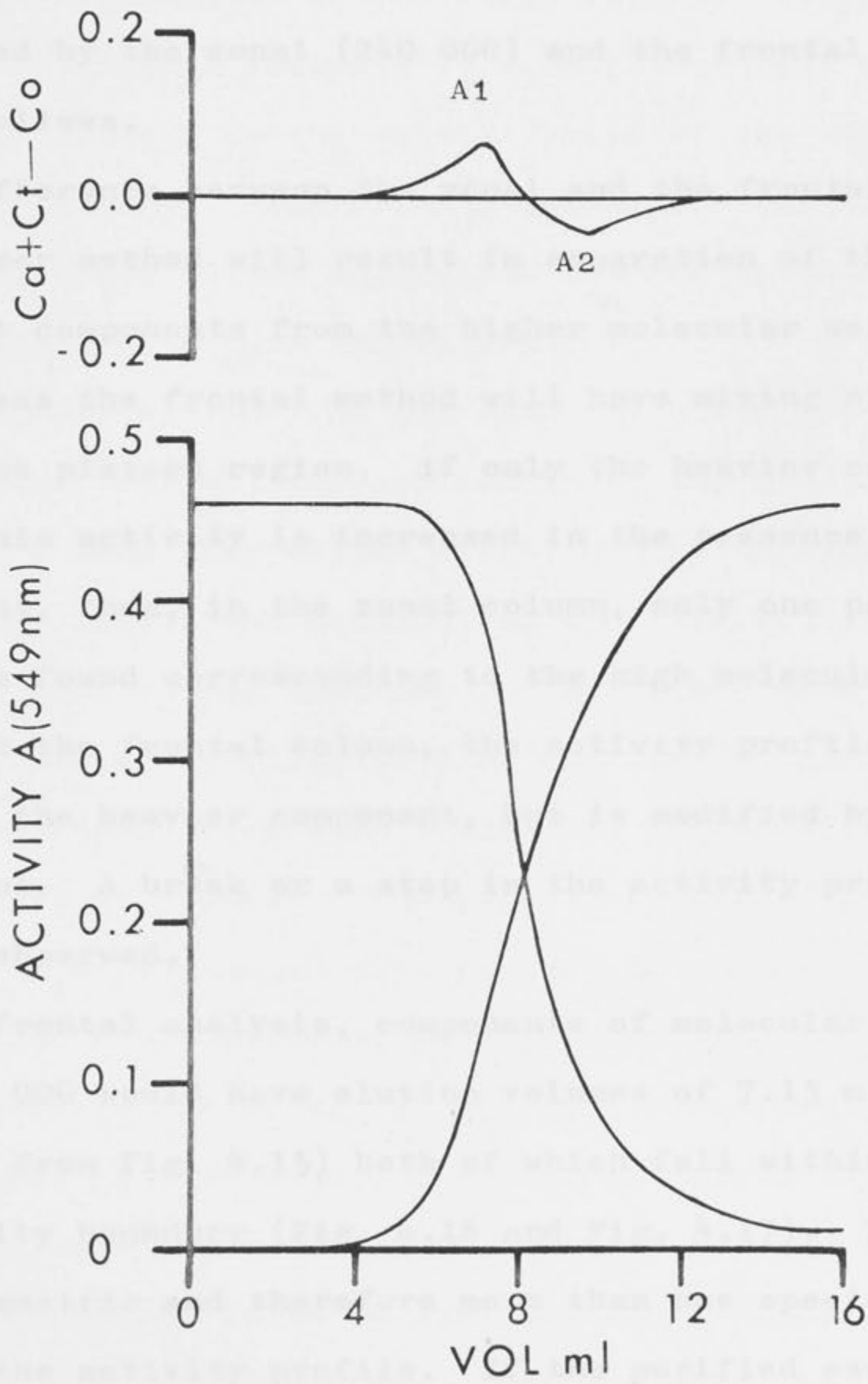
Fig. 4.18 Test for enantiography of the elution profile from Fig. 4.17.

Ca = activity of advancing profile

Ct = activity of trailing profile

Co = activity of applied sample

A positive area for A1 indicates that the advancing profile is more diffuse than its trailing counterpart.



that the profile is nonenantiographic. The positive value of the first area (A_1) implies that the advancing boundary is more diffuse than its trailing counterpart, a situation resulting from positive concentration dependence, that is, the elution decreases as the concentration is decreased, indicating physical interaction of the species present (Ronalds and Winzor, 1969).

One possible explanation for the difference in molecular weight determined by the zonal (240 000) and the frontal (165 000) methods is as follows.

The difference between the zonal and the frontal methods is that the former method will result in separation of the lower molecular weight components from the higher molecular weight components whereas the frontal method will have mixing of the components in the plateau region. If only the heavier component is active and this activity is increased in the presence of the smaller component, then, in the zonal column, only one peak of activity will be found corresponding to the high molecular weight form, whereas in the frontal column, the activity profile will not only follow the heavier component, but is modified by the smaller component. A break or a step in the activity profile may not be readily observed.

In the frontal analysis, components of molecular weight 240 000 and 110 000 would have elution volumes of 7.15 ml and 8.8 ml (as read from Fig. 4.15) both of which fall within the advancing activity boundary (Fig. 4.16 and Fig. 4.17). This boundary is asymmetric and therefore more than one species contributes to the activity profile. If the purified sample contains some 110 000 proteins either as contaminant in the isolation procedure or may be a dissociation product from the 240 000 molecules, and if these 110 000 proteins can increase

the activity of the heavier active enzyme, the activity profile from the frontal column will be distorted and the estimation of molecular weight will be lower since the activity profile is a composite of the effect of two different sizes molecules. Since the column size is small, a slight distortion of the activity profile will greatly affect the estimation of molecular weight. The molecular weight obtained by frontal analysis is a weight-average quantity in this example depending on the effectiveness of the smaller component on the activity. The value obtained of approximately 165 000 is intermediate to the values 240 000 and 110 000 and is therefore consistent with this interpretation.

When a sample eluted from the DEAE-cellulose column (Step 6 in the purification) was applied on to the frontal analysis column, a molecular weight of 240 000 is obtained. The elution profile is smooth but nonenantiographic (Fig. 4.19). The boundary is more symmetrical than for the purified enzyme. The concentration of protein applied (1.5 mg/ml) was 30 fold higher than used for the enzyme from Step 8 and also proteins of a large range of sizes will be present. The effect of protein concentration on enzyme activity will be evenly distributed across the activity profile so that the elution volume as read from the activity profile would indicate the value for the heavier active form.

The elution profile with a more diffuse advancing edge than the trailing edge means that the enzyme is not undergoing association on the column in which case one would expect to find a sharp advancing edge with a diffuse trailing edge.

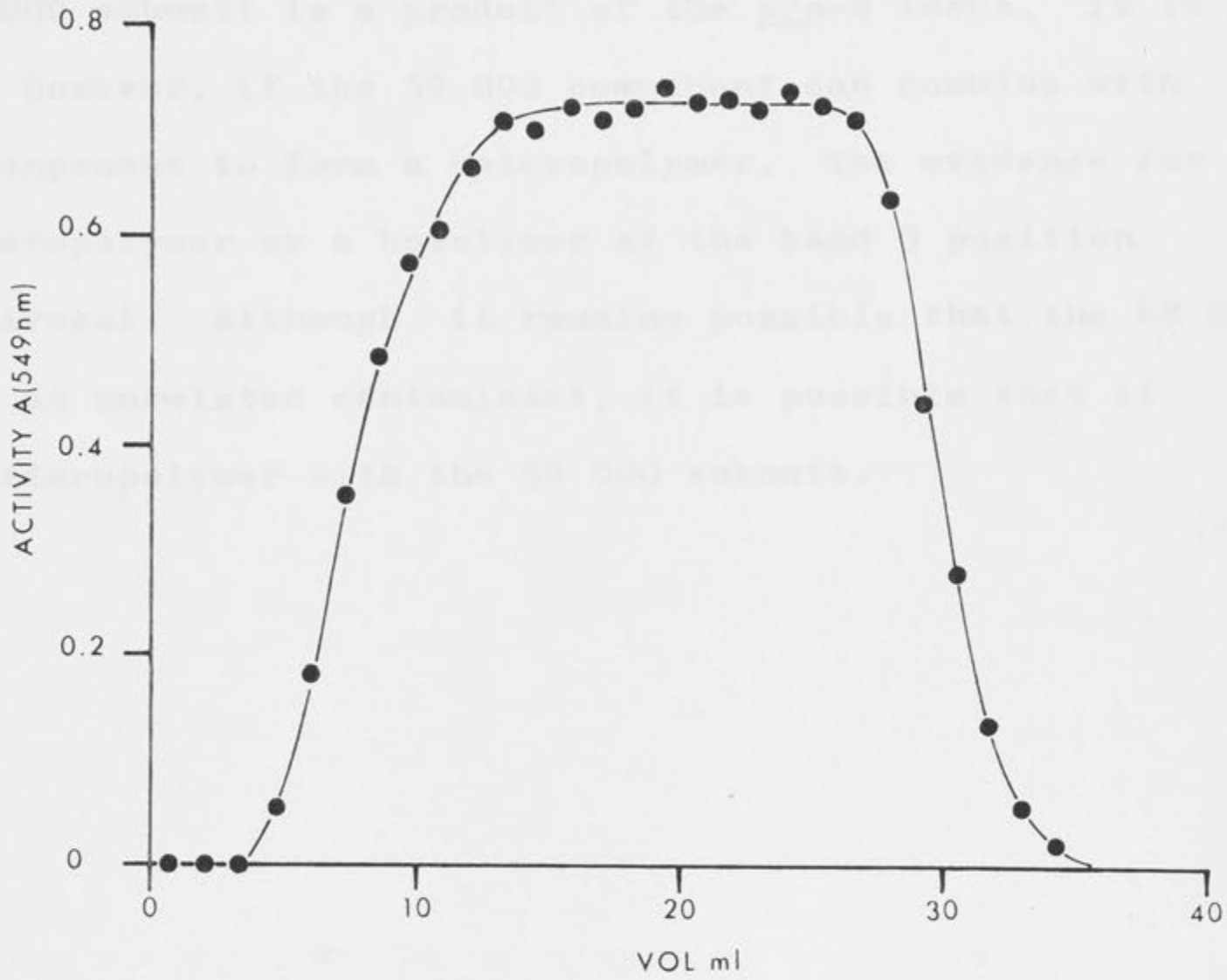


Fig. 4.19 Elution profile of DAHP synthase (Trp) activity from the small frontal column (13.5 ml bed volume) for sample from the DEAE-cellulose column.

The column was eluted at 20°C and a sample volume of 22 ml (1.5 mg protein/ml) was applied.

The molecular weight values obtained by various experimental technigues are summarised in Table 4.2. These values are consistent with the presence of tetramers made up of the 59 000, 48 000 or perhaps the 44 000 subunits. In particular, band 1 with DAHP synthase (Trp) activity of about 240 000 is probably a homopolymer of 4 x 59 000 subunits. It is considered that the 59 000 subunit is a product of the aro-8 locus. It is not certain, however, if the 59 000 component can combine with the 48 000 component to form a heteropolymer. The evidence for either a heteropolymer or a homolymer at the band 3 position is not unequivocal. Although it remains possible that the 48 000 component is an unrelated contaminant, it is possible that it can form a heteropolymer with the 59 000 subunit.

Table 4.2. Summary of the molecular weights obtained by polyacrylamide gel electrophoresis and gel filtration.

	Band 1 (With DAHP synthase (Trp) activity)	Band 3 (No DAHP synthase (Trp) activity)
1. Polyacrylamide gel electrophoresis		
(a) Method of Zwaan	204 000	156 000
(b) Method of Hedrick and Smith	204 000	168 000
(c) Gradient gel	235 000	210 000

	DAHP synthase (Trp) activity
2. Gel filtration on G200 columns	
(a) Zonal method	240 000
(b) Frontal method	
*(i) Sample from Step 8	165 000
*(ii) Sample from Step 6	240 000

*Step referred to steps in the purification procedures (Table 3.1).

VII. SAMPLES FROM HYDROCYLAPATITE (STEP 7) AND SEPHADEX G200 (STEP 8) COLUMNS

Samples eluting from the hydroxylapatite and the G200 columns were initially analysed as a preliminary investigation to decide on the purity of the products and what experimental step to follow. Results of these analyses when taken together appear to indicate some degree of protein interaction.

The elution profile from the hydroxylapatite column consists of multiple protein peaks and enzymic activity is associated with peaks 2 and 3, and sometimes peak 4 as well (Fig. 4.20).

Polyacrylamide gel electrophoresis of samples from peaks 1, 2, 3 and 4 (Fig. 4.21) shows that peaks 2 and 3 are almost identical except that peak 3 contains band 3 (Rm=0.2) which is absent in peak 2. However, band 3 can be observed after sieving when either peak 2 or peak 3 is applied to the G200 column. This suggests that a change in molecular forms might have occurred and that band 3 can be generated from proteins that are present in peak 2. Band 4 (Rm=0.25) which is sometimes found in small amount in the leading and trailing fractions from the G200 column (see Section II, (b)) is present in large quantity in peaks 2, 3 and 4 (Fig. 4.21). Apart from bands 1, 2, 3 and 4, peak 1 is made up from a majority of band 5 (Rm=0.35) and peak 4 is made up from a majority of band 6 (Rm=0,5) together with other bands.

Peaks 2 and 3 (Fig. 4.20) when applied to the G200 column separately, give the same result in terms of purity of the final enzyme preparation. Initially, the pooled sample from peaks 2 and 3 when examined in the ultracentrifuge gave a single peak when a synthetic boundary, capillary type, double sector cell was used (Fig. 4.22a). However, when an ordinary single sector

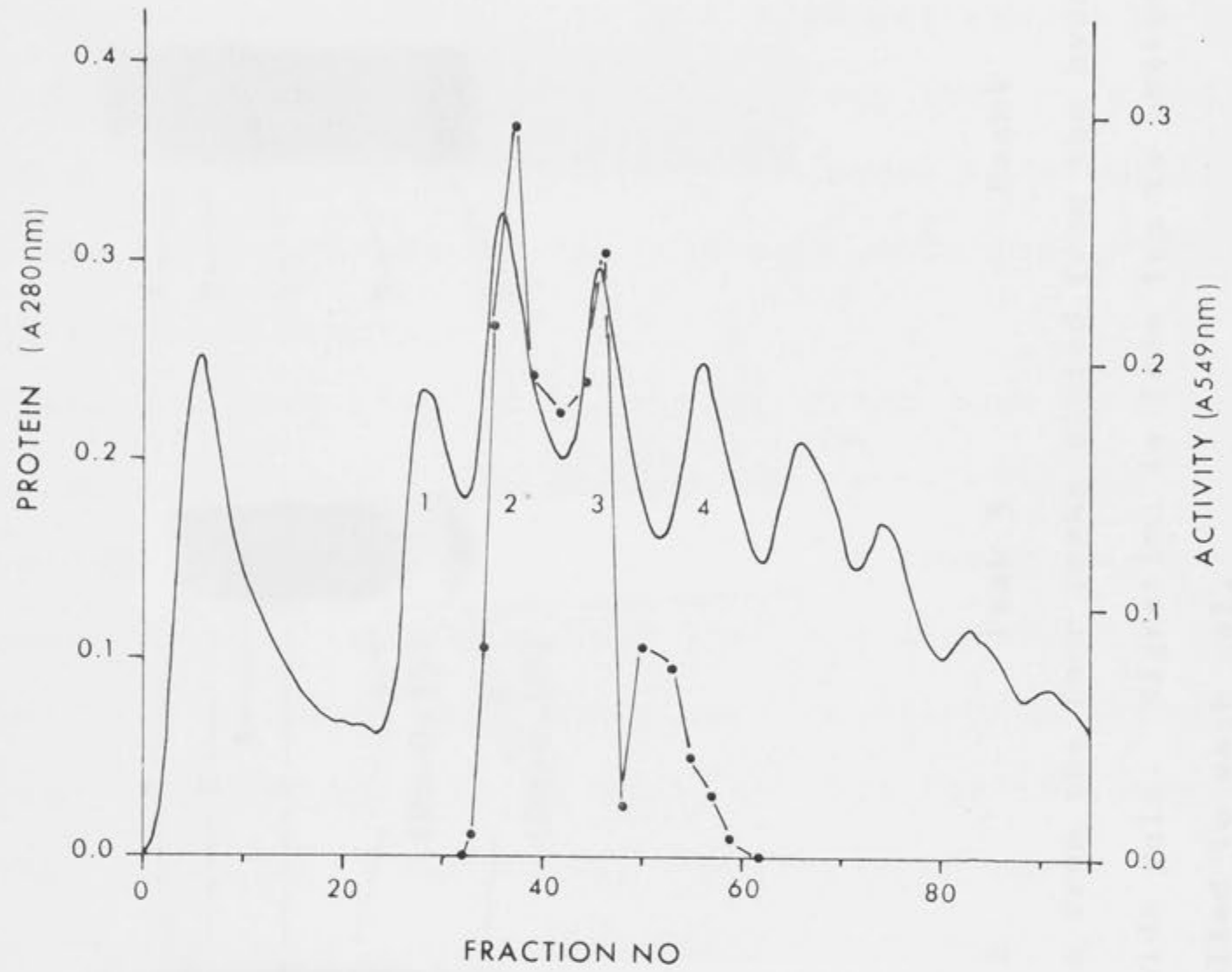


Fig. 4.20 Elution profile from the hydroxylapatite column.

Experimental details are described in Chapter 3. Note the relative position of the four numbered protein peaks and the two activity peaks.

●———● DAHP synthase activity;———protein.

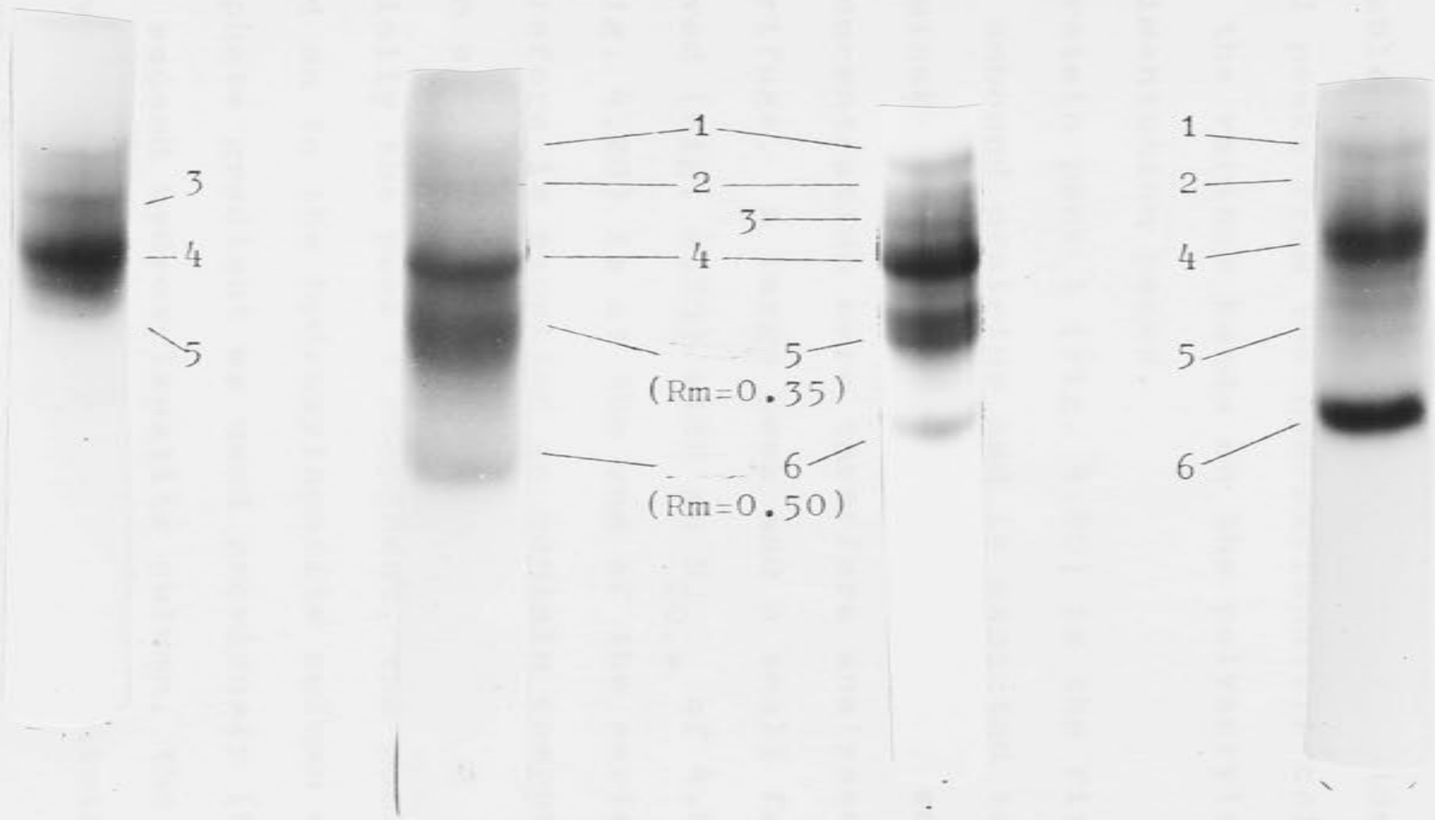


Fig. 4.21 Electrophoresis of samples from the four peaks eluted from the hydroxylapatite column (Fig. 4.20) on 7% polyacrylamide gels. Migration is from top to bottom. Approximately 100 µg protein was applied to each gel.

cell was used instead, three peaks were resolved (Fig. 4.22b). The synthetic boundary, capillary type, double sector cell, because of the short column length is therefore unsuitable if more than one species is present. The three peaks have $S_{20,w}$ of 9.57S, 6.26 S and 3.56 S. These results mean that separation can be achieved by molecular sievings and that G200 gel should be the most suitable gel to use. It was also decided then to examine individual peaks from the hydroxylapatite column in an attempt to assign the various bands on the polyacrylamide gel to the three sedimentation peaks.

Protein peak 1 (Fig. 4.20) is the first peak to elute after the unbound proteins and is expected to be relatively free of "contaminants" from peaks 2 and 3. Pooled fractions from peak 1 after concentration were therefore analysed directly in the ultracentrifuge. A large peak and a small faster migrating peak are observed (Fig. 4.23) with an $S_{20,w}$ of 4.0S for the larger peak. Peak 4 (Fig. 4.20) is at the end of the series of peaks 1, 2 and 3 and therefore is expected to contain components of the earlier peaks. In order to clean up the sample more so that it consists of essentially the peak 4 component, the pooled fractions were re-applied on to the hydroxylapatite column and eluted by the same phosphate gradient as used previously (0.02M to 0.35M). From this second hydroxylapatite column, the pooled fractions from peak 4 gave a single peak in the ultracentrifuge (Fig. 4.24) with an $S_{20,w}$ of 6.0S.

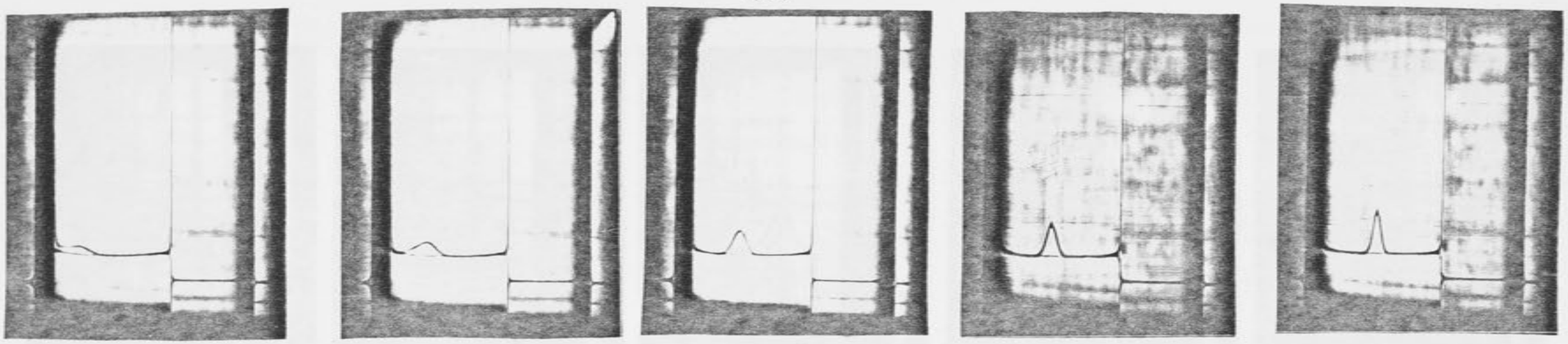
Comparison of the ultracentrifuge results with that of the polyacrylamide gel results indicates that band 5, the major band in peak 1, has an S value of 4.0S and that band 6, the major band in peak 4, has an S value of 6.0S.

Fig. 4.22 (a) Sedimentation of peaks 2 and 3 from hydroxylapatite column (Fig. 4.20) in a double sector synthetic boundary cell. Speed = 59 900 rpm. Phase angle = 70°. Temperature = 2.5°C.

(b) Same sample as in (a) except a single sector cell was used. Temperature = 2.8°C. Phase angle = 60°. Speed = 60 000 rpm.

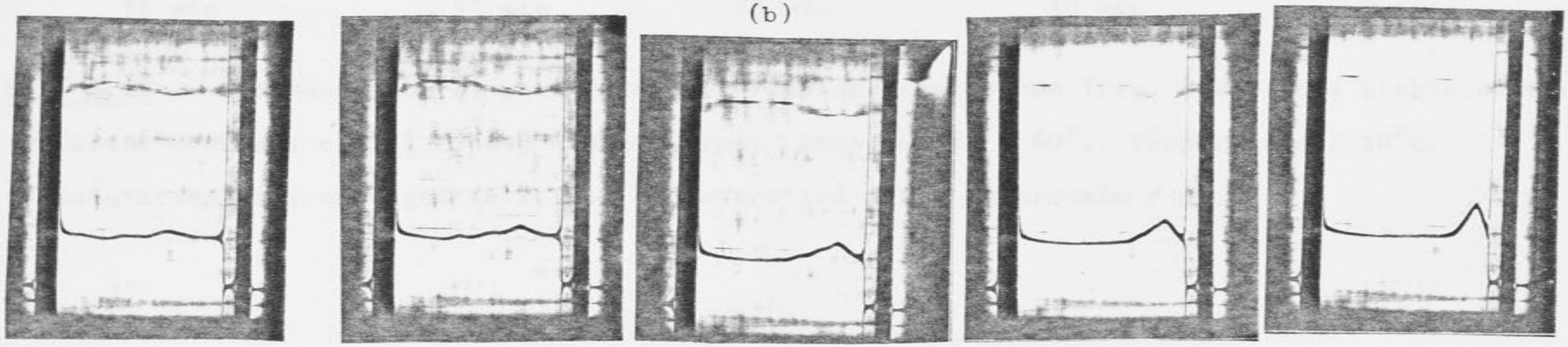
Sedimentation is from right to left. Concentration = 2.5 mg protein / ml.

(a)

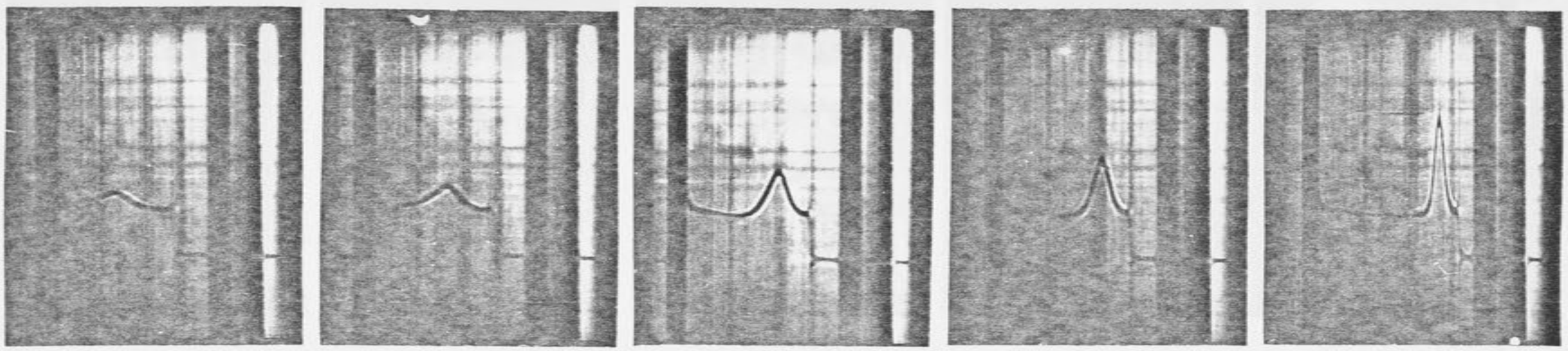


50 min 26 min 10 min 5 min 0 min

(b)

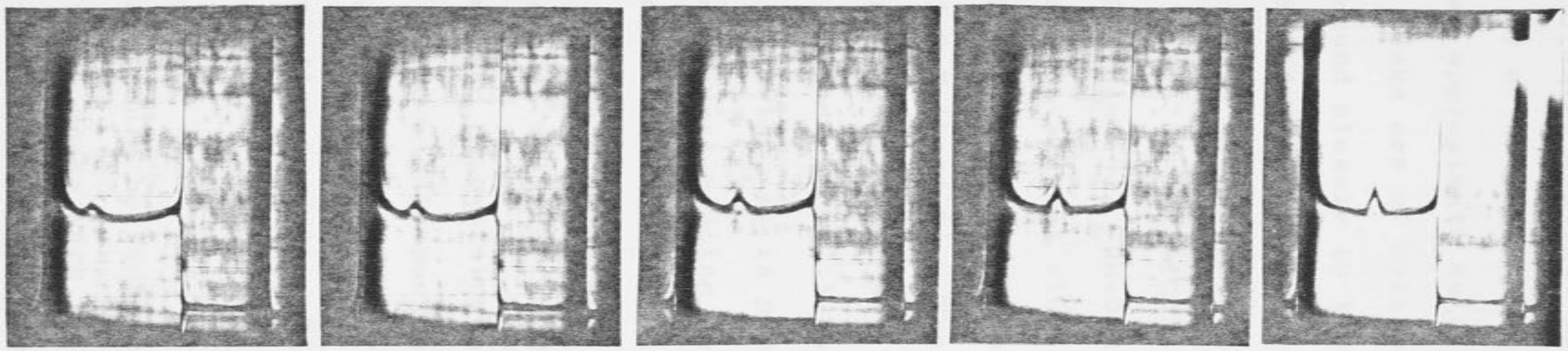


106 min 74 min 50 min 26 min 10 min



55 min 35 min 15 min 10 min 0 min

Fig. 4.23 Sedimentation of peak 1 from hydroxylapatite column (Fig. 4.20) in a single sector synthetic boundary cell. Speed = 59 700 rpm. Phase angle = 60°. Temperature = 20°C. Sedimentation is from right to left. Concentration = 2.5 mg protein / ml.



32 min 24 min 16 min 8 min 0 min

Fig. 4.24 Sedimentation of peak 4 from hydroxylapatite columns (Fig. 4.2c) in a double sector synthetic boundary cell. Speed = 60 000 rpm. Phase angle = 40° . Temperature = 3.6°C. Sedimentation is from right to left. Concentration = 0.8 mg protein / ml.

Three distinct protein peaks elute from the G200 column (Step 8) of which the enzyme is the first to elute (Fig. 3.3) with an approximate molecular weight of about 240 000. The other peaks are of molecular weights 110 000 and 48 000 which correspond closely to the molecular weights attributed to the 6.26 S and 3.56 S components when the sample before sieving was analysed in the ultracentrifuge. Polyacrylamide gel electrophoresis (Fig. 4.25) of the middle eluting peak shows three bands of Rm=0.25 (band 4), Rm=0.35 (band 5) and Rm=0.5 (band 6); whereas that of the slowest eluting peak shows a preponderance of band 4 and band 5 with a few minor bands of Rms, 0.1 (band 1), 0.2 (band 3) and 0.5 (band 6). When the pooled samples were concentrated, a very low activity was detected from the slowest eluting peak. Since a band of Rm=0.1 has been identified to have DAHP synthase (Trp) activity, it is possible that the protein of Rm=0.1 as found in the slowest eluting peak is the same protein as found earlier in the analysis of the activity peak. The presence of bands 1 and 3 in the fastest and the slowest eluting peaks but not in the middle peak from the G200 column seems to suggest that some interaction of proteins has occurred.

The middle peak of about 110 000 is about half the molecular weight of the fastest eluting peak and is probably a dissociation product. Similarly, the slowest peak is about half the molecular weight of the middle peak. However, when individual peaks (Fig. 3.3) were re-applied on to the same G200 column, only one peak could be found corresponding in position to where the individual peaks were taken originally.

All three protein peaks give multiple bands on polyacrylamide gels. Whereas band 4 is present in major proportion both in the middle and the slowest eluting peaks from the G200 column, the

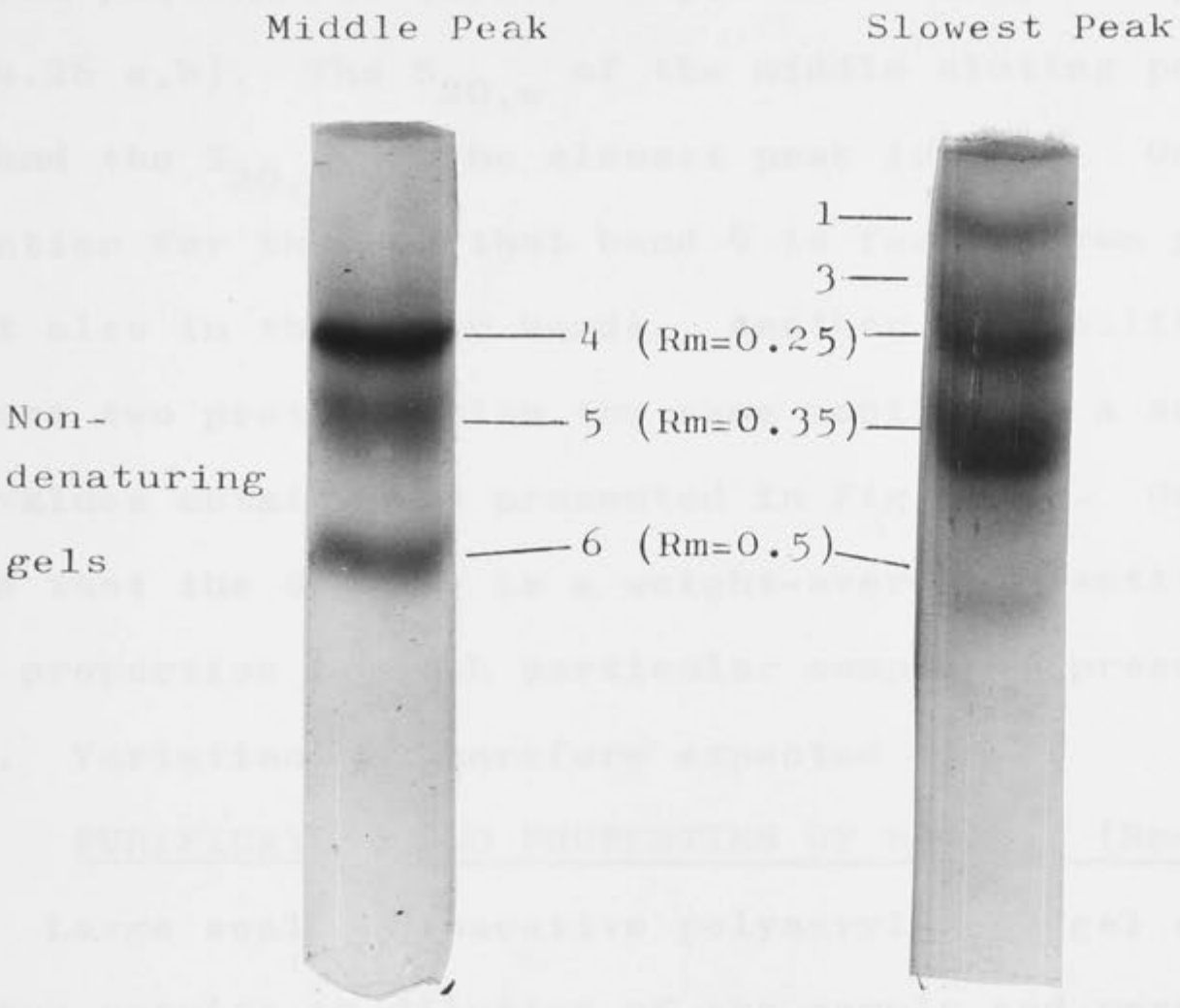


Fig. 4.25 Electrophoresis of samples from G200 columns on 7% polyacrylamide gels.

Migration is from top to bottom.

The TEA-TES system (Orr, 1969) of buffer was used with a running pH of 7.0. Approximately 100 µg protein was applied to each gel.

schlieren pattern for either sample shows only one peak (Fig. 4.26 a,b). The $S_{20,w}$ of the middle eluting peak is 5.78S and the $S_{20,w}$ of the slowest peak is 3.2S. One possible explanation for this is that band 4 is formed from proteins present also in the other bands. Another possibility is that there are two proteins with the same mobility. A summary of the S values obtained is presented in Fig. 4.27. One must realise that the S value is a weight-average quantity depending on the proportion of each particular component present in the sample. Variation is therefore expected

VIII. PURIFICATION AND PROPERTIES OF BAND 4 (Rm=0.25)

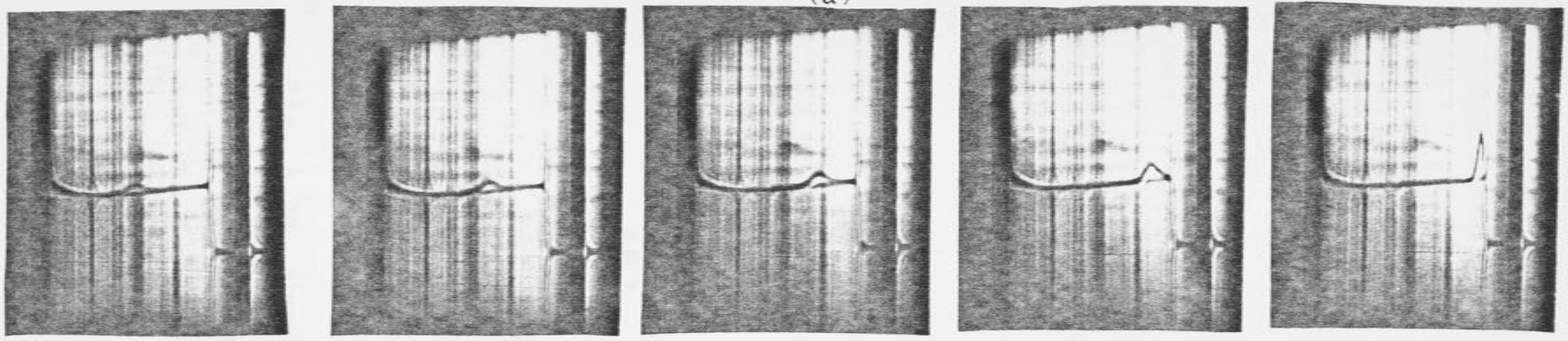
Large scale preparative polyacrylamide gel electrophoresis apparatus results in dilution of the sample and recovery is low. Since dilution is undesirable for this sample, a semi-preparative method was used utilising the analytical apparatus. The method consists of attaching a dialysis bag to the bottom of the gel tube and sample is collected as it elutes from the end. Eight gels (3 cm of 7% resolving gel and 1 cm of 3% stacking gel each) were run at the same time and one of them was stained with Coomassie brilliant blue G250 (which gave identification of bands within 10 min) when the dye (bromophenol blue) had migrated to within 3 mm from the bottom of the gel column. Meanwhile, the time taken for the dye to travel a marked distance on the gel tube was taken. A dialysis bag was attached to the bottom of each of the remaining gels at this time. The zero time was taken as the time when electrophoresis was recommenced. The time for band 4 to migrate to the bottom of the gel column from zero time could be calculated from the Rm of the band determined in the same experiment. The dialysis bag was removed and a new one put on when the time had come to within 5 min before the elution of the band and

Fig. 4. 26 (a) Sedimentation of middle peak from the G200 column (Fig. 3. 3 in a double sector cell. Speed = 59 700 rpm. Phase angle = 50°. Temperature = 20°C. Concentration is 1.5 mg protein per ml.

(b) Sedimentation of slowest eluting peak from the G200 column (Fig. 3.3) in a double sector cell. Speed = 59 700 rpm. Phase angle = 50°. Temperature = 20°C. Concentration = 1.5 mg protein / ml.

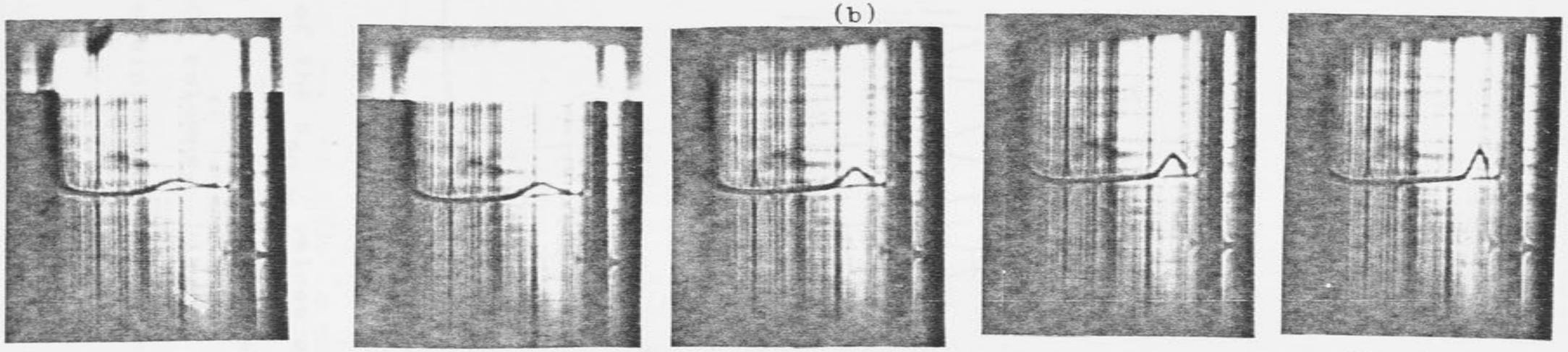
Sedimentation is from right to left.

(a)



64 min | 48 min | 32 min | 16 min | 0 min

(b)



75 min | 55 min | 35 min | 25 min | 15 min

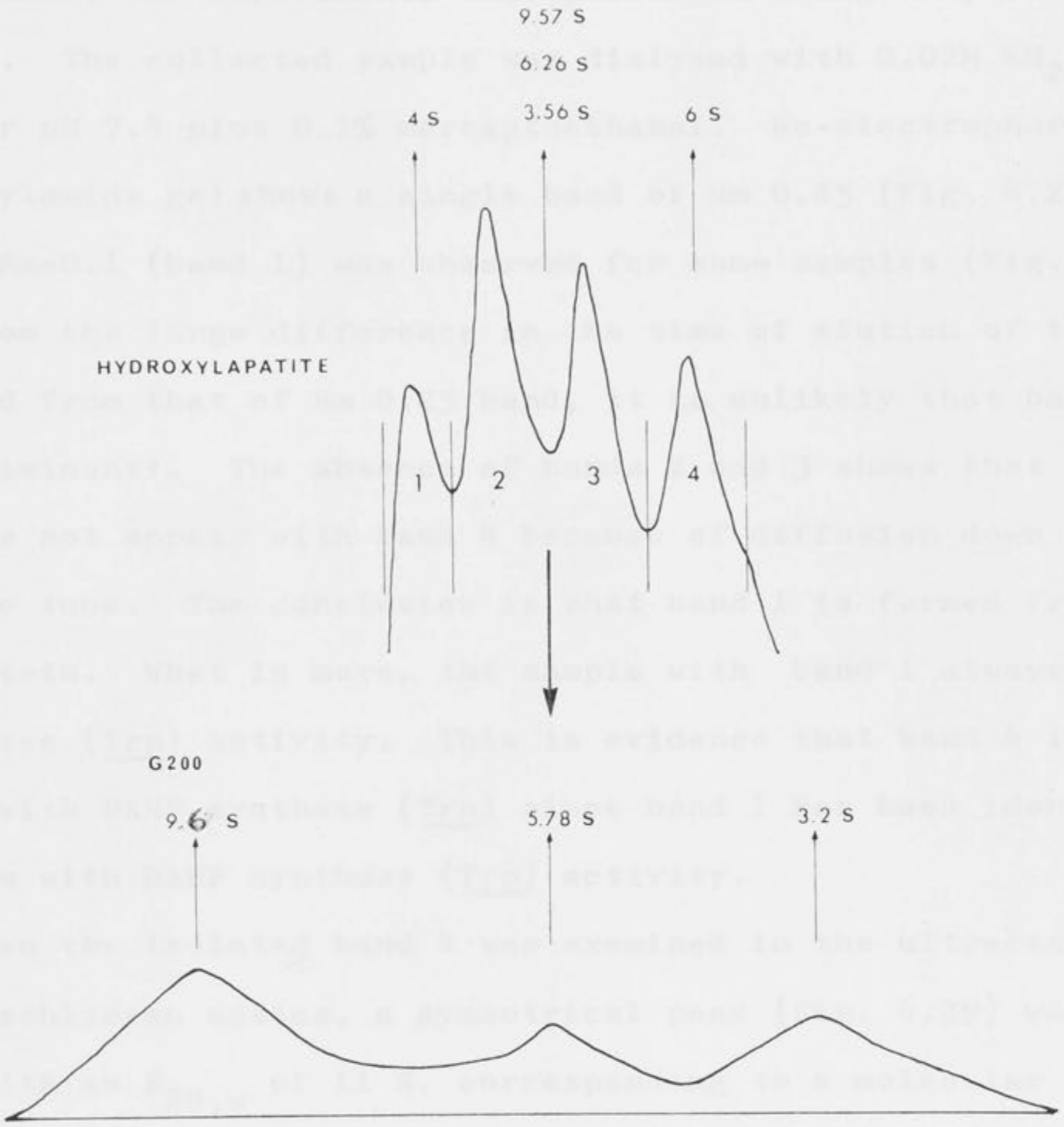


Fig. 4.27 Summary of the $S_{20,w}$ values obtained for various samples from the hydroxylapatite and the Sephadex G200 columns. Diagram shows the elution profiles (protein) from the two columns.

electrophoresis was continued until 15 min after elution of the band (the actual time was dependent on the Rms of the bands in front of and behind the required band).

Since band 4 is most abundant in samples from the hydroxyl-apatite column, the experiments were performed using enzyme from that stage. The collected sample was dialysed with 0.02M KH_2PO_4-NaOH buffer pH 7.4 plus 0.1% mercaptoethanol. Re-electrophoresis on polyacrylamide gel shows a single band of Rm 0.25 (Fig. 4.28a). A band of Rm=0.1 (band 1) was observed for some samples (Fig. 4.28b). Judging from the large difference in the time of elution of the Rm 0.1 band from that of Rm 0.25 band, it is unlikely that band 1 is a "contaminant". The absence of bands 2 and 3 shows that band 1 does not appear with band 4 because of diffusion down the side of the tube. The conclusion is that band 1 is formed from band 4 protein. What is more, the sample with band 1 always has DAHP synthase (Trp) activity. This is evidence that band 4 is connected with DAHP synthase (Trp) since band 1 has been identified as the form with DAHP synthase (Trp) activity.

When the isolated band 4 was examined in the ultracentrifuge using the schlieren optics, a symmetrical peak (Fig. 4.29) was obtained with an $S_{20,w}$ of 11 S, corresponding to a molecular weight of about 300 000. Yet band 4 can be found in the middle and the slowest eluting peaks from the G200 column corresponding to molecular weights of 110 000 and 48 000 respectively. When band 4 was examined in the gradient gel, a single band of molecular weight of about 140 000 was obtained. This would seem to suggest that the protein in band 4 has polymerised after isolation from the gel and when examined in the ultracentrifuge. These results are inconsistent with the possibility that there are two proteins having similar mobility on polyacrylamide gels (see Section VII).

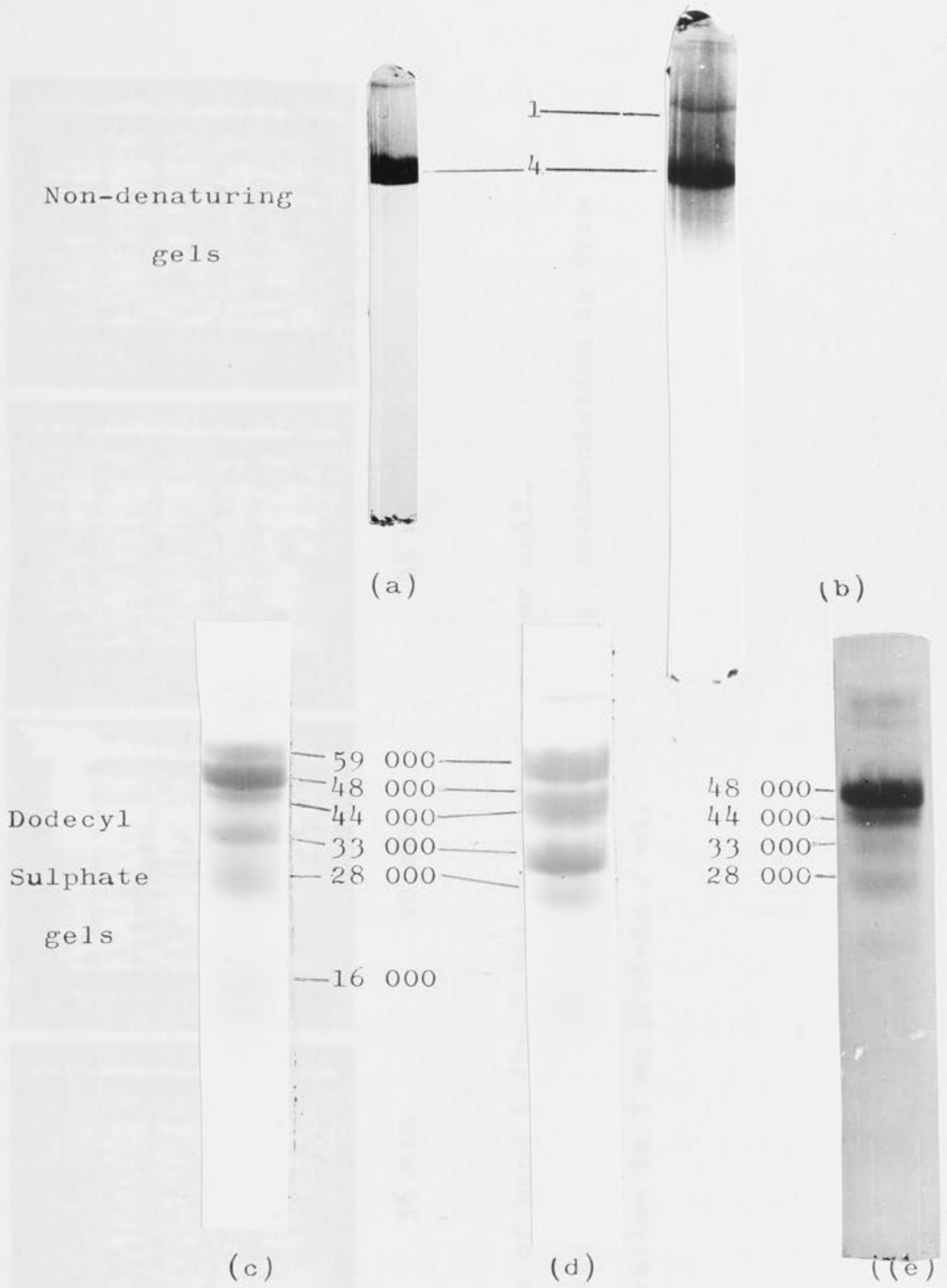
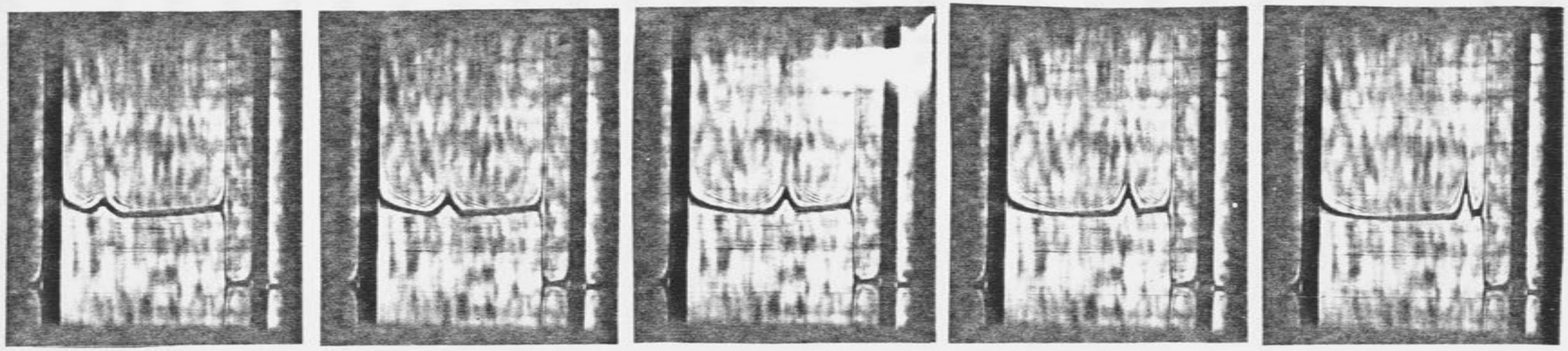


Fig. 4.2.8 (a) Nondenaturing polyacrylamide gel of band 4 isolated by preparative gel electrophoresis showing single band. (b) Same as in (a) but contains band 1 in addition to band 4. (c) Dodecyl sulphate gel of band 4. (d) Dodecyl sulphate gel of band 5. (e) Dodecyl sulphate gel band 6.

Approximately 100 µg protein was applied to each gel.



72 min 56 min 40 min 24 min 8 min

Fig. 4.29 Sedimentation of band 4 (Rm=0.25) in a single sector cell. Speed = 60 700 rpm. Phase angle = 50° . Temperature = 4.0°C. Sedimentation is from right to left. Concentration is 1 mg protein / ml.

It is possible that band 4 is formed from proteins that are present also in bands 5 and 6 (Fig. 4.21).

Dodecyl sulphate gel electrophoresis of band 4 (Fig. 4.28c) shows a preponderance of the 48 000 component and to a lesser extent a 33 000 component. In addition, the 59 000, 44 000, 28 000 and 16 000 components are present. It is possible that the 59 000 and the 48 000 components as found in band 4 are not the same components as found in band 1 and band 3. However, the fact that band 1, with enzyme activity, can be obtained from a sample which is purified for band 4 protein on the gel seems to suggest that the 59 000 component as found in band 4 is the same 59 000 component as found in protein 1 which has DAHP synthase (Trp) activity.

Bands 5 and 6 can be isolated by cutting the analytical gel and eluting from the gel slices. Careful examination shows that band 5 region contains two bands. These two bands were eluted together from the gel slices. Dodecyl sulphate gel electrophoresis shows (Fig. 4.28d) that band 5 consists of the 59 000, 48 000, 44 000 and 33 000 components present in approximately equal proportion with lesser amount of the 28 000 components. Dodecyl sulphate gel of band 6 (Fig. 4.28e) shows a preponderance of the 48 000 component with components of 44 000, 33 000 and 28 000 present. The 59 000 component is not observed. It is possible that the 59 000 and the 48 000 components as found in band 5 and the 48 000 component as found in band 6 are the same components as found in band 1 and band 3.

The fact that the 59 000 component can migrate with the 48 000 and the 44 000 components on the gels as in bands 3 and 4

seems to suggest that they can exist as an aggregate. Formation of these aggregates probably is a function of the experimental condition and the composition of subunit proteins that are present. These possibly "unnatural" complexes may restrict the expression of DAHP synthase (Trp) activity and therefore result in loss in activity. However, the fact that these proteins are able to combine with one another *in vitro* may imply that they can also combine with one another *in vivo* but in a different proportion.

These analyses, therefore, show that the four protein peaks from the hydroxylapatite column (Fig. 4.20) and the three protein peaks from the G200 column (Fig. 3.3) may contain components that can associate with active forms of DAHP synthase (Trp).

IX. AFFINITY CHROMATOGRAPHY

Of the purification procedures currently being used, affinity chromatography is probably the least destructive in terms of disruption of multi-polypeptide complexes. Attempt to use an affinity column with Trp bound directly to the agarose gel through the amino group was unsuccessful (see Chapter 3, Section III). In addition, two other affinity columns were prepared with Phe and Trp bound through the carboxyl group to a six-carbon arm linked to the agarose gel. The procedures are described in Chapter 7, Section IV.

A sample after the pH changes (Step 4) was adjusted to pH 6.4 and applied to a Sepharose-6C-Trp column. About 60% of the total activity eluted unadsorbed. Before this column, the sample was 53% inhibited by Trp and 5% inhibited by Phe. The unbound activity was 83% inhibited by Trp and 3% by Phe. Part

of the bound activity could be eluted by raising the buffer to pH 7.4. Elution was gradual and spread over a large volume. Complete elution was obtained by 0.2M NaCl. When the unbound fraction was re-applied to the same affinity column, almost 92% of the activity was adsorbed. The bound and subsequently eluted sample showed more than twelve protein bands on polyacrylamide gel.

A crude extract after removal of nucleic acid by protamine sulphate was dialysed against 0.02M KH_2PO_4-NaOH buffer pH 6.4 plus 0.1mM PEP. The resulting solution was inhibited 60% by Phe. When this sample was applied to a Sepharose-6C-Phe affinity column, 80% of the activity was adsorbed and the unbound activity was inhibited 72% by Phe. Very little of the bound activity could be eluted by raising the buffer to pH 7.4. About 20% of the activity could be recovered by 0.2M NaCl.

Another sample was obtained by molecular sieving of the crude extract (after removal of nucleic acid) on an agarose column. This sample was inhibited 41% by Trp and slightly activated by Phe and Tyr. When this sample was applied to a Sepharose-6C-Phe column, about 50% of the activity was adsorbed which could be eluted by 0.2M NaCl. Almost all of the activity was adsorbed or some activity was retarded when the unbound fraction was re-applied to the same column.

These are preliminary results. A more thorough investigation has still to be carried out. Although preliminary, the results show that when first applied to affinity columns with either Trp or Phe as the binding ligand, DAHP synthase (Trp) tends not to adsorb but adsorbs on subsequent passage through the same affinity column. It may be that other proteins have a greater affinity for the column or that DAHP synthase (Trp) is

modified by the initial passage. Furthermore, it seems that DAHP synthase (Phe) and DAHP synthase (Trp) will bind to an affinity column with either Trp or Phe as the binding ligand.

X. SUBUNIT COMPOSITION AND ORGANISATION OF DAHP SYNTHASE (Trp)

DAHP synthase (Trp) in N. crassa is encoded in the genetic locus aro-8. Protein 1 (Rm=0.1) as detected on polyacrylamide gel has both DAHP synthase activity and is inhibited by Trp (Section II). This protein has a molecular weight of about 235 000 (Section IV) and is made up from four molecules of a 59 000 component. This 59 000 component as detected on dodecyl sulphate gel is, therefore, likely to be the product of aro-8. Other protein bands, for example bands 2, 3 and 4, as detected on non-denaturing gel may also contain this 59 000 component (Section II, Section VIII). There are some evidence which shows that this 59 000 component can become associated with a 48 000 component, for example in band 4 (Section VIII). The fact that band 1 can change and migrate together with band 3 on gels also suggests possible association between the two polypeptides (Section II). Therefore, it is possible that there is a much higher degree of organisation than would be expected from the simple subunit structure of protein 1 which has the DAHP synthase (Trp) activity.

DAHP synthase (Trp) is a metalloenzyme (Chapter 3, Section VIII). Co^{2+} is important for both activity and inhibition. The presence of Zn^{2+} results in deactivation of the enzyme and changes in inhibition (Chapter 3, Section IV). A divalent metal ion, therefore, is important in the organisation of the polypeptide subunits into specific conformations which may be active and inhibitable or less active and noninhibitable. Under conditions that will result in dissociation and re-association of polypeptides,

e.g. $(NH_4)_2SO_4$ precipitation followed by removal of $(NH_4)_2SO_4$, the activity and inhibition also change (Chapter 3, Section III). This change shows that there is a high degree of organisation of the polypeptides that are associated with DAHP synthase (Trp) activity. DAHP synthase activity is not associated with the purified common path aggregate specified by the aro-gene cluster (Doy, personal communication). However, it remains possible that each of the DAHP synthase isoenzymes may contain components that belong to the other isoenzymes. Results of works by Halsall (1969) and Hoffmann (1971) showed the complexities of DAHP synthase. These works show the biochemical and genetical relatedness of DAHP synthases (Phe) and (Tyr). In particular, highly purified forms of DAHP synthase (Phe) could be obtained that are more than 90% inhibited by tyrosine as well as phenylalanine (Hoffmann, 1971). Futhermore, purified DAHP synthase (Tyr) which gave one band on non-denaturing gel gave as many as twelve bands on dodecyl sulphate gels (Hoffmann, 1971). Some of the bands detected from these highly purified DAHP synthases (Tyr) and (Phe) may be identical with the bands detected for DAHP synthase (Trp) but in different proportion. For example, one of the minor band from DAHP synthase (Tyr) is assigned the molecular weight 59 000 and bands of 44 000 - 50 000 also occur.

The results from affinity chromatography (Section IX) are consistent with the view that the gene products of aro-6, aro-7 and aro-8 contribute to all three classes of isoenzymes. The interaction between DAHP synthase (Phe) and DAHP synthase (Trp) isoenzymes (Chapter 3, Section IV) also suggests that the aro-7 gene product might contribute to DAHP synthase (Trp). While it remains possible that the 48 000 component is completely unrelated to DAHP synthase (Trp) either in vitro or in vivo,

it seems reasonable to speculate that it might be a product of either aro-6 or aro-7.

Protein 1 can be purified to homogeneity if so desired by elution from polyacrylamide gel slices. This treatment results in instability and large loss of activity. With the present purification procedure, in terms of specific activity, the purification is only about 400 fold whereas in terms of protein recovery, the purification is about 17 000 fold (Table 3.1).

The only other DAHP synthase (Trp) that has been studied in some detail is that of E.coli K12. In the report of Camakaris and Pittard (1974), no information on the homogeneity of the preparation is given apart from a statement that the material showed at least three major bands on polyacrylamide gel, none of which could be shown to be active.

In the work of Simpson and colleagues (1971), the intracellular levels of DAHP synthase (Phe) in a lysogenised strain of E.coli K-12 was increased 15-fold over levels found in the wild type strain by incorporating the structural gene for DAHP synthase (Phe) into the genome of a heat-inducible mutant of phage lambda. Using this strain of E.coli, the DAHP synthase (Phe) was purified 2 000-fold compared with the wild type. However, electrophoresis of the native enzyme in polyacrylamide gels revealed a number of protein components and denaturing gels (8M urea, pH 3.65) gave two protein components.

Other than the results reported in this work, no DAHP synthase from any organism has ever been purified to give one band on denaturing polyacrylamide gels (Staub and Denes, 1969a; Simpson et al., 1971; Hoffmann, 1971; Camakaris and Pittard, 1974). This may be an indication of the complexities of DAHP synthases. I am hopeful that further studies of the organisation of DAHP

synthases with the other enzymes in the aromatic pathway would add to our understanding of the *in vivo* compartmentation and channeling of biochemical intermediates.

CHAPTER 5

KINETIC PROPERTIES OF DAHP SYNTHASE (TRP)

DAHP synthase is a key enzyme in the control of the biosynthesis of aromatic amino acids. The five steps after DAHP synthase that are catalysed by the aro aggregate are not regulated by the end products. The kinetic properties of purified DAHP synthase (Tyr) were studied by Hoffmann (1971). However, DAHP synthase (Tyr) is not particularly suitable for kinetic studies because it is unstable in the absence of PEP, one of its substrates. The enzyme used usually contains some unknown quantity of bound PEP which makes interpretation of results difficult. However, for DAHP synthase (Trp) the presence of PEP is not essential for reasonable stability.

One of the factors which limits studies of the kinetic properties of DAHP synthase is the enzyme assay. The present enzyme assay procedure (see Chapter 7, Section II) does not allow direct observation of the change in rate of the reaction with time. Also, the number of steps involved in developing the chromagen increases the probability of error. The enzymatic method for the determination of inorganic phosphate (Hwang and Cha, 1973) is unsuitable as an indicator in a dynamic coupled assay because of the length of time (14 min) for the velocity to reach 99% of the true value at a reasonable concentration of the purine nucleoside phosphorylase used.

To reduce this time, a large amount of the coupled enzyme has to be used and the assay becomes too costly.

In this study of the kinetic properties of DAHP synthase (Trp), the initial velocity of the reaction at the steady state is determined from the plot of the amount of DAHP formed (in terms of $A_{549\ nm}$) versus time of incubation of enzyme with the assay mixture. Five different times of incubation of enzyme with the assay mixture were taken per initial velocity determination. The rate of reaction is linear for at least five minutes. The initial velocity is calculated from the velocity at zero time and expressed as $A_{549\ nm}$ corresponding to a 10 min reaction time. Purified active enzyme from the final step, pooled activity peak fractions from the G200 column was used in this study.

I. EFFECT OF pH ON ACTIVITY AND INHIBITION

In 5 mM KH_2PO_4-NaOH buffer, the activity of the Trp-inhibited isoenzyme rises from pH 6.0 to an optimum at pH 7.0 (Fig. 5.1) and then declines slowly. However, above pH 8.0, the buffering capacity is low and therefore this portion of the curve may be unreliable. Inhibition by 4 μM Trp has a broad optimum from pH 6.0 to pH 6.8 which does not coincide with the activity optimum (Fig. 5.1).

In 0.02M Tris-maleate buffer, the activity shows a peak at pH 6.8 and another peak at pH 7.3 and then declines, faster

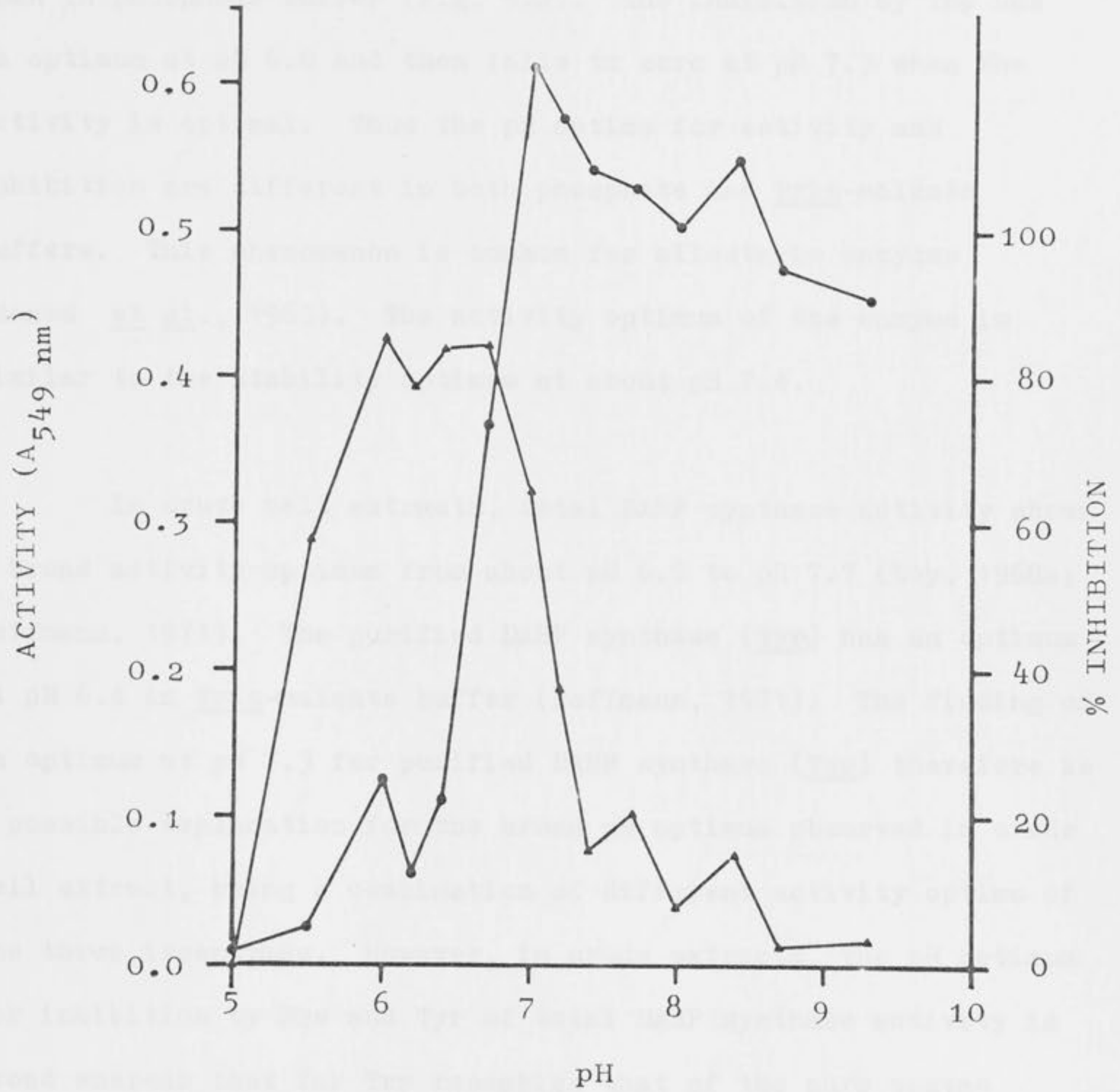


Fig. 5.1 The pH dependence of the activity (●) and of Trp inhibition (▲) of DAHP synthase (Trp) in 5 mM KH_2PO_4-NaOH buffer.

Composition of reaction mixtures was described in Chapter 7 and 4 μM Trp was included where indicated; 0.3 μg of proteinwas used for each assay.

than in phosphate buffer (Fig. 5.2). The inhibition by Trp has an optimum at pH 6.0 and then falls to zero at pH 7.3 when the activity is optimal. Thus the pH optima for activity and inhibition are different in both phosphate and Tris-maleate buffers. This phenomenon is common for allosteric enzymes (Monod et al., 1963). The activity optimum of the enzyme is similar to the stability optimum at about pH 7.4.

In crude cell extracts, total DAHP synthase activity shows a broad activity optimum from about pH 6.5 to pH 7.7 (Doy, 1968a; Hoffmann, 1971). The purified DAHP synthase (Tyr) has an optimum at pH 6.4 in Tris-maleate buffer (Hoffmann, 1971). The finding of an optimum at pH 7.3 for purified DAHP synthase (Trp) therefore is a possible explanation for the broad pH optimum observed in crude cell extract, being a combination of different activity optima of the three isoenzymes. However, in crude extracts, the pH optimum for inhibition by Phe and Tyr of total DAHP synthase activity is broad whereas that for Trp resembles that of the pure enzyme (Halsall, 1969).

II. EFFECT OF LIGANDS ON STABILITY TO HEAT DENATURATION

The enzyme was dialysed against 0.02M Tris-HCl buffer pH 7.4 plus 0.1% mercaptoethanol. The ability of PEP, E4P, Pi and Trp to protect the enzyme against heat denaturation was tested by incubating the enzyme with each of the ligands at 37°C for various lengths of time. The results are illustrated in Fig. 5.3. In the presence of PEP, the activity of the enzyme remains

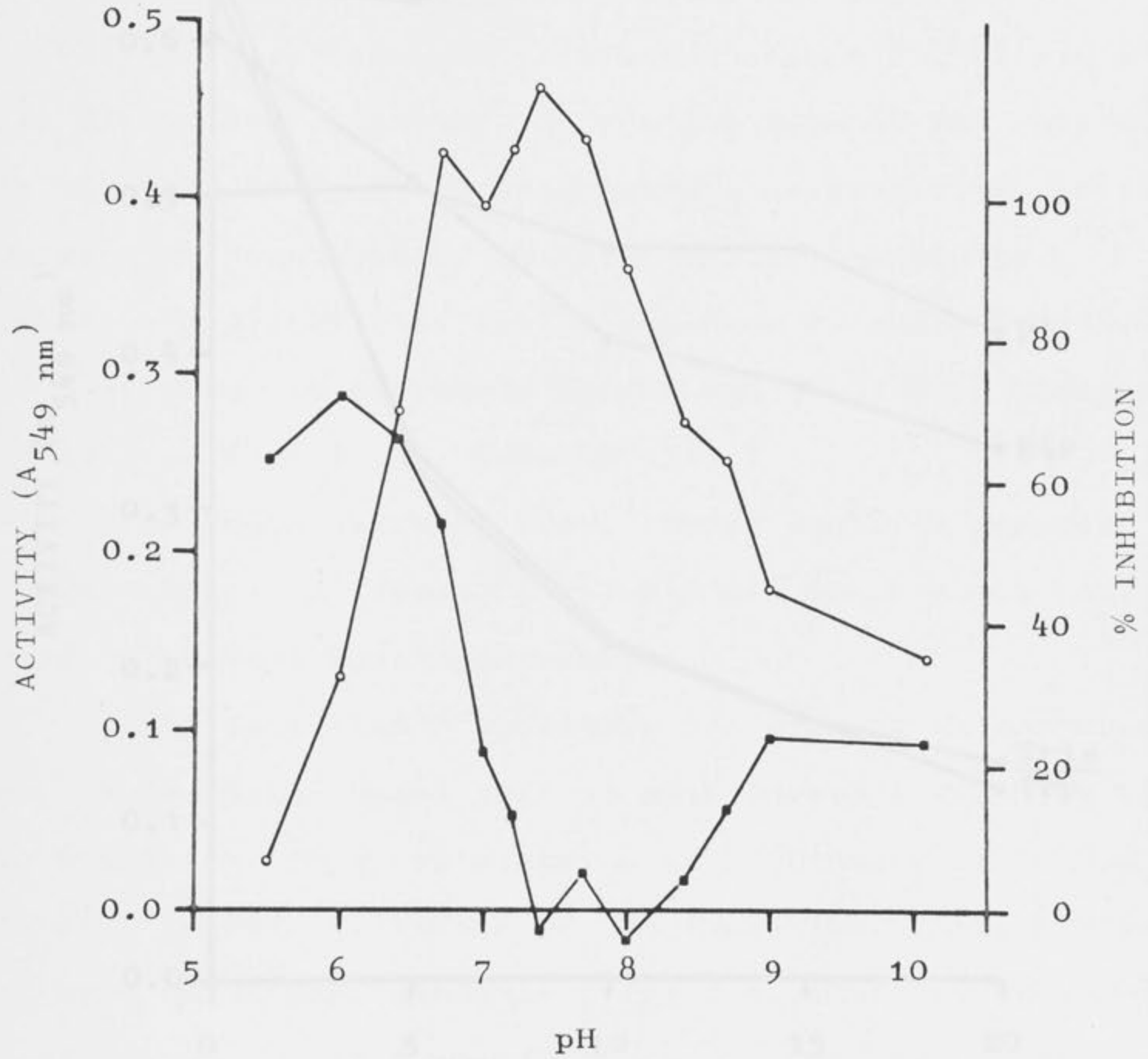


Fig. 5.2 The pH dependence of the activity (O) and of Trp inhibition (■) of DAHP synthase (Trp) in 0.02M Tris-maleate buffer.
Other conditions were the same as Fig. 5.1.

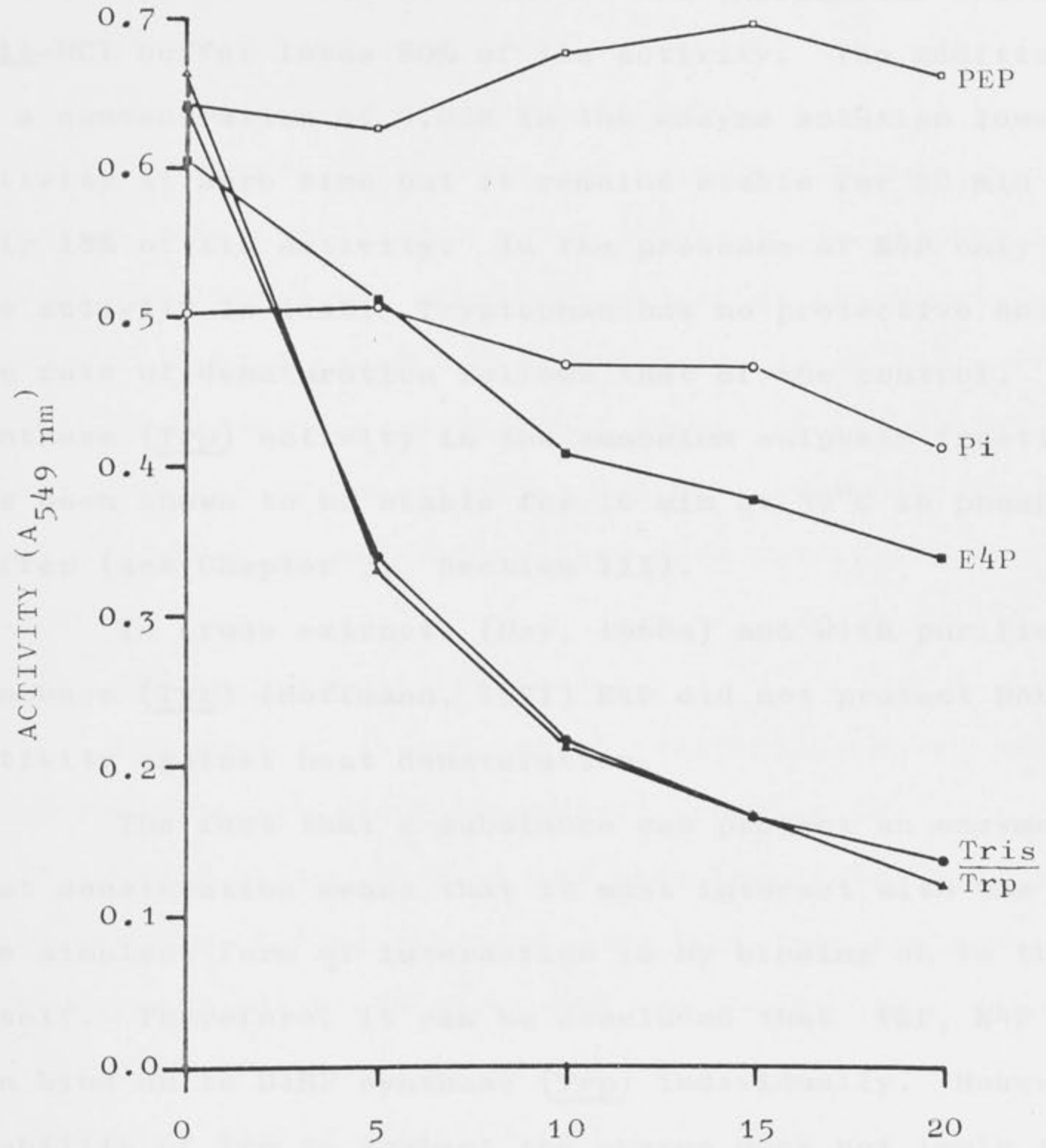


Fig. 5.3 The ability of various ligands to protect DAHP synthase (Trp) against heat denaturation at 37°C. Concentrations of ligands were PEP, 0.2 mM; E4P, 0.2 mM; Trp, 1 μm; Pi, 0.02M. Assay tubes were prepared as described in Chapter 7 and 1 μg of protein was used for each assay. Samples were incubated with ligands for time period indicated and then assayed for activity in mixture containing 0.5 mM each of PEP and E4P.

relatively stable for at least 20 min whereas the control in Tris-HCl buffer loses 80% of its activity. The addition of Pi to a concentration of 0.02M in the enzyme solution lowers the activity at zero time but it remains stable for 20 min losing only 18% of its activity. In the presence of E4P only 40% of the activity is lost. Tryptophan has no protective ability and the rate of denaturation follows that of the control. DAHP synthase (Trp) activity in the ammonium sulphate fraction also has been shown to be stable for 10 min at 39°C in phosphate buffer (see Chapter 3, Section III).

In crude extracts (Doy, 1968a) and with purified DAHP synthase (Tyr) (Hoffmann, 1971) E4P did not protect DAHP synthase activity against heat denaturation.

The fact that a substance can protect an enzyme against heat denaturation means that it must interact with the protein. The simplest form of interaction is by binding on to the enzyme itself. Therefore, it can be concluded that PEP, E4P and Pi can bind on to DAHP synthase (Trp) individually. However, the inability of Trp to protect the enzyme does not imply that Trp cannot bind on to the enzyme.

III. KINETIC PROPERTIES

The study of the kinetics of enzymic reaction is aimed at obtaining information on the mechanism of the reaction including the regulatory properties of an enzyme. DAHP synthase (Trp) is an allosteric enzyme feedback inhibited by an end product of aromatic biosynthesis, tryptophan. This coupled with the fact that the enzyme catalyses a two-substrate-two-product

reaction suggests that the enzyme has great regulatory potential (Atkinson and Walton, 1965).

(a) Effect of enzyme concentration

Increasing the enzyme concentration results in a linear increase in activity. For a few preparations, a non-linear increase was observed. These preparations presumably contained a component which could affect the activity of the enzyme when present in higher concentration. Since this result of non-linear response was not typical for DAHP synthase (Trp), preparations which showed this result were not used for the kinetic studies.

(b) Initial velocity studies

The general theory for the identification of the reaction mechanism is described in Chapter 7, Section IX.

Using crude extracts and measuring total DAHP synthase activity, Doy (1968a) reported curvature (concave upward) in the double-reciprocal plots when E^4P was the variable substrate at different fixed concentrations of PEP. A set of parallel lines was obtained when PEP was the variable substrate, indicating that the reaction probably proceeds via a ping-pong bi bi mechanism. Curvature (concave upward) indicates positive co-operativity between E^4P binding sites. Hill plots of the crude extract saturation data (Doy, 1968a) gave values of m=1.7 for PEP and m=1.8 for E^4P indicating homotropic co-operativity and at least two binding sites for each substrate.

Preliminary investigation

Similar experiments were performed with purified DAHP synthase (Trp). Assays were done in Tris-maleate buffer pH 6.4 and the enzyme was in phosphate buffer. The final phosphate

concentration was 1.2 mM. When PEP is the variable substrate, the double-reciprocal plots are linear for four fixed concentrations of E4P (50 μM, 100 μM, 175 μM and 250 μM) (Fig. 5.4). Whereas the two lines at 50 μM and 100 μM fixed concentration of E4P are parallel, the plots at higher E4P conentration show increasing slopes and the activity at 250 μM E4P and 50 μM PEP is lower than that at 175 μM E4P and 50 μM PEP. This indicates inhibition by high concentrations of E4P. The double-reciprocal plots when E4P is the variable substrate show a series of curves (concave upward) (Fig. 5.5). Curvature of this kind in double-reciprocal plot can mean positive co-operativity of E4P binding to the enzyme or, alternatively, if the plot curves such that the ordinate becomes the asymptote to the curve, substrate inhibition is indicated (Cleland, 1967). The plot at 50 μM fixed concentration of PEP suggests substrate inhibition by E4P.

For further studies, two modifications were made. The concentration of E4P was lowered so that it is no longer inhibitory. The enzyme was dialysed with Tris-maleate buffer pH 7.4 and 0.1% mercaptoethanol to remove the Pi (being one of the products it may affect the reaction).

Assay at pH 6.4

The double-reciprocal plots when E4P is the variable substrate are linear and parallel from 12.5 μM to 100 μM E4P (Fig. 5.6) at fixed concentrations of 50 μM, 100 μM and 175 μM PEP concentration. Curvature occurs at higher E4P concentration and high PEP concentration. The double-reciprocal plots when PEP is the variable substrate show curvature (concave downward) at low E4P concentration becoming linear above 100 μM fixed concen-

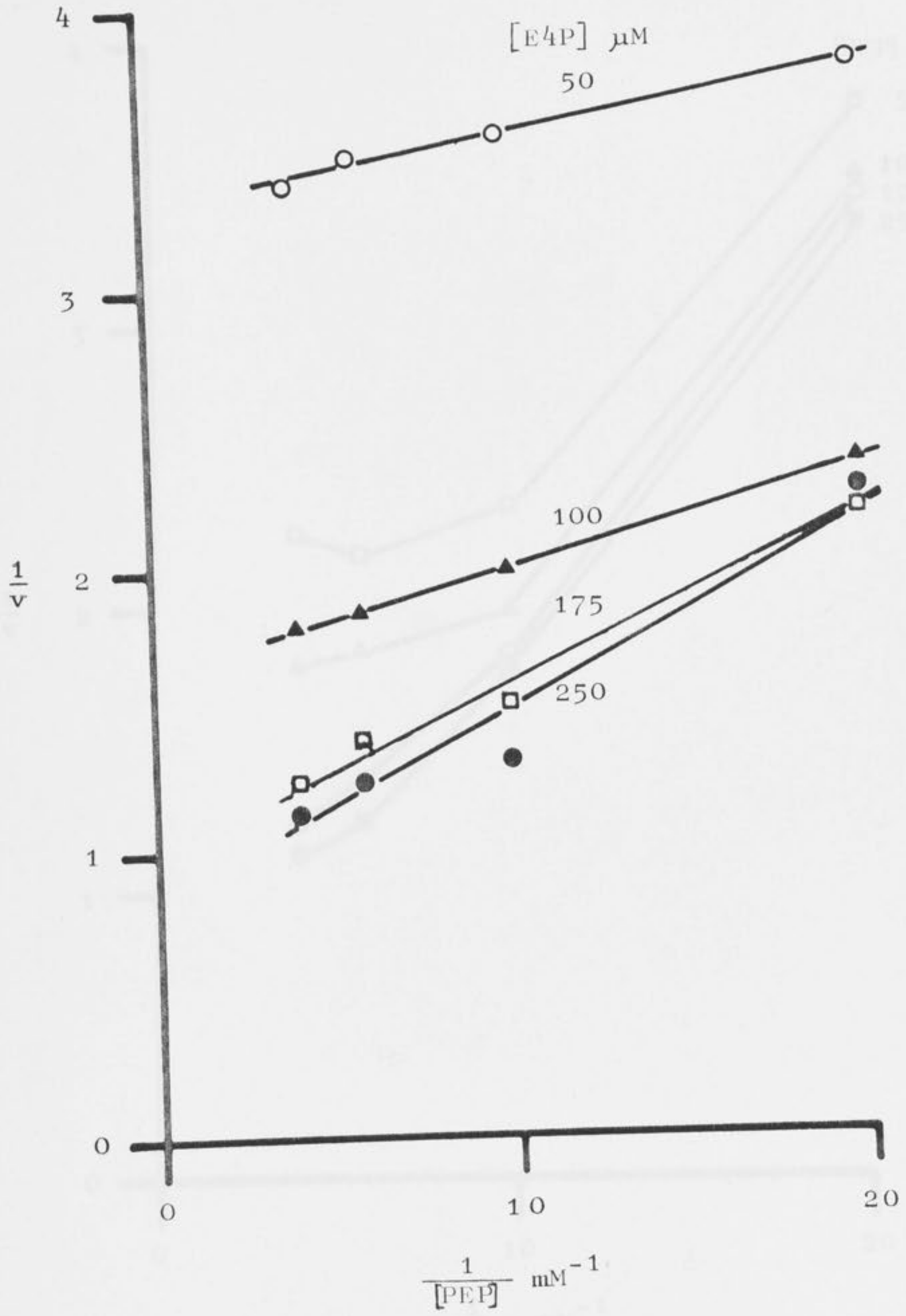


Fig. 5.4 Double-reciprocal plots with PEP as the variable substrate at different fixed concentrations of E4P as indicated.

Assays were done in 0.02M Tris-maleate buffer pH 6.4 and 1 µg of protein was used per assay.

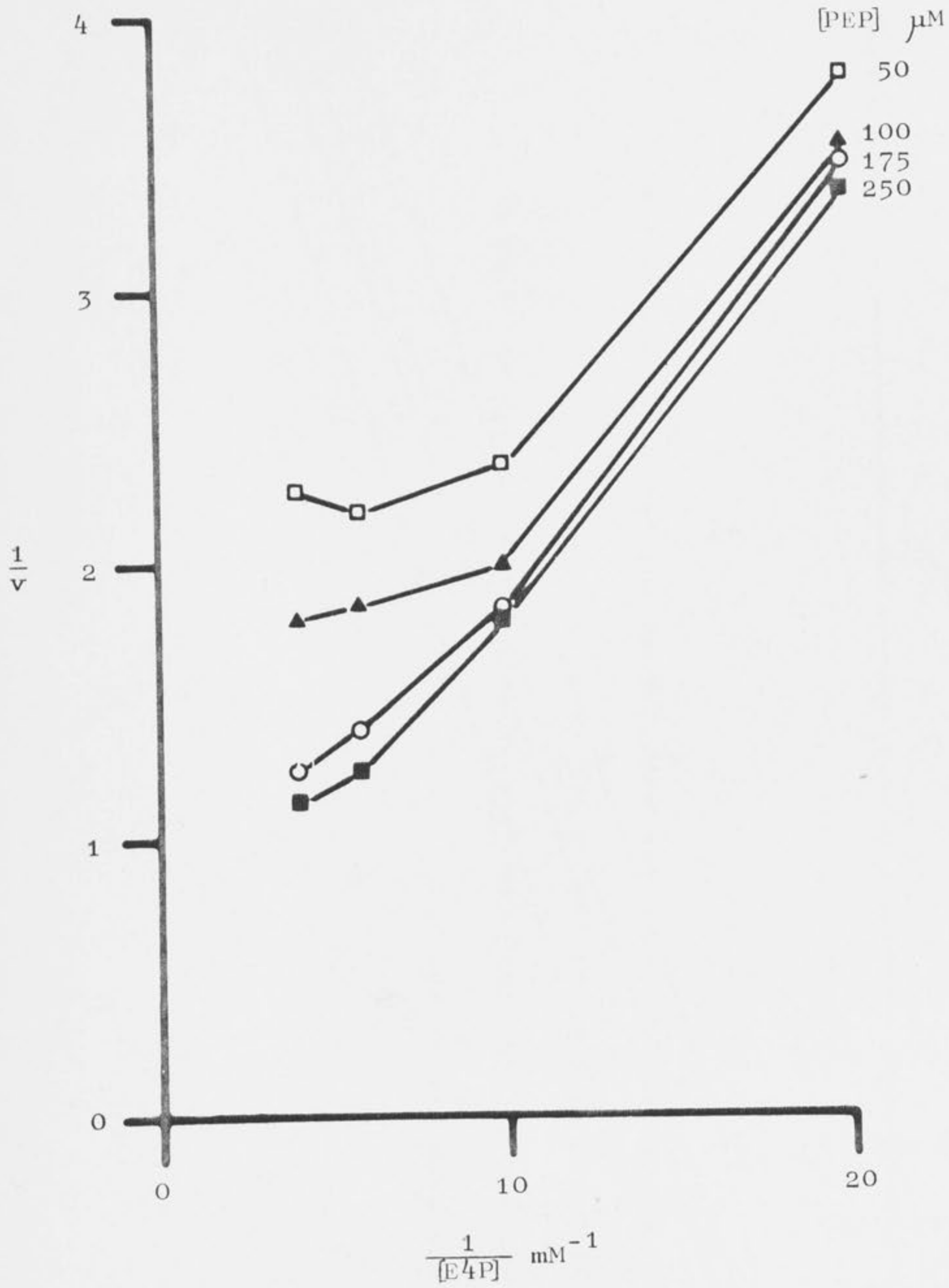
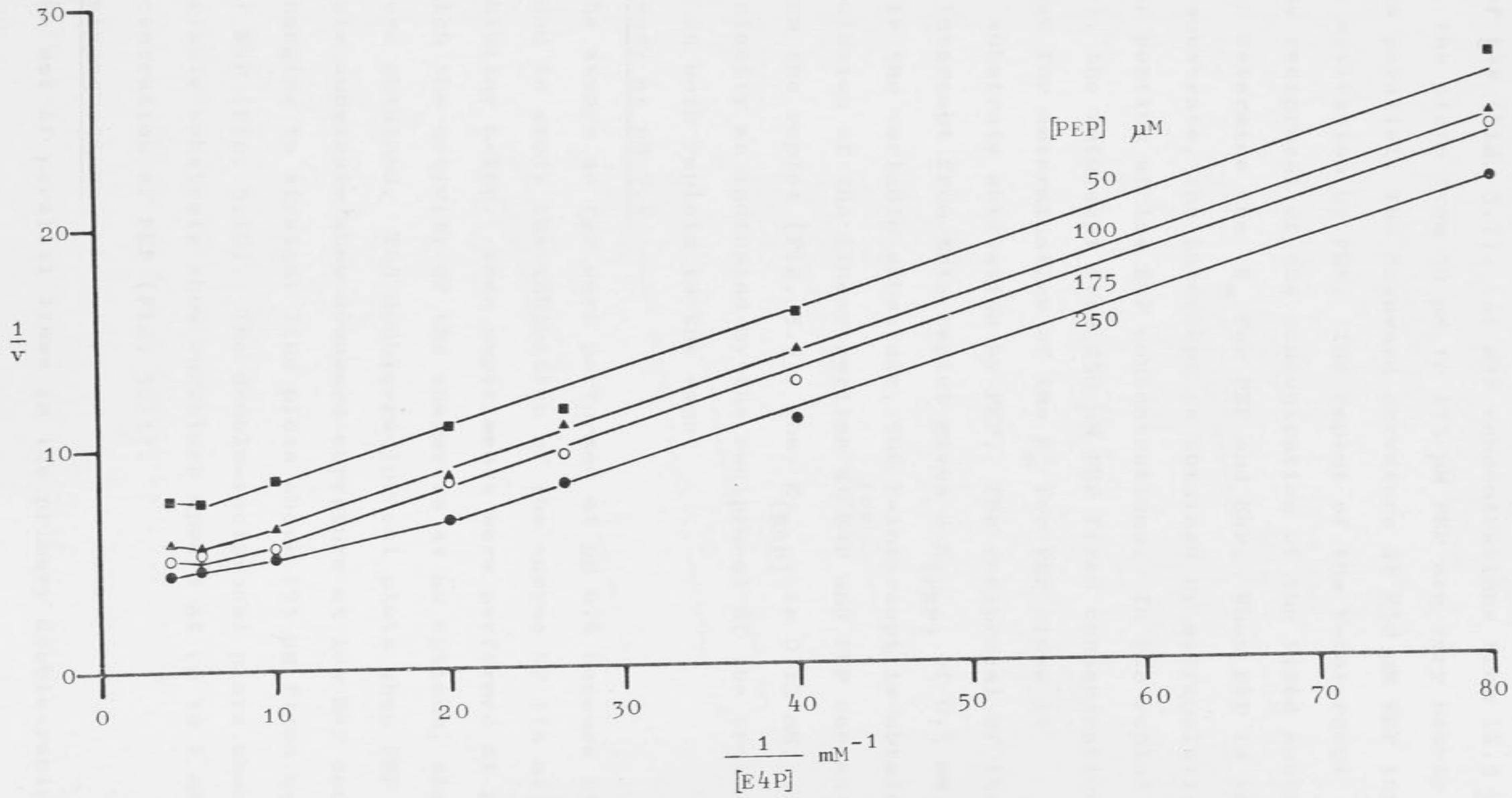


Fig. 5.5 Double-reciprocal plots with E4P as the variable substrate at different fixed concentrations of PEP as indicated.

Assays were done in 0.02M Tris-maleate buffer pH 6.4 and 1 μg of protein was used per assay.

Fig. 5.6 Double-reciprocal plots with E4P as the variable substrate at different fixed concentrations of PEP as indicated.

Assays were done in 0.02M Tris-maleate buffer pH 6.4 and 2 µg of protein was used per assay.


[PEP] μM
50
100
175
250
1/v
30
20
10
0
0
10
20
30
40
50
60
70
80
1/[E4P] mM⁻¹

tration of E4P (Fig. 5.7). At E4P concentrations from 12.5 μM to 50 μM, the plots from 50 μM to 175 μM PEP are very nearly linear and parallel. The downward curvature at 250 μM PEP indicates substrate activation by PEP. The replot of the Y-intercept versus the reciprocal of the concentration of the fixed substrate is used to determine the K_m for PEP and E4P. When E4P is the variable substrate, the intercept is obtained by extrapolation of the linear portion at low E4P concentrations. In the replot (Fig. 5.8), the intercept from 250 μM PEP fixed concentration is not used for determination of the K_m for PEP since it indicates substrate activation by PEP. The reciprocal of the abscissa-intercept from this replot gives a $K_{(PEP)}$ of 0.1 mM. When PEP is the variable substrate, the Y-intercept is obtained by extrapolation of the linear portion at low E4P and PEP concentrations. From the replot (Fig. 5.9), the $K_{(E4P)}$ is 0.13 mM. The maximum velocity as indicated by the reciprocal of the ordinate-intercept in both replots is the same.

Assay at pH 7.4

The assays so far were performed at pH 6.4 because it was intended to study the inhibition of the enzyme by its allosteric inhibitor L-Trp. When experiments were performed at pH 7.4 in which the activity of the enzyme is at an optimum, similar results were obtained. The double-reciprocal plots when PEP is the variable substrate show downward curvature at low E4P concentration changing to straight line plots above 175 μM fixed concentration of E4P (Fig. 5.10). The double-reciprocal plots when E4P is the variable substrate show curvature upward at up to 1 mM fixed concentration of PEP (Fig. 5.11).

Rate equations

The set of parallel lines in the primary double-reciprocal

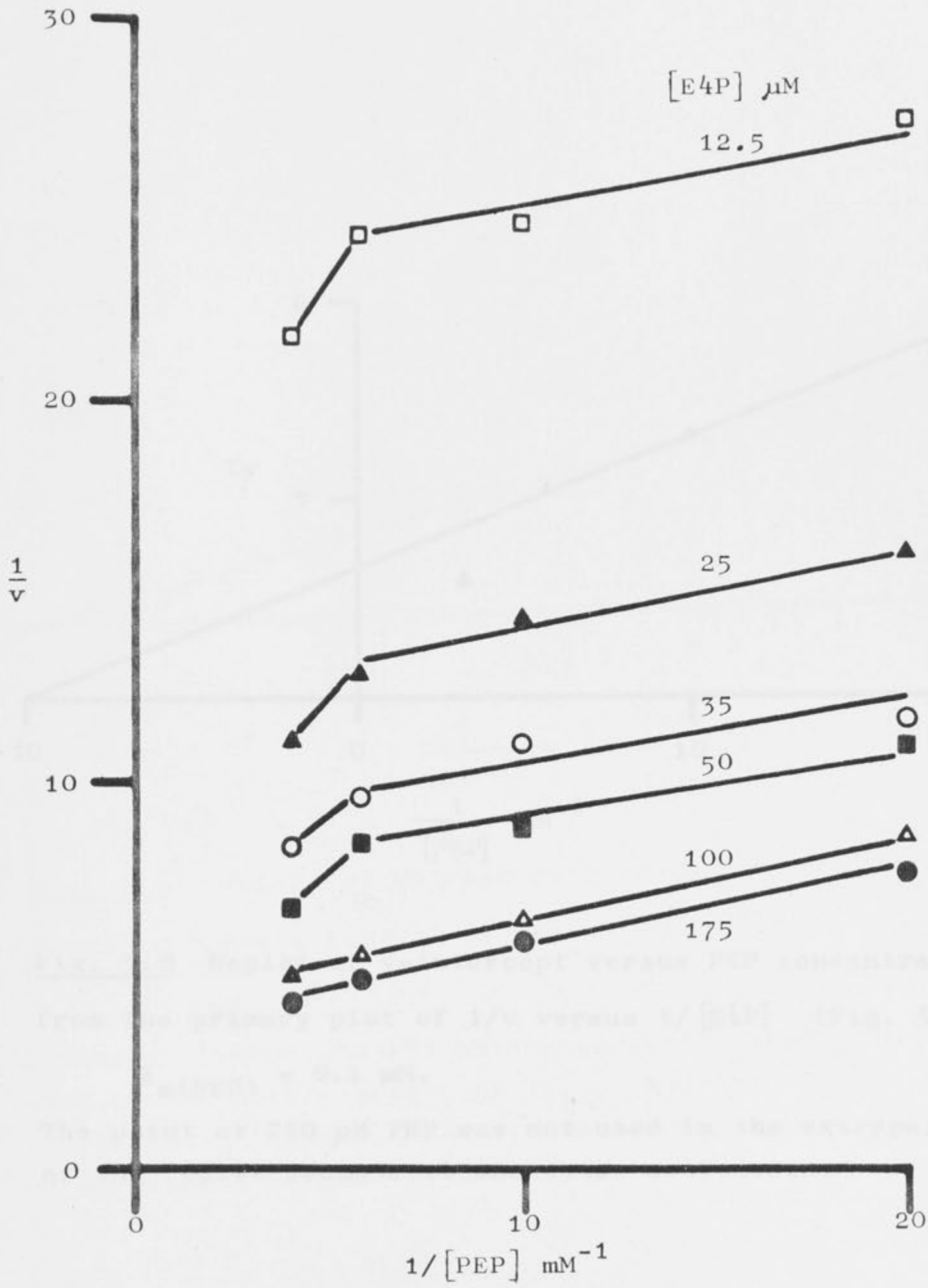


Fig. 5.7 Double-reciprocal plots with PEP as the variable substrate at different fixed concentrations of E4P as indicated. Assays were done in 0.02M Tris-maleate buffer pH 6.4 and 2 µg of protein was used per assay.

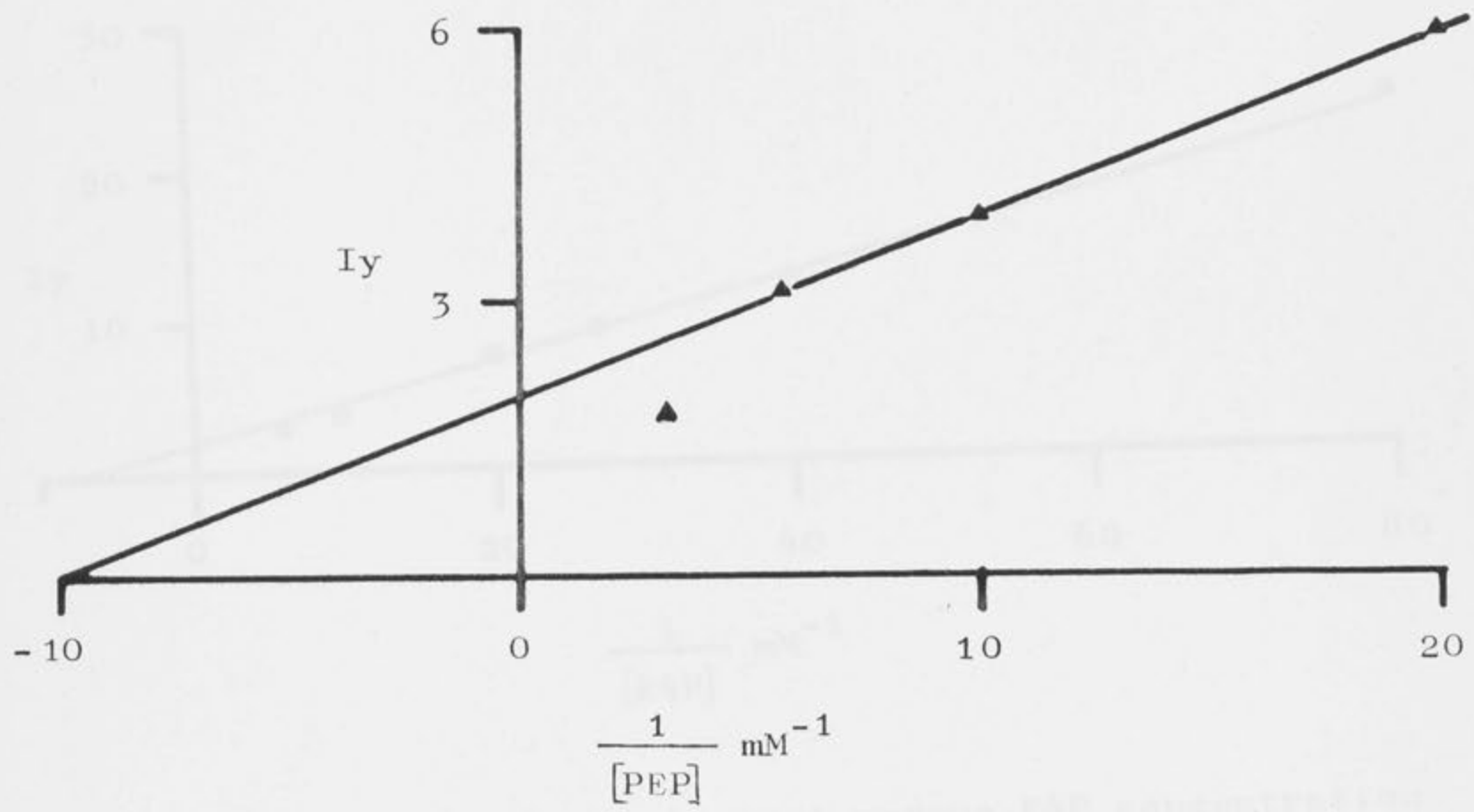


Fig. 5.8 Replot of y-intercept versus PEP concentrations from the primary plot of 1/v versus 1/[E4P] (Fig. 5.6).

$K_{m(PEP)}$ = 0.1 mM.

The point at 250 μM PEP was not used in the extrapolation of the replot because it indicates activation.

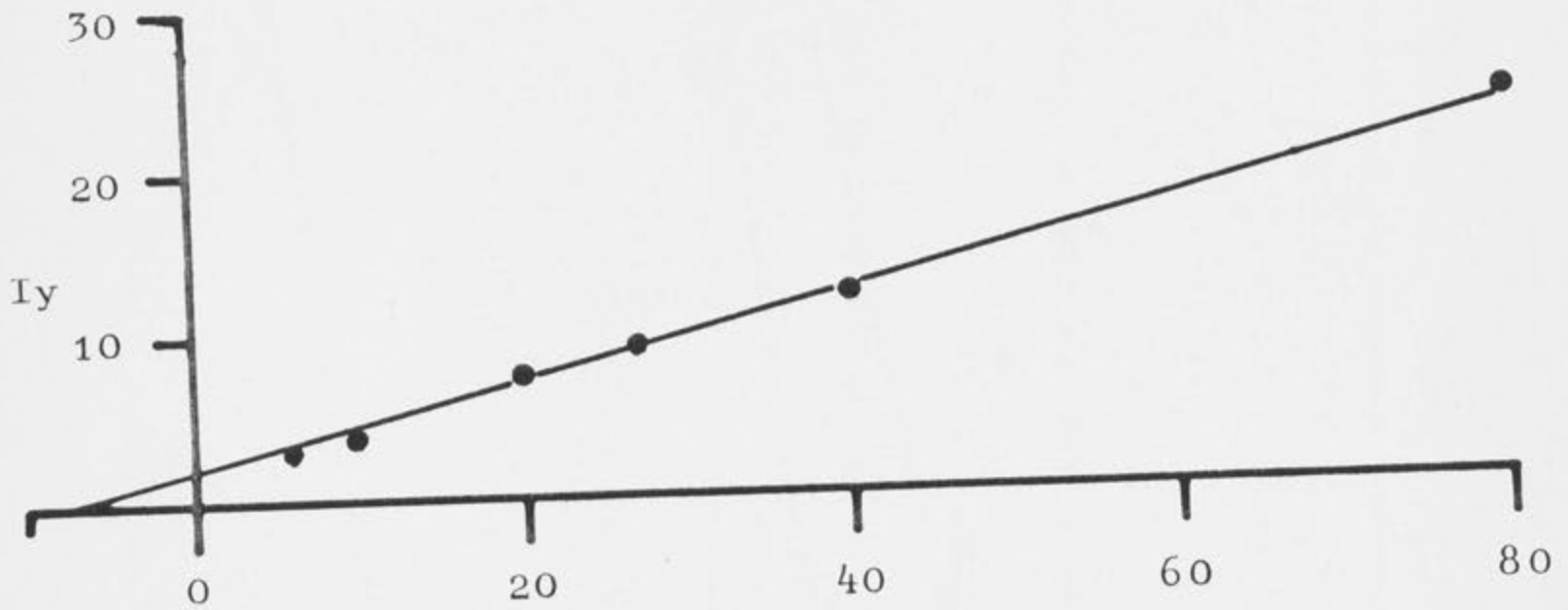


Fig. 5.9 Replot of y-intercept versus E4P concentration from the primary plot of 1/v versus 1/[PEP] (Fig. 5.7)

$K_{(E4P)} = 0.13$ mM.

Points at 100 uM and 175 uM E4P concentrations were not used in the determination of $K_{(E4P)}$ because of substrate inhibition. However, the differences are too small to be observable on this graph.

Fig. 5.10 Double-reciprocal plots with PEP as the variable substrate at different fixed concentrations of E4P.

Assays were done in 0.02M Tris-maleate buffer pH 7.4 and 1.5 µg of protein was used per assay.

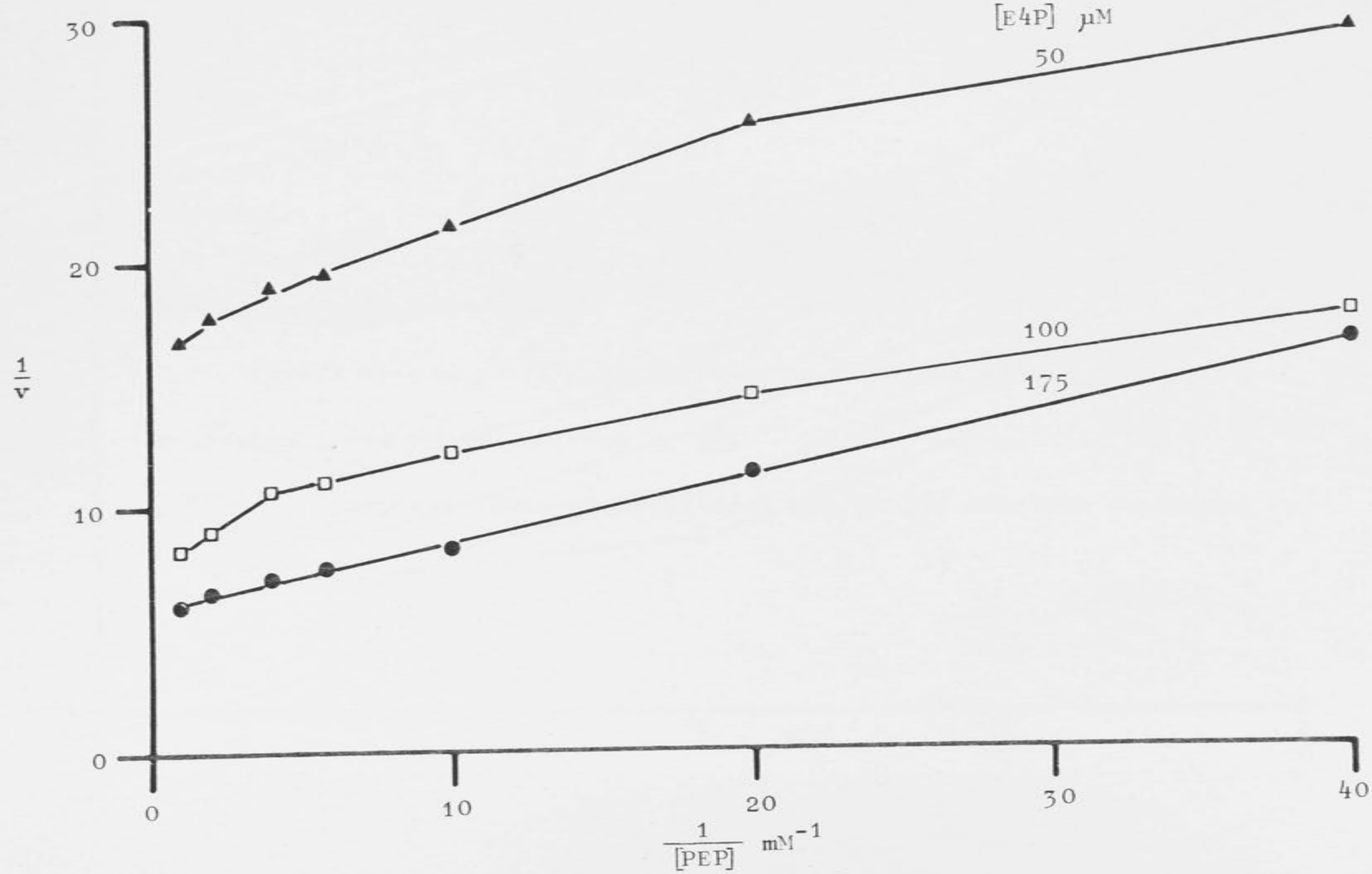
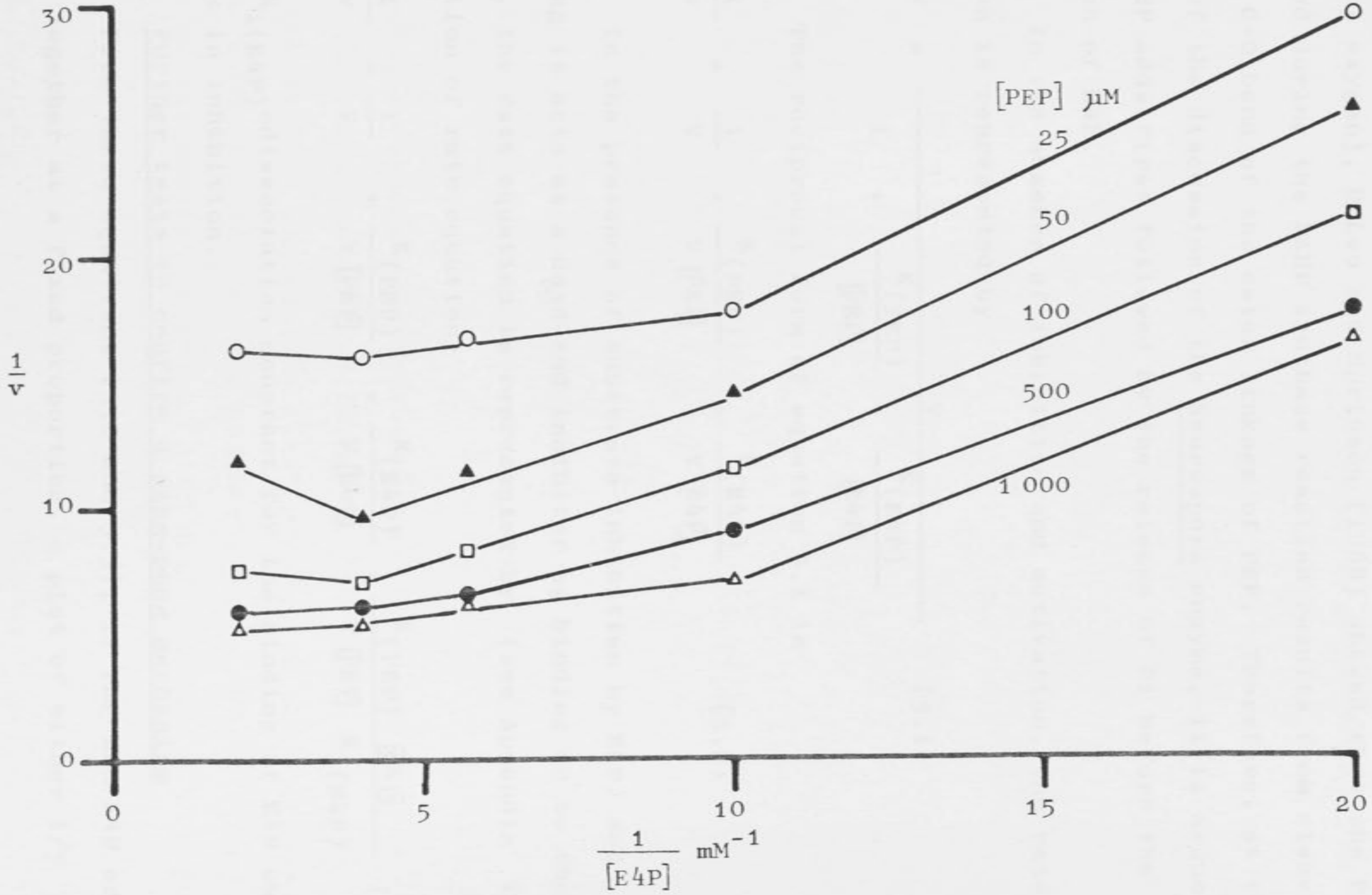

[E4P] μM
50
100
175
30
20
10
0
$\frac{1}{v}$
0
10
20
30
40
$\frac{1}{[PEP]}$ mM^{-1}

Fig. 5.11 Double-reciprocal plots with E4P as the variable substrate at different fixed concentrations of PEP.

Assays were done in 0.02M Tris-maleate buffer pH 7.4 and 1.5 µg of protein was used per assay.


30
20
10
0
$\frac{1}{v}$
[PEP] μM
25
50
100
500
1 000
0
5
10
15
20
$\frac{1}{[E4P]}$ mM^{-1}

plots suggests that the reaction proceeds via a ping-pong bi bi mechanism at low concentrations of PEP and E4P.

In Salmonella typhimurium, using ^{18}O-PEP (labelled at the enol oxygen), DeLeo and Sprinson (1968) showed that the Pi released during the DAHP synthase reaction results from cleavage of the C-O bond of the ester linkage of PEP. Therefore, at this stage of the discussion of the Neurospora enzyme, it is assumed that PEP adds first followed by the release of Pi before the addition of E4P.

In the absence of inhibition and activation, the rate equation is represented by

$$v = \frac{V}{1 + \frac{K_{(PEP)}}{[\bar{P}EP]} + \frac{K_{(E4P)}}{[\bar{E}4P]}} \qquad (5.1)$$

The reciprocal form of equation 5.1 is

$$\frac{1}{v} = \frac{1}{V} + \frac{K_{(PEP)}}{V\,[\bar{P}EP]} + \frac{K_{(E4P)}}{V\,[\bar{E}4P]} \qquad (5.2)$$

In the presence of substrate inhibition by E4P, and assuming it acts as a dead-end inhibitor by binding on to the enzyme, the rate equation is represented by (see Appendix for derivation of rate equation)

$$\frac{1}{v} = \frac{1}{V} + \frac{K_{(PEP)}}{V\,[\bar{P}EP]} + \frac{K_{(E4P)}}{V\,[\bar{E}4P]} + \frac{K_{(PEP)}\,[\bar{E}4P]}{V\,[\bar{P}EP]\;K_{i(E4P)}} \qquad (5.3)$$

where $K_{i(E4P)}$=dissociation constant for the binding of E4P which results in inhibition.

Further tests to confirm a ping-pong mechanism

From these equations (5.2 and 5.3), if PEP and E4P are varied together at a fixed proportion, a plot of either 1/v

versus 1/PEP or 1/v versus 1/E4P will be linear. In the absence of substrate inhibition, the Y-intercept will always be the same (1/V), independent of the proportion of PEP and E4P. In the presence of substrate inhibition, the Y-intercept will not be equal to 1/V but will vary with different proportion of PEP and E4P.

For example,

if $[PEP] = [E4P]$,

$$Iy = \frac{1}{V} + \frac{K_{(PEP)}}{V\ K_{i\,(E4P)}} \qquad (5.4)$$

if $[PEP] = 2[E4P]$,

$$Iy = \frac{1}{V} + \frac{K_{(PEP)}}{2V\ K_{i\,(E4P)}} \qquad (5.5)$$

and if

$2[PEP] = [E4P]$,

$$Iy = \frac{1}{V} + \frac{2K_{(PEP)}}{V\ K_{i\,(E4P)}} \qquad (5.6)$$

The intercept is the same for a given ratio of PEP to E4P when 1/v is plotted against either 1/PEP or 1/E4P.

In the sequential reaction mechanism, a square term of substrate concentration will be introduced in the rate equation when PEP and E4P are varied at a fixed proportion and the double-reciprocal plot will be a curve, concave upward. Therefore, linearity of the double-reciprocal plot will indicate a ping-pong

bi bi mechanism and variation of the Y-intercept at different PEP to E4P ratio will indicate substrate inhibition.

When the PEP/E4P is fixed at 1, the double-reciprocal plot is linear (Fig. 5.12). When the PEP/E4P is fixed at 0.5, the double-reciprocal plots for both 1/v versus 1/PEP and 1/v versus 1/E4P are linear and intersect at one point on the Y-axis (Fig. 5.13). When the PEP/E4P is fixed at 2, the two double-reciprocal plots are again linear and intersect at one point on the Y-axis (Fig. 5.14). The intercepts for the three ratios of PEP and E4P are different showing deviation from a basic ping-pong bi bi reaction in the manner expected for the assumed pattern of substrate inhibition.

It was intended to test the type of inhibition by E4P with respect to PEP. However, increasing the concentration of E4P results in interference with the enzyme assay presumably because the -CHO group of E4P reduces the periodate that is added to develop the chromagen. The inhibition by E4P is therefore not tested. However, it will be interesting to test if E4P inhibition is competitive with respect to PEP.

(c) Product Inhibition

The two reaction products are Pi and DAHP. Since the assay measures the amount of DAHP produced, the effect of inhibition by DAHP is not tested. It should, however, be done by assaying for Pi if an adequate supply of DAHP became available.

At a concentration of 0.5 mM for both PEP and E4P, the plot of v versus Pi (Fig. 5.15) is hyperbolic. Inhibition at 0.8 mM Pi is negligible. The plot of percent inhibition versus Pi shows a maximum inhibition of about 60% with half maximal inhibition corresponding to a Pi concentration of

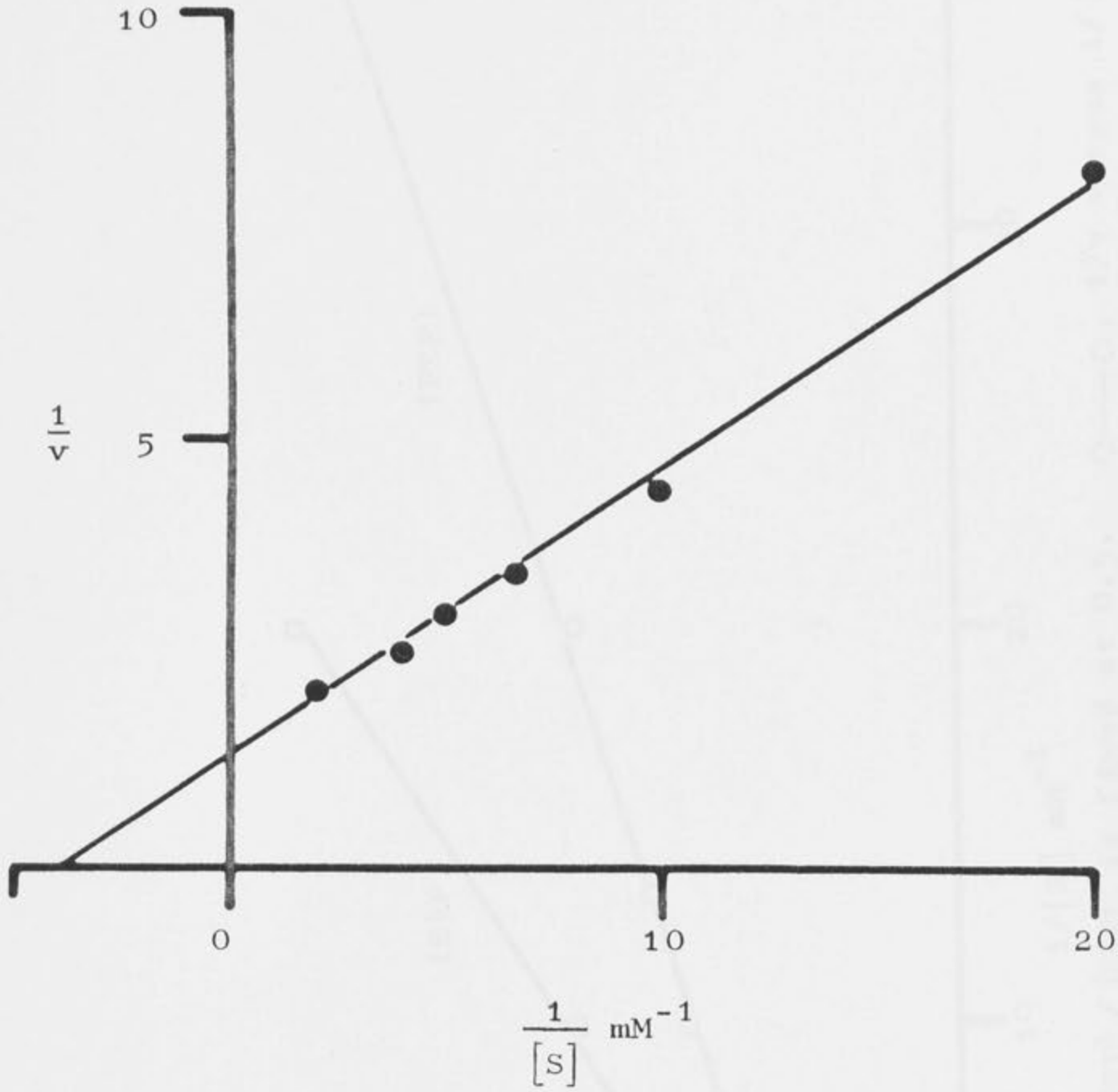


Fig. 5.12 Double-reciprocal plots when $[PEP]/[E4P]$ is fixed at 1.

Assays were done in 0.02M Tris-maleate buffer pH 6.4 and 1 µg of protein was used per assay.

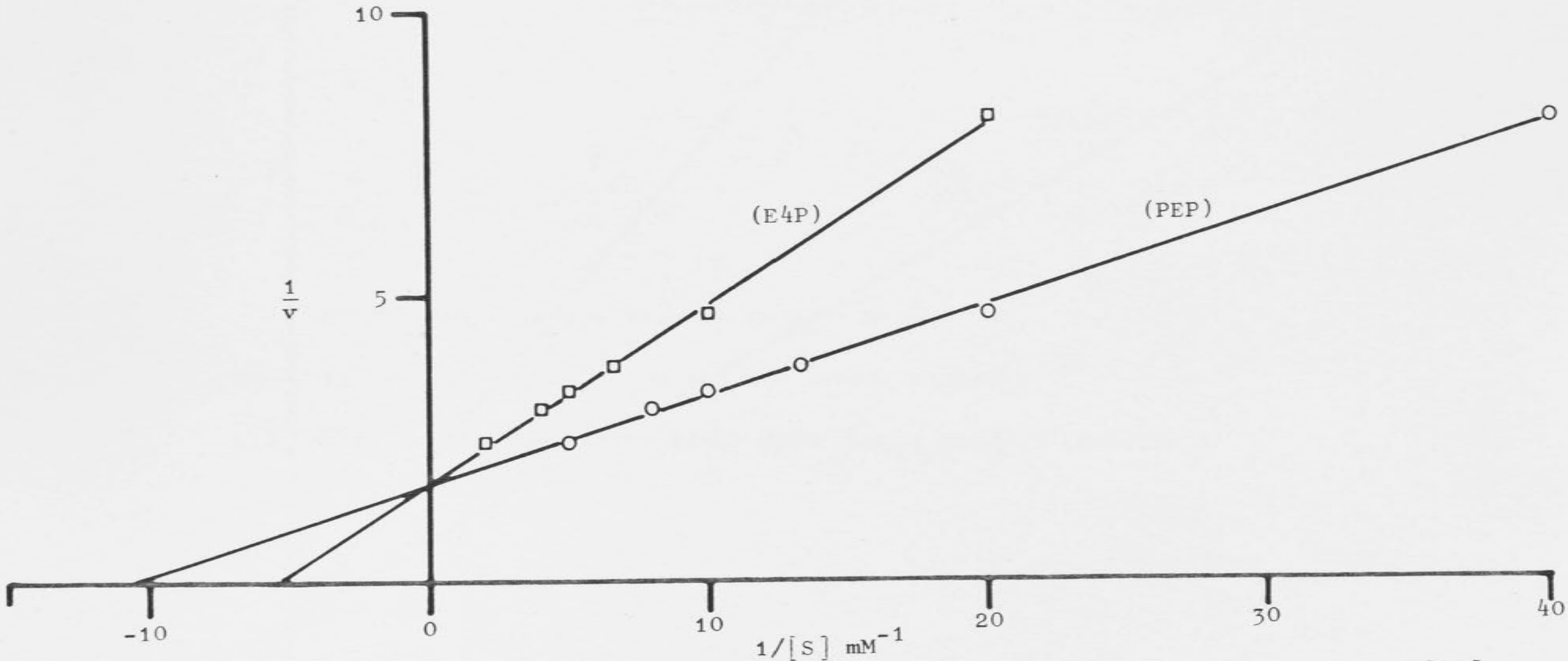
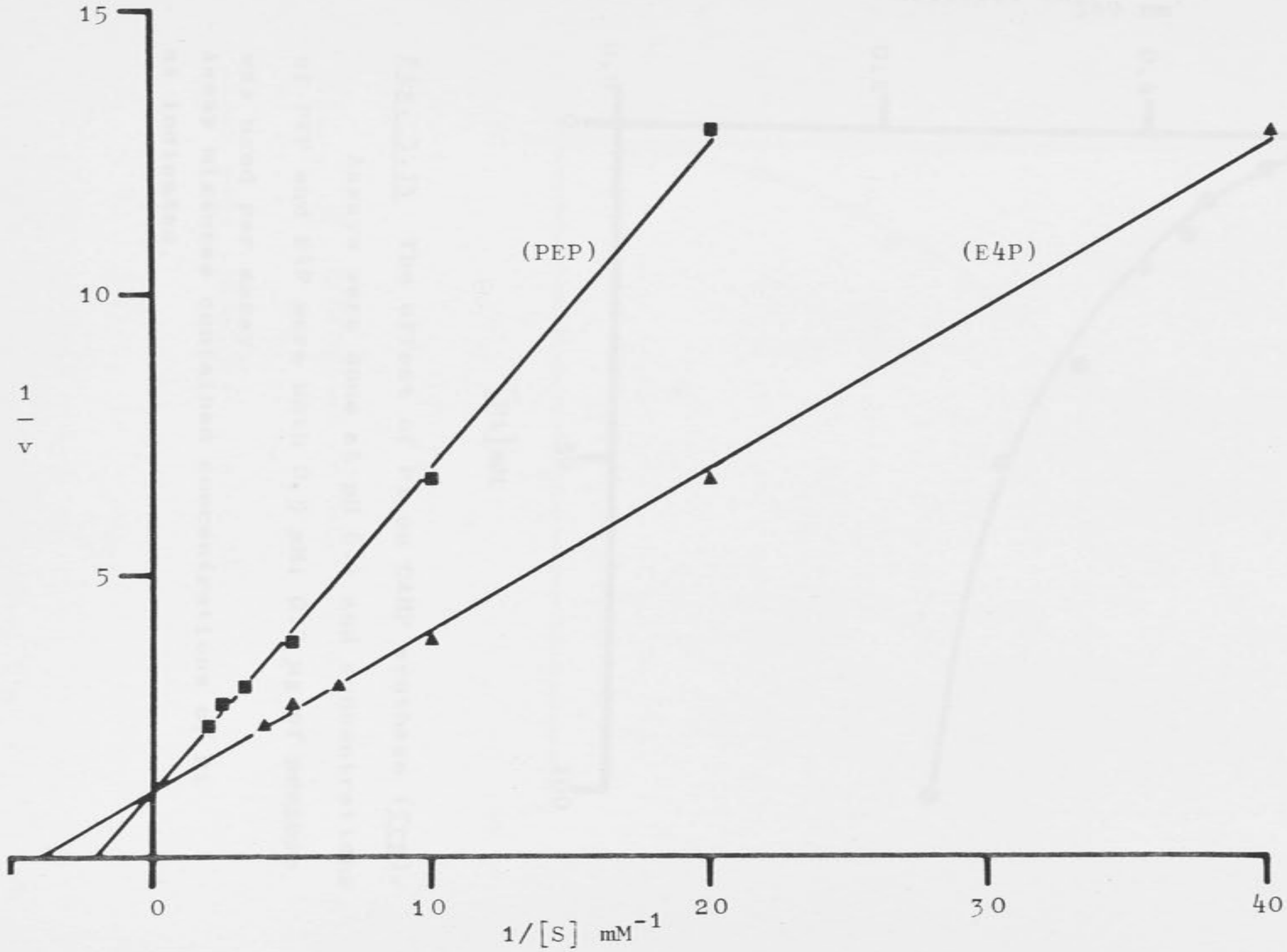


Fig. 5.13 Double-reciprocal plots when [PEP] / [E4P] is fixed at 0.5. O——O, 1/v versus 1/[PEP]; □——□, 1/v versus 1/[E4P]. Conditions were the same as in Fig. 5.12.

Fig. 5.14 Double-reciprocal plots when [PEP]/[E4P] is fixed at 2.

■—■, 1/v versus 1/[PEP] ; ▲—▲, 1/v versus 1/[E4P].

Conditions were the same as in Fig. 5.12.


15
10
5
1
—
v
(PEP)
(E4P)
0
10
20
30
40
1/[S] mM^{-1}

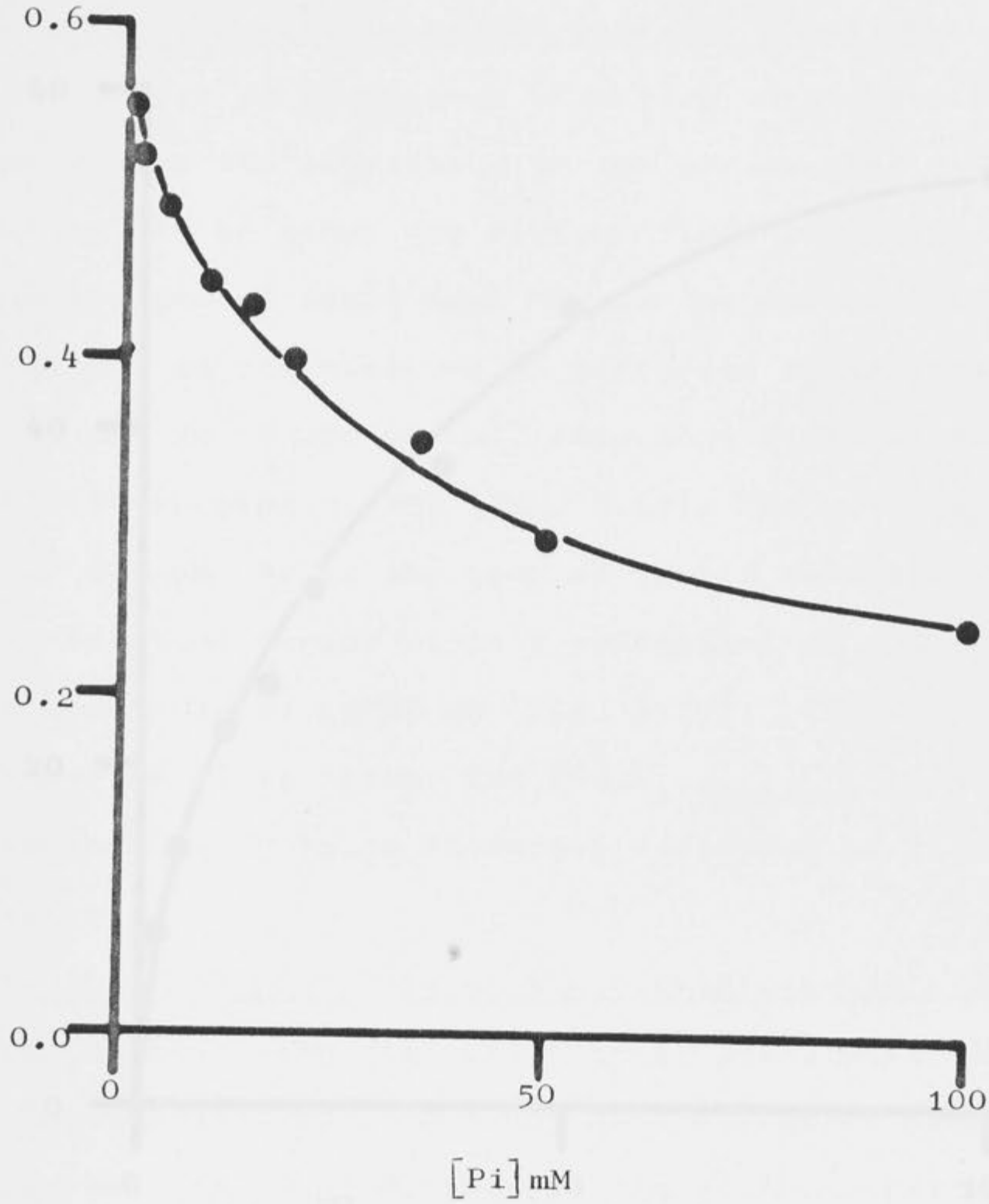


Fig. 5.15 The effect of Pi on DAHP synthase (Trp).

Assays were done at pH 6.4 and concentrations of PEP and E4P were both 0.5 mM; 0.7 μg of protein was used per assay.
Assay mixtures contained concentrations of Pi as indicated.

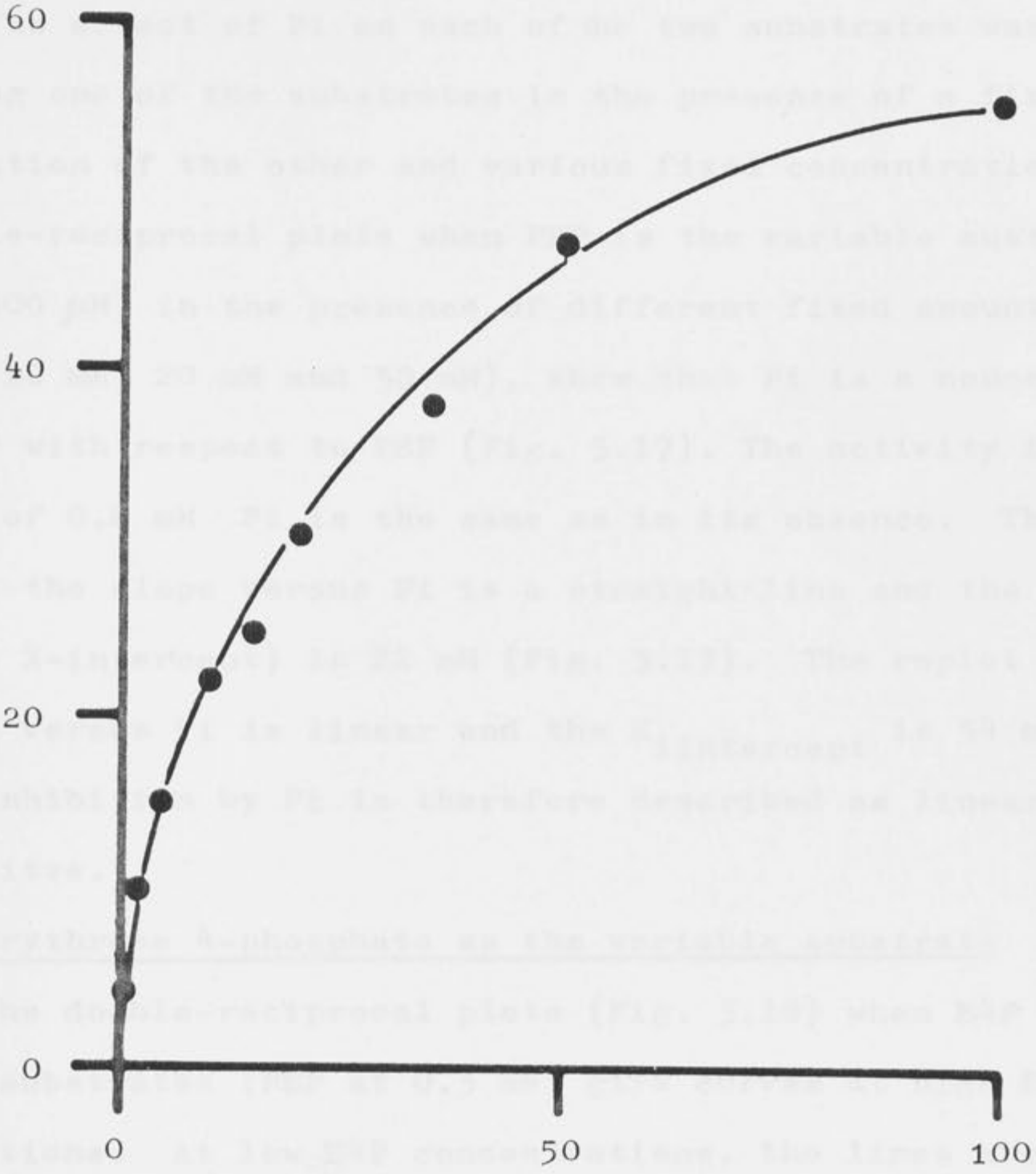


Fig. 5.16 Inhibition of DAHP synthase (Trp) by Pi.

Data were taken from Fig. 5.15.

21 mM (Fig. 5.16).

Phosphoenolpyruvate as the variable substrate

The effect of Pi on each of the two substrates was tested by varying one of the substrates in the presence of a fixed concentration of the other and various fixed concentrations of Pi. The double-reciprocal plots when PEP is the variable sustrate (E4P at 500 μM) in the presence of different fixed amount of Pi (0.8 mM, 10 mM, 20 mM and 50 mM), show that Pi is a noncompetitive inhibitor with respect to PEP (Fig. 5.17). The activity in the presence of 0.8 mM Pi is the same as in its absence. The replot of the slope versus Pi is a straight line and the K_{islope} (from the X-intercept) is 22 mM (Fig. 5.17). The replot of intercept versus Pi is linear and the $K_{iintercept}$ is 54 mM (Fig. 5.17). Inhibition by Pi is therefore described as linear noncompetitve.

Erythrose 4-phosphate as the variable substrate

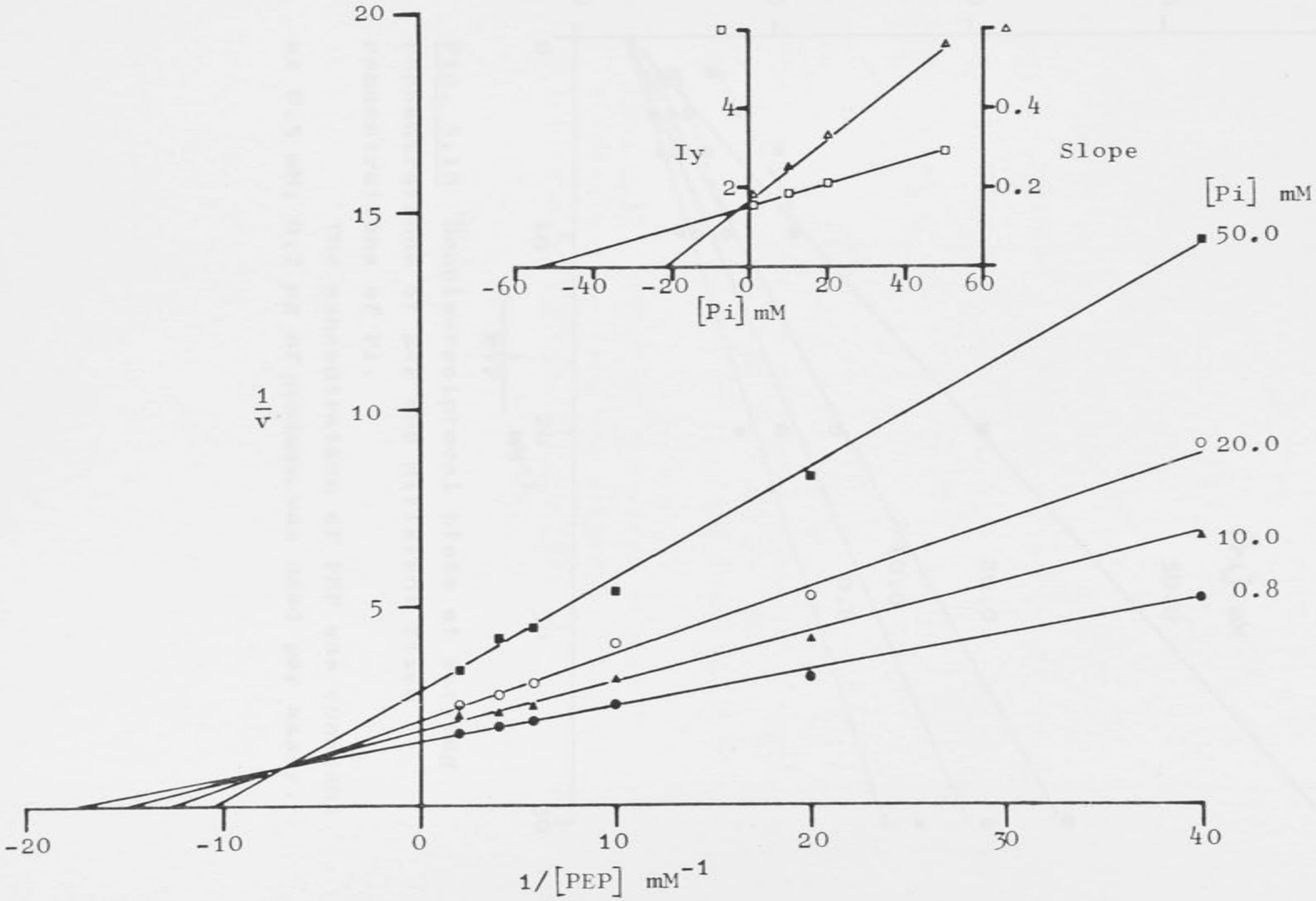
The double-reciprocal plots (Fig. 5.18) when E4P is the variable substrates (PEP at 0.5 mM) give curves at high E4P concentrations. At low E4P concentrations, the lines are straight and intersect on the ordinate indicating competitive inhibition, The replot (Fig. 5.19) of the slope versus phosphate concentration is linear (K_{islope} 25mM) indicating overlapping sites for the binding of Pi and E4P. Since Pi and E4P are sterically different, it is unlikely that they will occupy the same site. The reaction is therefore a two sites ping-pong instead of a one site ping-pong,

The fact that Pi is a noncompetitive inhibitor with respect to PEP is consistent with the ping-pong bi bi mechanism in which PEP adds first and Pi is released before the addition of E4P. In the notation of Cleland (1963) the reaction sequence can be represented by

Fig. 5.17 Double-reciprocal plots at varying concentrations of PEP and different fixed concentrations of Pi.

The concentration of E4P was constant at 0.5 mM; 0.7 µg of protein was used per assay.

"Insert": Secondary plot of slopes and y-intercepts in the primary plot versus the Pi concentration, for the graphical determination of K_{islope} and $K_{iintercept}$.


20
15
10
5
1
v
-20
-10
0
10
20
30
40
1/[PEP] mM^{-1}
[Pi] mM
50.0
20.0
10.0
0.8
Iy
Slope
4
2
0.4
0.2
-60
-40
-20
0
20
40
60
[Pi] mM

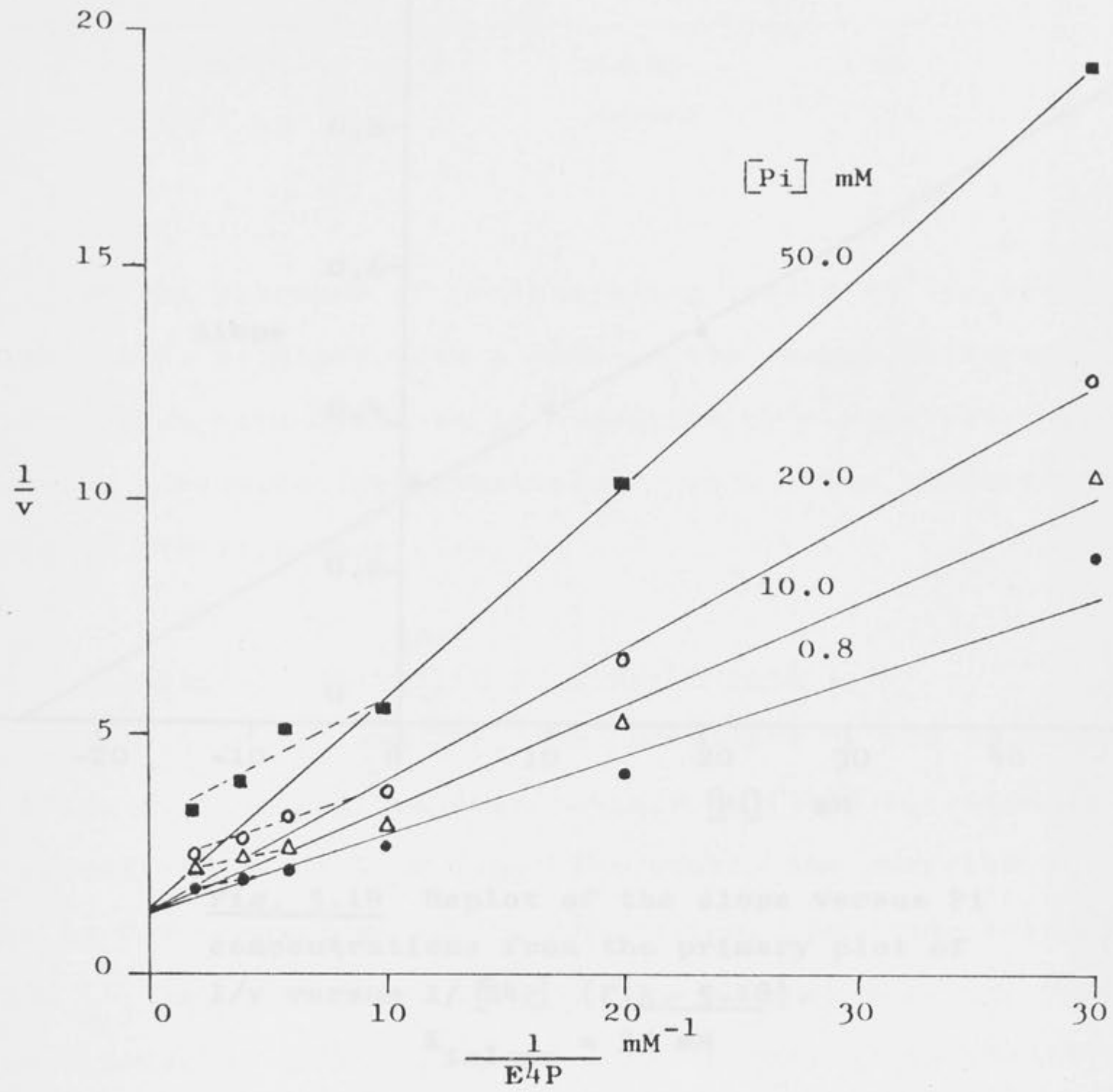


Fig. 5.18 Double-reciprocal plots at varying concentrations of E4P and different fixed concentrations of Pi.

The concentration of PEP was constant at 0.5 mM; 0.7 μg of protein was used per assay.

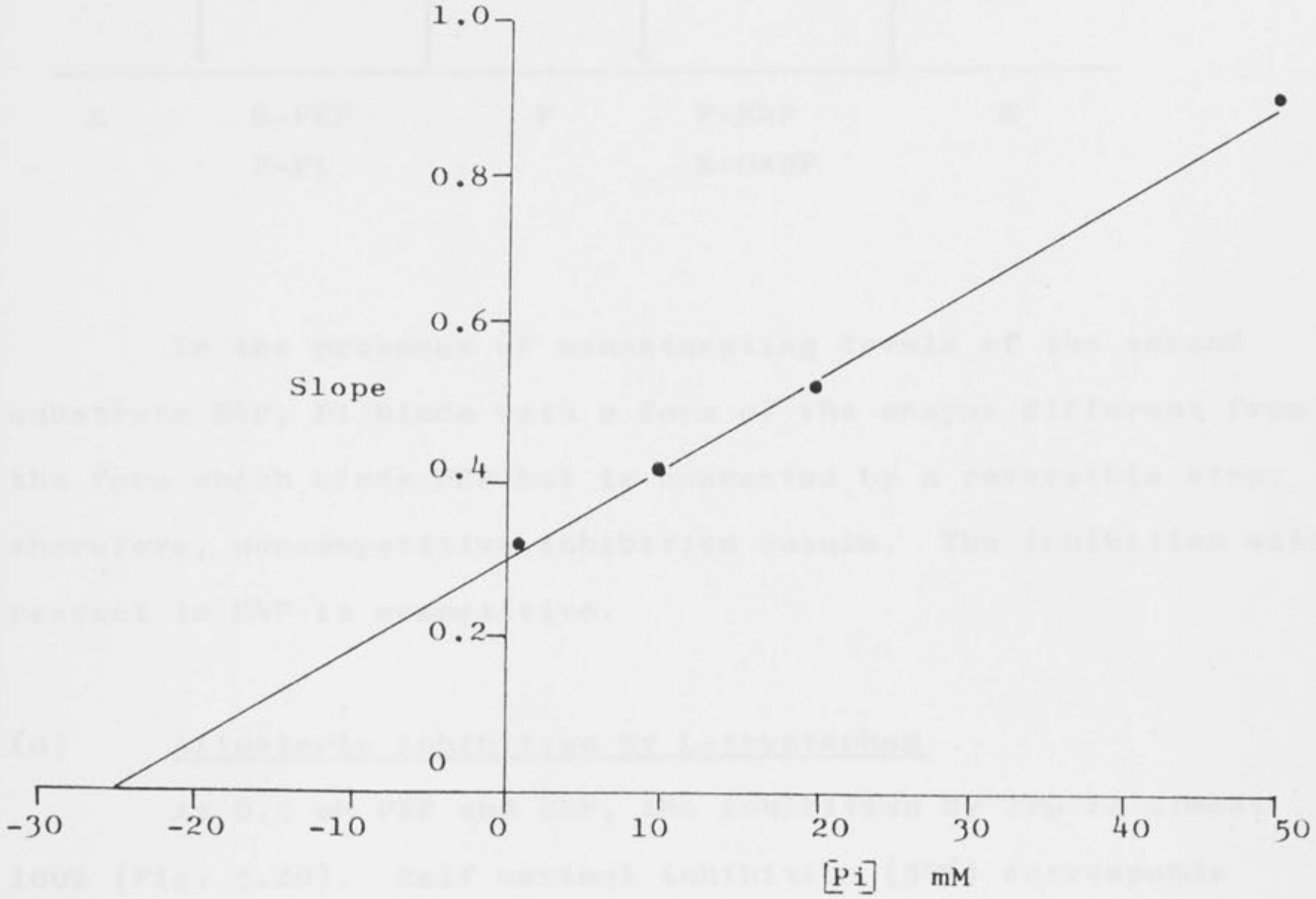
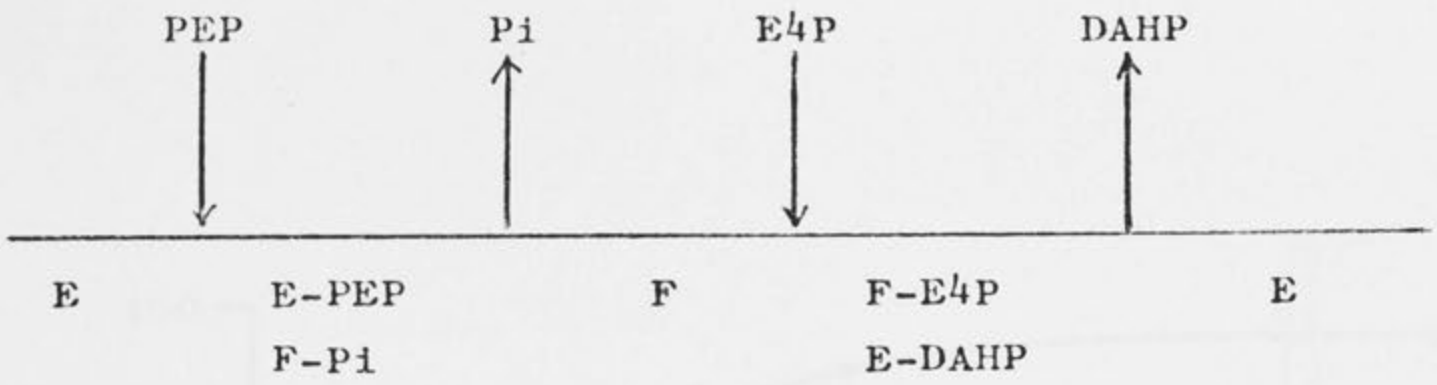


Fig. 5.19 Replot of the slope versus Pi concentrations from the primary plot of 1/v versus 1/ [E4P] (Fig. 5.18).

K_{islope} = 25 mM



In the presence of nonsaturating levels of the second substrate E4P, Pi binds with a form of the enzyme different from the form which binds PEP but is connected by a reversible step, therefore, noncompetitive inhibition results. The inhibition with respect to E4P is competitive.

(d) Allosteric inhibition by L-tryptophan

At 0.5 mM PEP and E4P, the inhibition by Trp is almost 100% (Fig. 5.20). Half maximal inhibition (50%) corresponds to a concentration of 4 μM Trp. Therefore, the purified enzyme is very sensitive to Trp inhibition. In crude extracts, the $Trp_{0.5}$ is 10 μM (Doy, 1968a). The plot of velocity versus Trp is hyperbolic (Fig. 5.21) but sigmoidicity is difficult to detect in this kind of plot.

Erythrose 4-phosphate as the variable substrate

The double-reciprocal plots with different fixed concentrations of Trp when E4P is the variable substrate (PEP at 750 μM) curve at high E4P concentrations at 0, 2 and 4 μM fixed concentrations of Trp (Fig. 5.22). At 6 μM Trp, the plot is linear. Extrapolation of the linear portions intersect on the Y-axis suggesting competitive inhibition by Trp. The curved portions also appear to intersect on the Y-axis suggesting

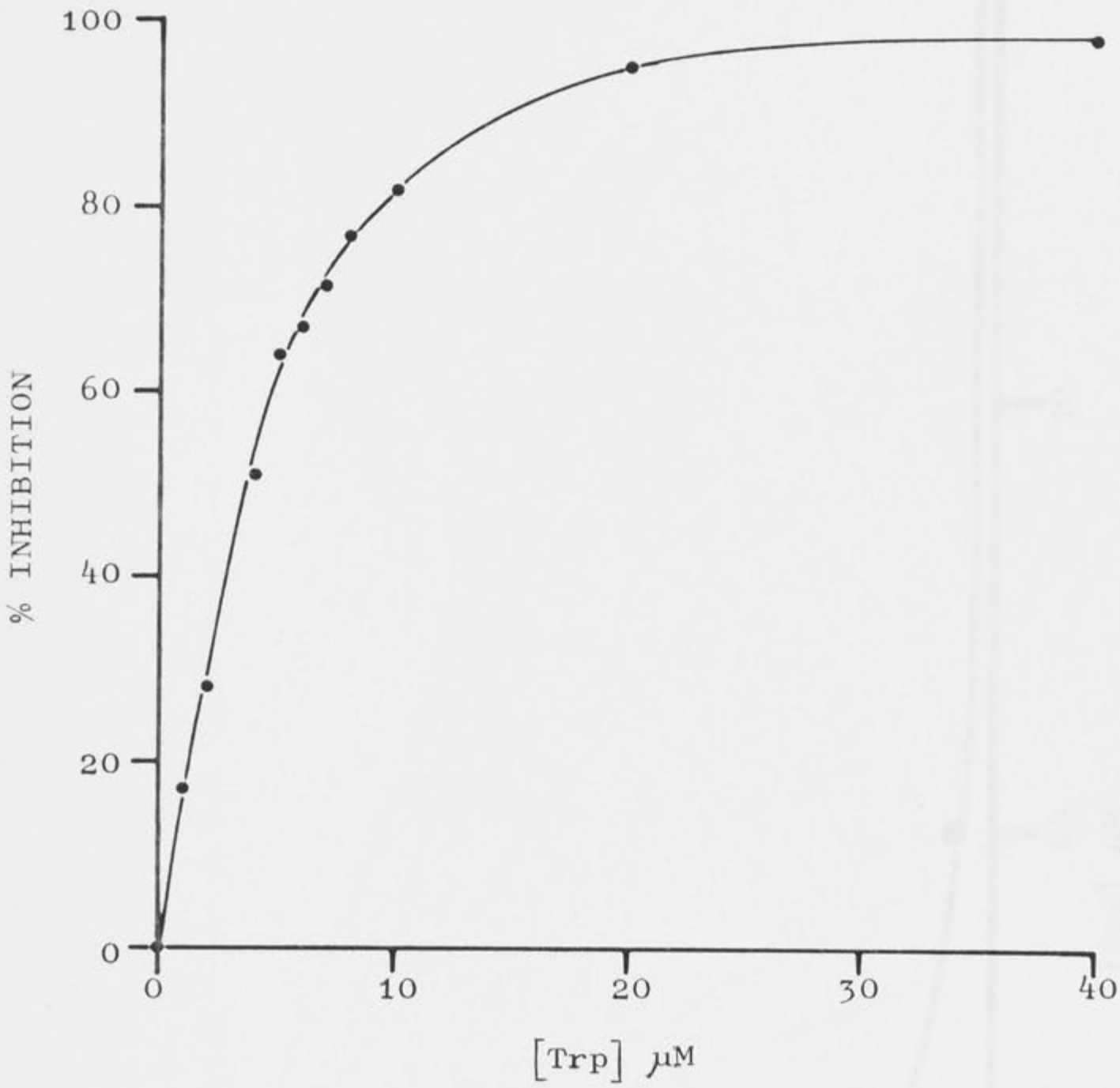


Fig. 5.20 The inhibition of DAHP synthase (Trp) by Trp.

Reaction mixtures contained 0.5 mM each of PEP and E4P, and inhibitor as indicated; 0.5 μg of protein was used per assay.

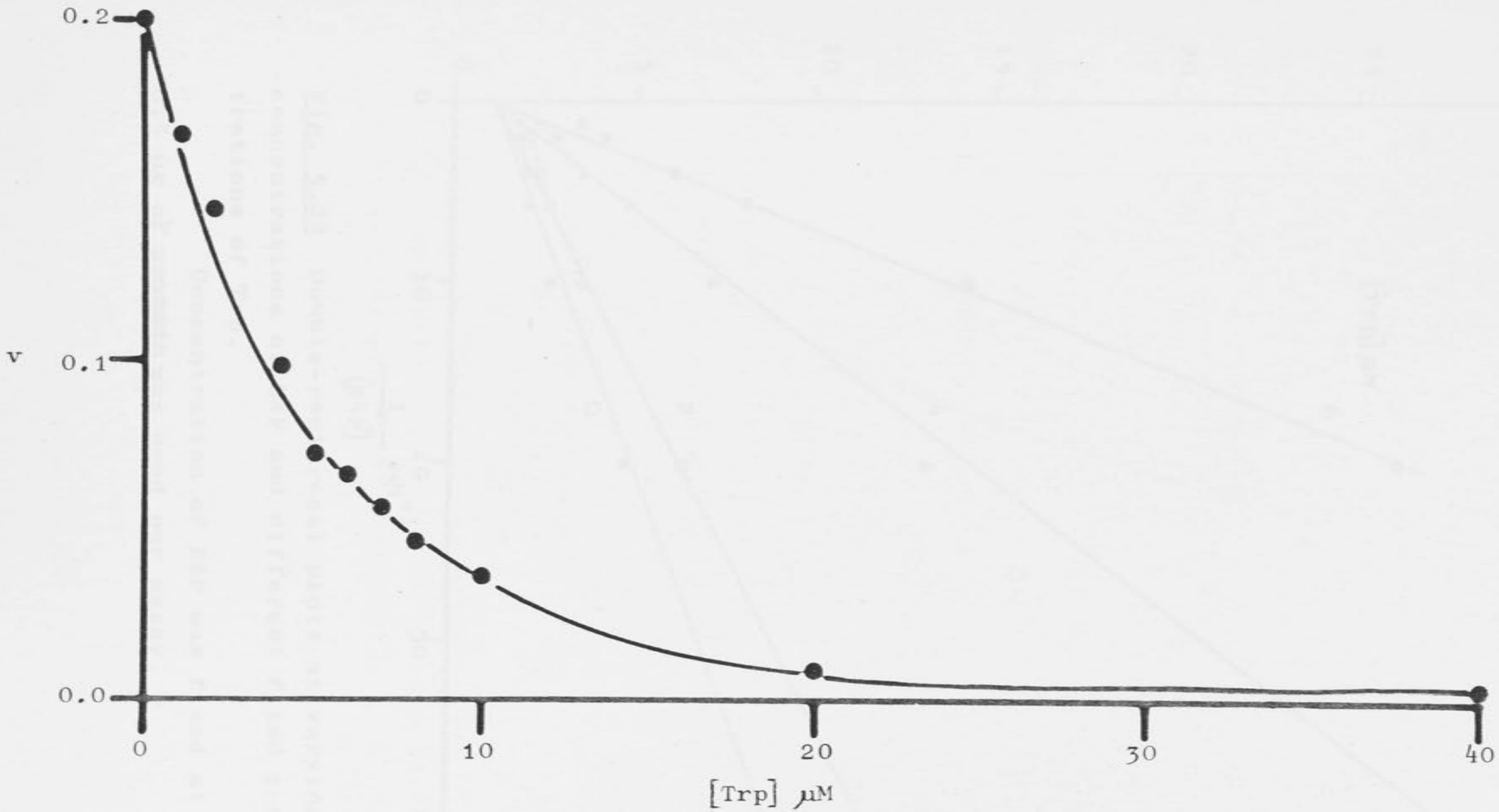


Fig. 5.21 The effect of Trp on DAHP synthase (Trp). Data are the same as in Fig. 5.20.

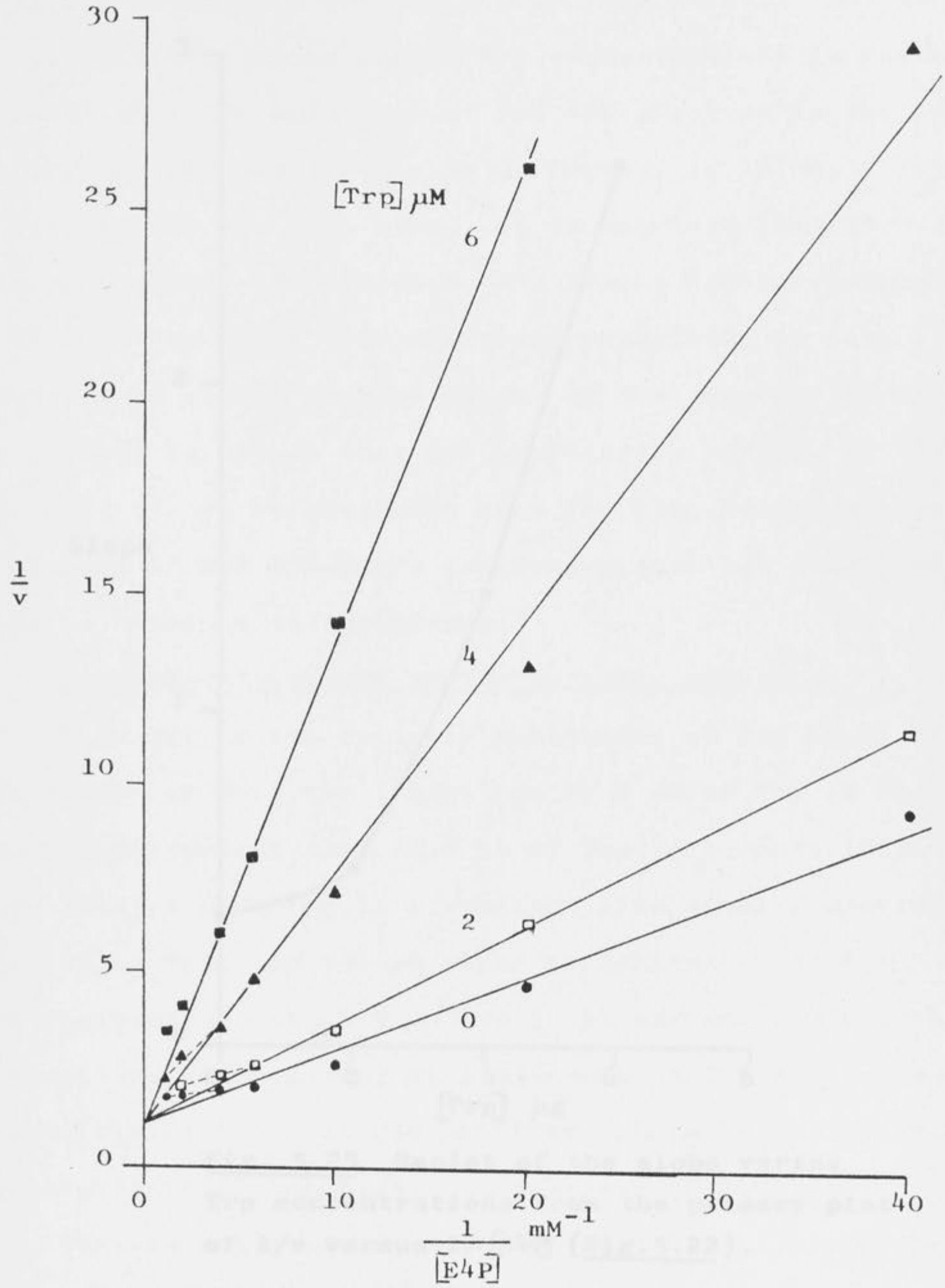


Fig. 5.22 Double-reciprocal plots at varying concentrations of E4P and different fixed concentrations of Trp.

Concentration of PEP was fixed at 0.75 mM; 0.5 μg of protein was used per assay.

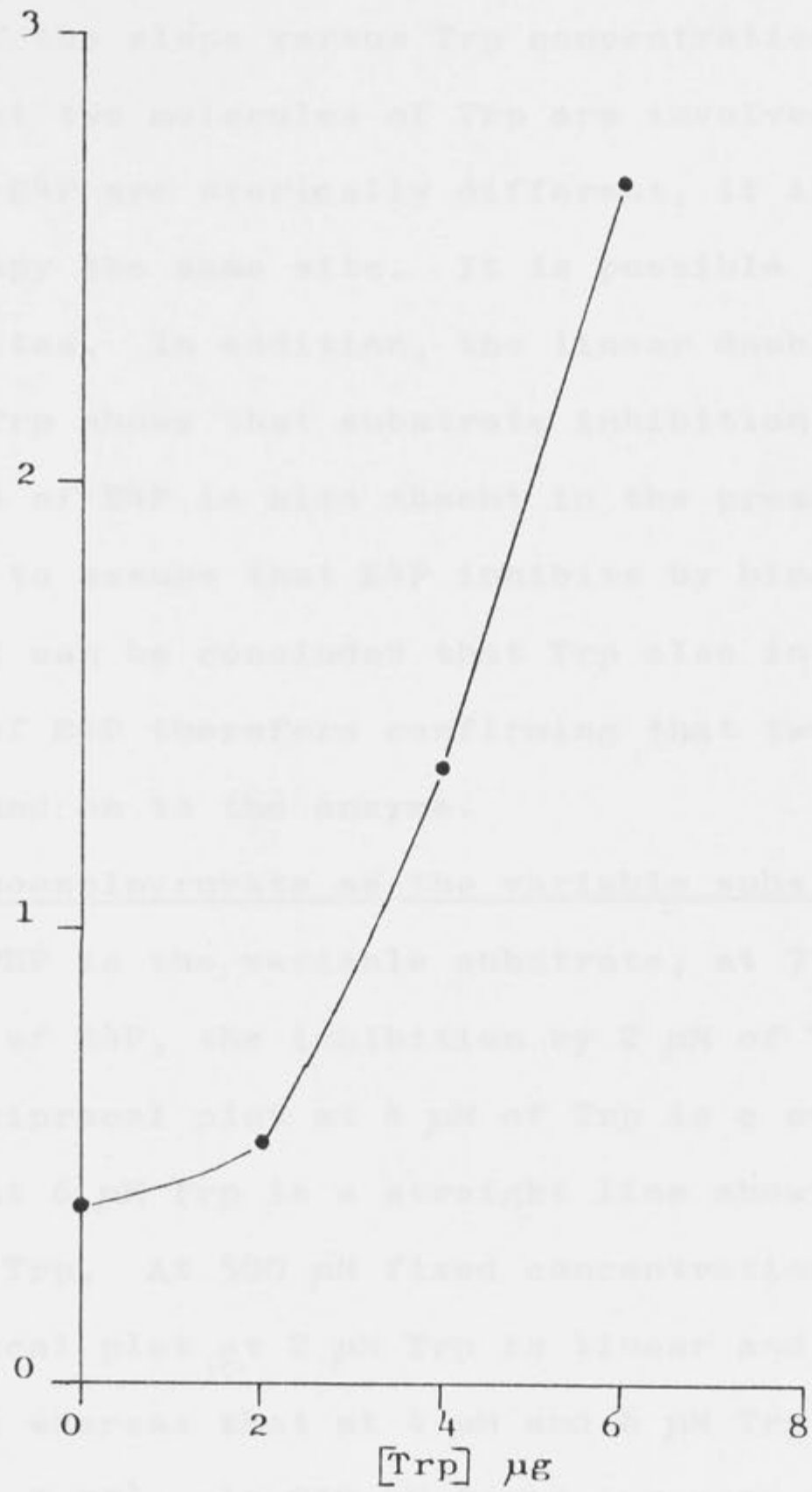


Fig. 5.23 Replot of the slope versus Trp concentrations from the primary plot of 1/v versus 1/ [E4P] (Fig.5.22).

competitive inhibition at high E4P concentrations. Replot (Fig. 5.23) of the slope versus Trp concentrations is parabolic indicating that two molecules of Trp are involved in the inhibition. Since Trp and E4P are sterically different, it is unlikely that they will occupy the same site. It is possible that they occupy overlapping sites. In addition, the linear double-reciprocal plot at 6 μM Trp shows that substrate inhibition by high concentrations of E4P is also absent in the presence of Trp. If it is correct to assume that E4P inhibits by binding to the enzyme before PEP, it can be concluded that Trp also interferes with this binding of E4P therefore confirming that two molecules of Trp may be bound on to the enzyme.

Phosphoenolpyruvate as the variable substrate

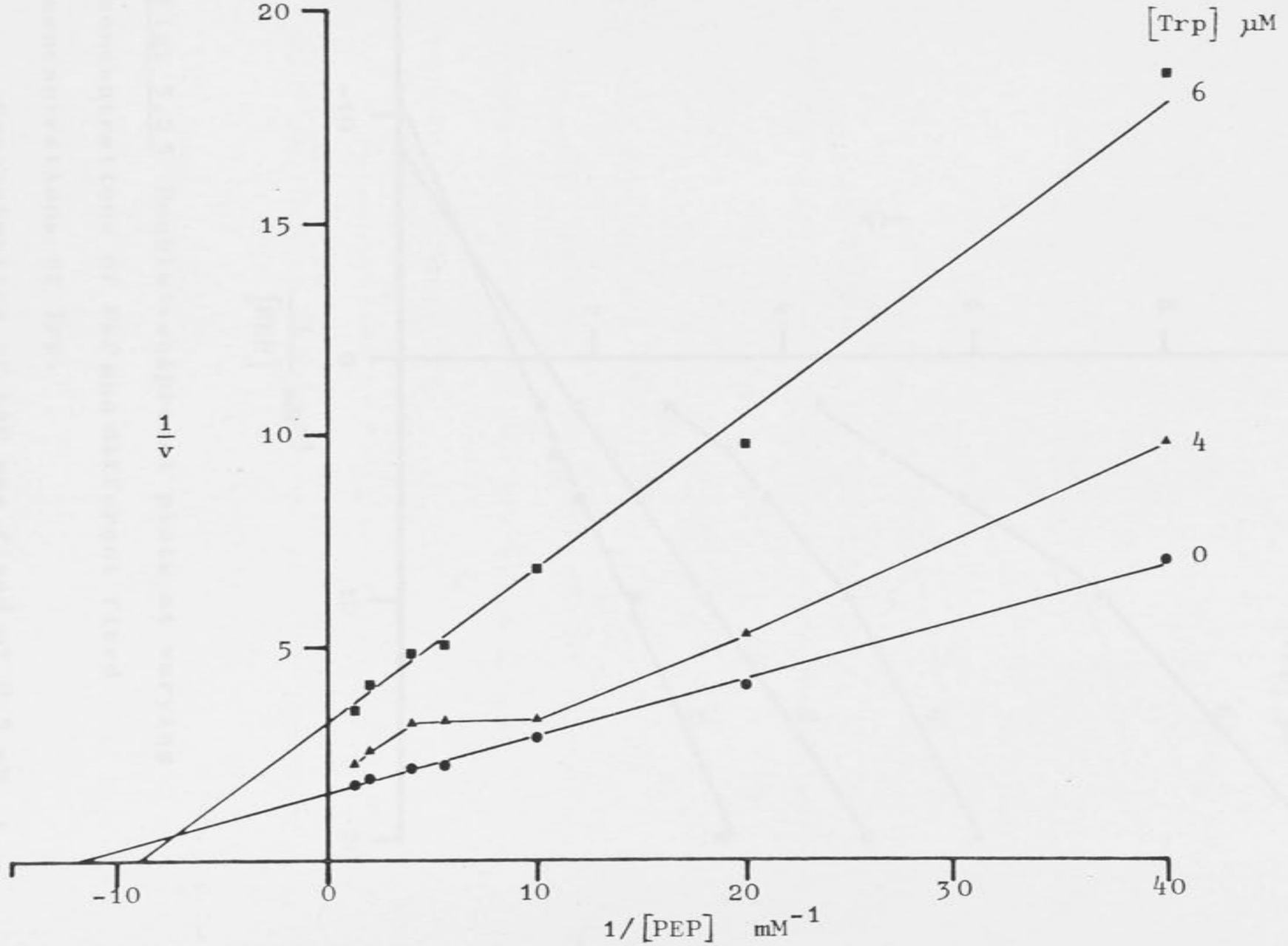
When PEP is the variable substrate, at 750 μM fixed concentration of E4P, the inhibition by 2 μM of Trp is negligible. The double-reciprocal plot at 4 μM of Trp is a curve (Fig. 5.24) whereas that at 6 μM Trp is a straight line showing noncompetitive inhibition by Trp. At 500 μM fixed concentration of E4P, the double-reciprocal plot at 2 μM Trp is linear and inhibition is noncompetitive whereas that at 4 μM and 6 μM Trp are curved downward (Fig. 5.25). At 250 μM fixed concentration of E4P the same pattern of inhibition is observed (Fig. 5.26).

Therefore it can be concluded that inhibition by Trp is noncompetitive with respect to PEP.

Since Trp is a competitive inhibitor of E4P (the second substrate to add in the reaction sequence) Trp must bind after the first substrate, PEP. Tryptophan, not being part of the reaction sequence, cannot reverse the reaction sequence, therefore

Fig. 5.24 Double-reciprocal plots at varying concentrations of PEP and different fixed concentrations of Trp.

Concentration of E4P was fixed at 0.75 mM; 0.5 µg of protein was used per assay.


20
15
10
5
1/v
[Trp] μM
6
4
0
-10
0
10
20
30
40
1/[PEP] mM⁻¹

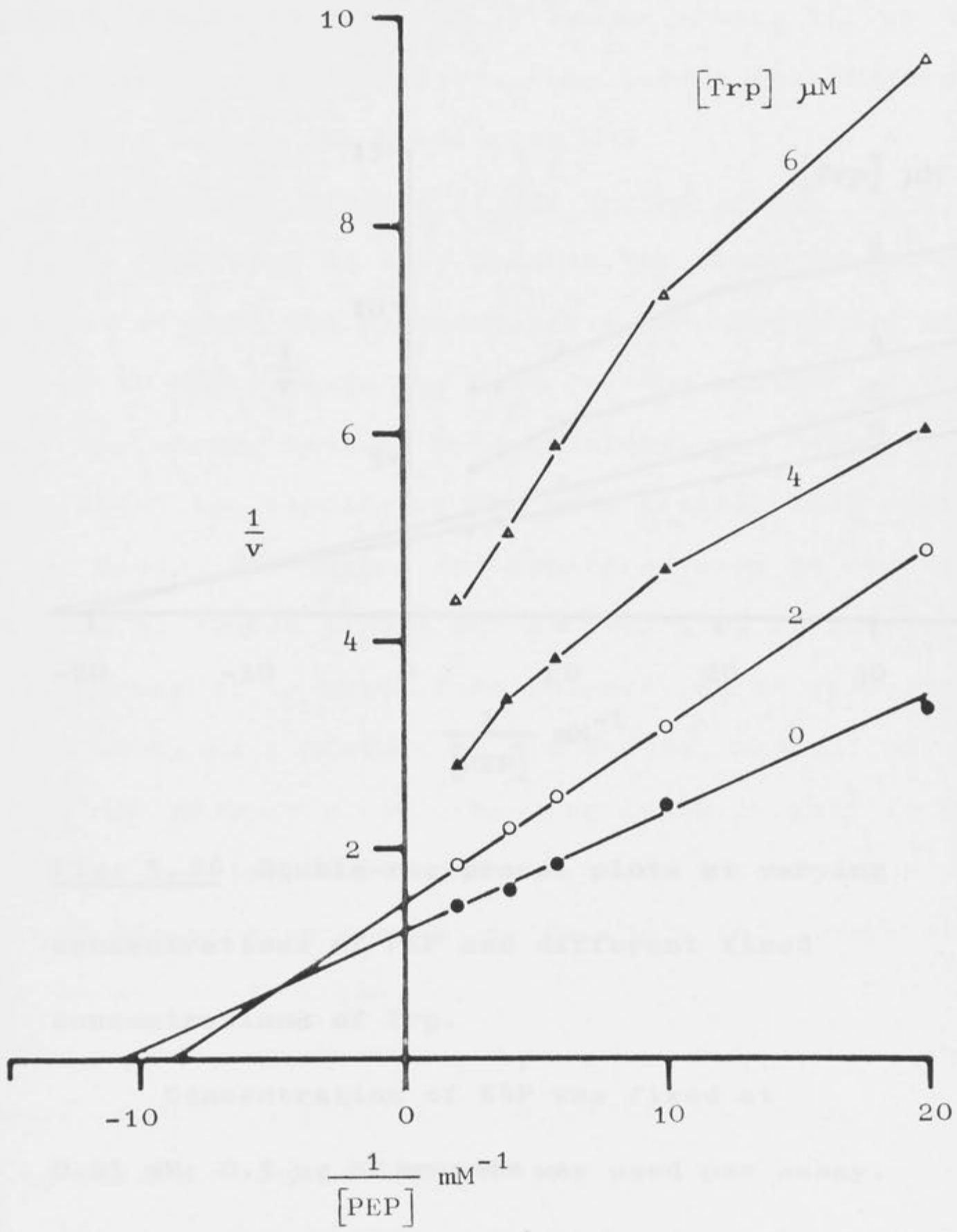


Fig. 5.25 Double-reciprocal plots at varying concentrations of PEP and different fixed concentrations of Trp.

Concentration of E4P was fixed at 0.5 mM ; 0.5 μg of protein was used per assay.

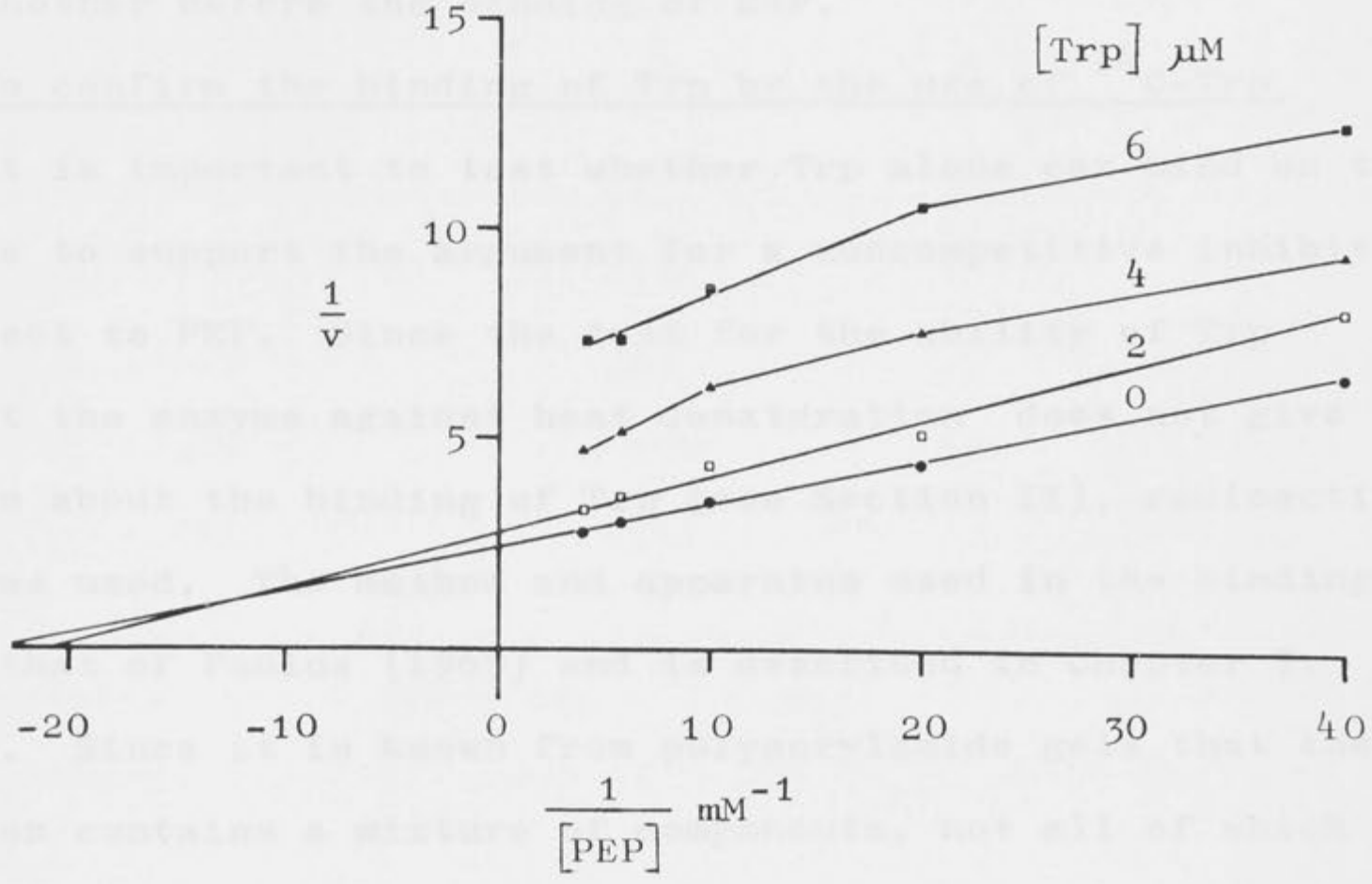


Fig. 5.26 Double-reciprocal plots at varying concentrations of PEP and different fixed concentrations of Trp.

Concentration of E4P was fixed at 0.25 mM; 0.5 μg of protein was used per assay.

if it binds after the substrate (PEP), the inhibition should be uncompetitive with that substrate (PEP). However, noncompetitive inhibition will be observed if two molecules of Trp bind on to the enzyme during inhibition, one before the binding of PEP and another before the binding of E4P.

IV. To confirm the binding of Trp by the use of ^{14}C-Trp

It is important to test whether Trp alone can bind on to the enzyme to support the argument for a noncompetitive inhibition with respect to PEP. Since the test for the ability of Trp to protect the enzyme against heat denaturation does not give a conclusion about the binding of Trp (see Section II), radioactive ^{14}C-Trp was used. The method and apparatus used in the binding study is that of Paulus (1969) and is described in Chapter 7, Section X. Since it is known from polyacrylamide gels that the preparation contains a mixture of components, not all of which are active, the purpose of this binding study is only to test the ability of Trp to bind to the enzyme in the presence of various ligands. No attempts were made to determine the number of Trp bound per molecule of enzyme.

The results are summarised in Table 5.1. Tryptophan, in the absence of PEP and E4P, can bind on to the enzyme and this binding is increased by 64% in the presence of PEP and decreased by 60% in the presence of E4P. In the presence of both PEP and E4P, binding increases by 59% over that in the absence of both substrates.

The decrease in binding of Trp in the presence of E4P suggests that E4P can compete with the binding of Trp in the absence of PEP. This in turn suggests that E4P can bind on to the enzyme in the absence of PEP which is consistent with the

TABLE 5.1

Binding of ^{14}C-Trp to purified DAHP synthase (Trp).

Ligands*	Radioactivity (c.p.m., net)	% Change in binding with respect to control when only ^{14}C-Trp is present
^{14}C-Trp	1731	-
^{14}C-Trp + PEP	2833	+63.7
^{14}C-Trp + E4P	700	-59.5
^{14}C-Trp + PEP + E4P	2749	+58.9

*Concentration of PEP = E4P = 0.2mM.

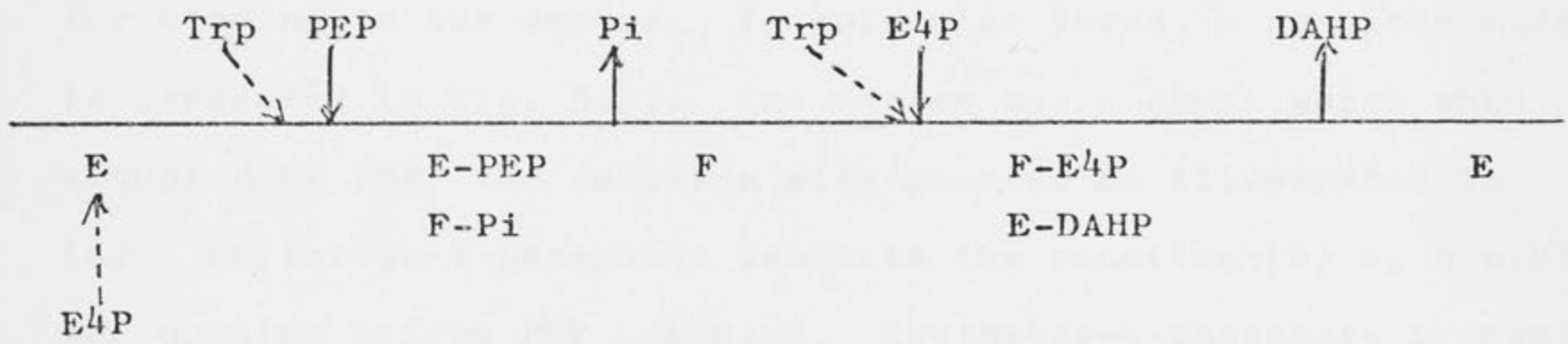
Experiments were performed in 0.02M KH_2PO_4-NaOH buffer pH 6.4.

conclusion drawn in Section II. This binding of E4P may result in the substrate inhibition observed at high E4P concentrations.

This binding study shows that Trp can bind on to the enzyme by itself and that PEP facilitates whereas E4P inhibits the binding of Trp. The results therefore confirm the inhibition characteristics of Trp with respect to the two substrates discussed in Section III (d).

V. DISCUSSION

The DAHP synthase reaction catalysed by the Trp-inhibitable isoenzyme probably proceeds via a ping-pong bi bi mechanism with PEP the first substrate to add. A reaction sequence is proposed as follows,



The sets of parallel lines in the double-reciprocal plots were obtained at low E4P and PEP concentrations, thus avoiding the complication of substrate inhibition by E4P and activation by PEP. The study of the reaction at low concentrations of substrates is made possible by the use of large amount of assay mixture and enzyme and extracting the chromagen into the same volume of cyclohexanone as used normally. The sensitivity of the assay can therefore be increased.

The linear double-reciprocal plots when PEP and E4P were varied together at a fixed ratio are consistent with a basic ping-pong bi bi mechanism. The inhibition patterns by Pi show that PEP is the first substrate to add. Inorganic

phosphate is a competitive inhibitor with respect to E4P. Two molecules of Trp can bind on to the enzyme. Binding of Trp is increased in the presence of PEP and decreased in the presence of E4P. Inhibition by Trp is S-parabolic competitive with respect to E4P and noncompetitive with respect to PEP. It seems unlikely that Trp is metabolised to some other compounds which then inhibit the enzyme. The reason is that the compounds indole, anthranilate, tryptamine and alanine, the likeliest degradation products of tryptophan do not inhibit the enzyme (see Chapter 3, Section IX).

Tryptophan, being an allosteric inhibitor, is sterically different from E4P and yet they can compete with one another for binding to the enzyme. In molecular terms, a possible model is presented in Fig. 5.27. The enzyme has a cleft which when occupied by PEP, the reaction will proceed as illustrated in (a). Erythrose-4-phosphate inhibits the reaction (b) by blocking the opening before PEP can bind. Erythrose-4-phosphate probably binds through the HC=O group on one side and the Pi on the other side of the cleft. Competitive inhibition by Pi with respect to E4P is therefore envisaged as preventing the proper binding of E4P. In the absence of PEP, only one molecule of Trp can be bound on to the opening of the cleft (c). The presence of E4P will therefore compete with Trp for binding on to the enzyme. In the presence of PEP, the opening is widened (d) and two molecules of Trp can now bind on to the enzyme, one on each side of the opening.

The presence kinetic data do not support the symmetrical model (Monod et al., 1965) which is a configurational rather than an interaction model. Whereas negative cooperativity

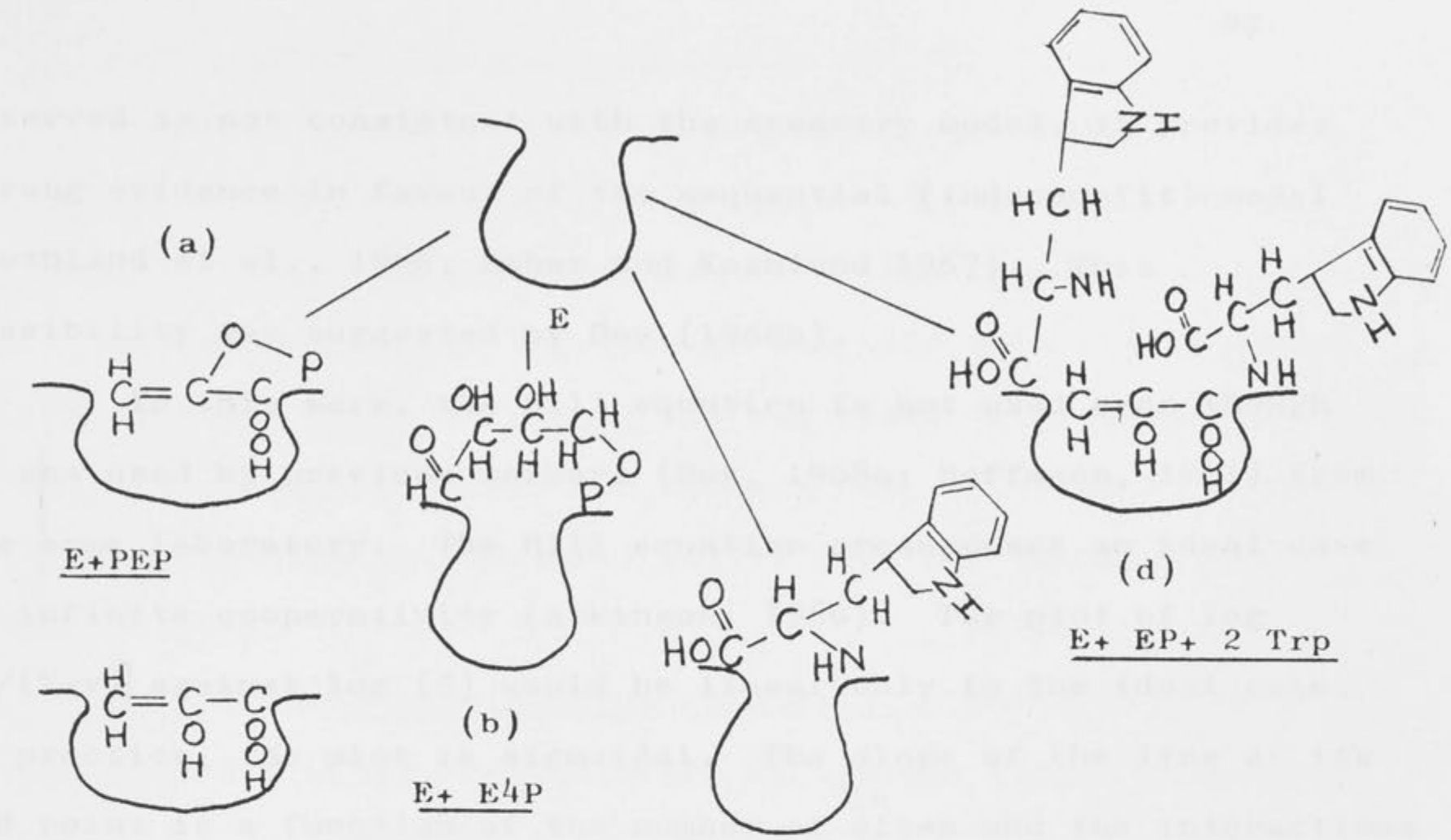


Fig. 5.27 A possible molecular model for

(a) Normal reaction sequence leading to the formation of DAHP.

(b) E4P binds to enzyme resulting in inhibition.

(c) Trp binds to enzyme resulting in inhibition.

(d) Trp binds to enzyme in the presence of enolpyruvate.

Symbols used are : PEP phosphoenolpyruvate; E4P erythrose-4 phosphate; EP enolpyruvate; E enzyme; Trp tryptophan; P phosphate.

Atoms and bond distance are not drawn to scale.

observed is not consistent with the symmetry model, it provides strong evidence in favour of the sequential (induced-fit) model (Koshland et al., 1966; Haber and Koshland 1967). This possibility was suggested by Doy (1968b).

In this work, the Hill equation is not used even though it was used by previous workers (Doy, 1968a; Hoffmann, 1971) from the same laboratory. The Hill equation presupposes an ideal case of infinite cooperativity (Atkinson, 1966). The plot of log $[v/(V-v)]$ against log (S) would be linear only in the ideal case. In practice, the plot is sigmoidal. The slope of the line at its mid point is a function of the number of sites and the interactions between sites. It is considered (Morrison, personal communication) that the use of the Hill equation does not provide any more information than that of the double-reciprocal plot. Cooperativity can be deduced from the shape of the double-reciprocal plot alone without the use of the Hill equation.

VI. MECHANISM OF THE DAHP SYNTHASE REACTION

The DAHP synthase reaction was first postulated by Srinivasan and Sprinson (1959) as a concerted reaction between PEP and E4P leading to the formation of orthophosphate and DAHP. This mechanism was regarded as unlikely when the reaction by DAHP synthase (Phe) in E.coli K12 was found to proceed via a ping-pong mechanism (Staub and Dénes, 1969a,b). The Pi released during the reaction was shown to derive from PEP (DeLeo and Sprinson, 1968).

Except for Rhodomicrobium vanniellii, kinetic results with enzymes from different organisms are consistent with a ping-pong mechanism. In R. vanniellii, the DAHP synthase reaction is claimed to proceed via a sequential mechanism and PEP as the first substrate is the kinetically preferred pathway (Jensen and Trentini, 1970). However, the enzyme exhibits substrate inhibition by E4P which can be overcome by increasing the PEP to E4P ratio. An optimum ratio of 2 PEP to 1 E4P was found. Using those E4P concentrations which were not inhibiting, the plots of 1/v versus 1/E4P at three different fixed concentrations of PEP intersected on the abscissa. The v versus PEP plots at four different fixed concentrations of E4P were sigmoidal (except at the lowest concentration of E4P used (0.02M)).

Substrate inhibition by E4P has also been demonstrated in Brevibacterium flavum (Shiio et al., 1974).

For DAHP synthase (Trp) in E.coli K12 (Camakaris and Pittard, 1974), the plot of 1/v versus 1/E4P with saturating concentration of PEP (1 mM) and 1 mM Co^{2+} was linear. When PEP was the variable substrate, the initial velocity did not vary over the range 0.06 mM to 0.9 mM PEP. The enzyme is only inhibited to a maximum of 56% by Trp. In the presence of 50 μM Trp, the

1/v versus 1/E4P plot was linear and both the slope and the intercept were altered indicating noncompetitive inhibition with respect to E4P. However, the use of 50 μM Trp resulted in 56% inhibition, i.e. maximum inhibition obtainable, throughout the range of E4P concentration tested. The possibility that there may be two forms of the enzyme, one of which is completely inhibitable by Trp and the other is not inhibitable, should be considered. In the absence of Trp, the kinetics observed could be a mixture of two enzymes whereas in the presence of a saturating amount of Trp, the kinetics observed represented that of the noninhibitable enzyme. The results, therefore, do not provide strong evidence for the mechanism of inhibition by Trp. More than one concentration of Trp should have been used, especially in this example where the inhibition is less than 100%.

Also in E.coli K12, the inhibition of activity of the DAHP synthase (Phe) by Phe is noncompetitive for both PEP and E4P (Staub and Dénes, 1969 b).

In N. crassa, evidence from crude extracts supports the ping-pong bi bi mechanism for DAHP synthase isoenzymes (Doy, 1968a). Using a partially purified DAHP synthase (Tyr), Hoffmann (1971) obtained a set of parallel lines in the 1/v versus 1/PEP plots at four different fixed concentrations of E4P. The 1/v versus 1/E4P plots show curvature, concave upward. However, with the purified Tyr-inhibited enzyme, Hoffmann found that PEP was saturating over the substrate concentration range used (50 μM to 400 μM). The 1/v versus 1/E4P plot is linear at 1.0 mM fixed PEP. The inhibition patterns by Pi for this enzyme are consistent with the ping-pong bi bi mechanism in which PEP is the first substrate to add. Inhibition by Tyr is uncompetitive

with respect to E4P.

DAHP synthase (Trp) from N. crassa, therefore, differs from the other DAHP synthases in that inhibition by the end product and allosteric ligand is competitive with respect to E4P.

VII. REGULATORY ASPECTS OF DAHP SYNTHASE (Trp)

DAHP synthases catalyse an essentially irreversible reaction (Jensen and Nester, 1966; Doy, 1968b). This is consistent with the regulatory role of the enzymes in the sense that they catalyse a non-equilibrium reaction whose activity is controlled by factors other than the substrate concentration. That does not mean, however, that the activity of these enzymes cannot be regulated by substrates. The existence of a control point in a biosynthetic pathway allows an organism to adjust to changes in the in vivo metabolic state and in the availability of the end product in the environment. Any system which incorporates control by a specific allosteric effector and at the same time allows a specific response to changes in substrate concentration would have a positive advantage over one which is regulated by a specific regulator alone. An increase in sensitivity to changes in substrates concentrations, either positive or negative, will also compensate for the "desensitisation" of the reaction pathway as a result of the incorporation of end product inhibition. Many allosteric enzymes do not exhibit the classical Michaelis-Menten kinetics and plots of velocity versus substrate concentration are sigmoidal. This behaviour implies that a threshold concentration of substrate exists, below which changes in concentration have relatively little effect on enzyme activity, yet when exceeded, slight changes in concentration have profound effects. Thus over quite a narrow range of substrate concentration, the

activity of the enzyme responds in a very sensitive fashion, a desirable feature in a regulatory enzyme.

Purified DAHP synthase (Trp) is sensitive to substrate inhibition by E4P and activation by PEP (Chap. 5, IIIb). The activity of the enzyme therefore varies with the ratio of PEP to E4P. Phosphoenolpyruvate, a substrate, is also an important high energy compound. A low PEP to E4P ratio would indicate a deficiency in intracellular energy and biosynthetic activity would be depressed whereas a high PEP to E4P ratio would indicate a favourable energy supply and biosynthesis could then be increased. Indeed Gaertner and Cole (1973) reported inhibition of the synthesis of 3-enolpyruvylshikimate 5-phosphate from shikimate by ADP. Inhibition can be eliminated by the addition of an ATP-regenerating system. This is consistent with the idea that the biosynthesis of aromatic amino acids is also controlled by the intracellular energy level.

The purified DAHP synthase (Trp) is very sensitive to inhibition by Trp. The fact that Trp is a competitive inhibitor with respect to E4P implies a double control of DAHP synthase (Trp) activity. If the ratio of PEP to E4P is low, the enzyme is inhibited by E4P whereas if the ratio is high, the enzyme in the presence of Trp will be inhibited by Trp.

CHAPTER 6

CONCLUDING REMARKS AND SUGGESTION FOR FURTHER WORK

Evidence in this work (Chapter 3, Section IV) shows that the Phe- and Trp-inhibited activities are related. DAHP synthase (Tyr) and DAHP synthase (Trp) can be purified whereas DAHP synthase (Phe) is very unstable. The best purified sample of DAHP synthase (Phe) still contained Tyr-inhibitable activity and the polyacrylamide gel shows a similar pattern to the purified DAHP synthase (Tyr) (Hoffmann, 1971). In my work, DAHP synthase (Phe) activity remains stable up to the DEAE-cellulose step in the absence of PEP and Phe. Further purification leads to instability and the activity remaining becomes entirely Trp-sensitive. It is possible that, for stability, DAHP synthase (Phe) activity requires to be associated with the other activities. My present view is that aro-7, the genetic locus for DAHP synthase (Phe) activity, was the first of the DAHP synthase loci to evolve and later gave rise to aro-6 and aro-8. This view is consistent with the finding that DAHP synthase (Phe) may be related to both DAHP synthase (Tyr) and DAHP synthase (Trp).

For further analysis, the aro-6 and aro-7 gene products will have to be identified. Immunological methods can be used utilising the isolated 59 000 and the 48 000 components as the antigens. Identification of the N- and C-terminal amino acids of the two purified isoenzymes should be informative. The peptide maps for the two purified isoenzymes will enable us to arrive at some conclusion about the structural relationship of the purified products. The ultimate objective would be to isolate the individual gene products of aro-6, aro-7 and aro-8 and obtain the amino acid sequence for a direct comparison.

To investigate the in vivo organisation of the aromatic

biosynthetic system, the properties of DAHP synthases from various mutants in the aro loci should be studied. The potential of the technique of affinity chromatography has to be explored further. It would be interesting to know if an affinity column designed to bind DAHP synthase or the aro multi-enzyme aggregate or even chorismate synthase will also bind any or all of the activities in the common aromatic pathway. The aro multi-enzyme complex coded for by the aro gene cluster can be disrupted giving rise to fragments with various combination of the five activities. DAHP synthase activity has not been detected in the aro multi-enzyme aggregate neither have the five activities in the aro multi-enzyme complex been detected in the purified DAHP synthase (Tyr) (Hoffmann, 1971). However, the purified product is clearly not the ideal material to look for in vivo organisation. It is, however, useful as a reference material for preparing antibody for immunological study. These antibodies can be coupled to an affinity column for use in the isolation of components that contain the particular polypeptide specific to the antibody.

The properties of the DAHP synthases from various aro-6, aro-7 and aro-8 mutants should be studied. The ideal mutants to look for would be mutants deleted for the various gene loci concerned with aromatic biosynthesis. If the genetic information is absent, there will be no question of its product being present and if the product is part of a structural aggregate the change can be detected in vitro. This is something which cannot be assumed to be true for a point mutant. Unfortunately, in Neurospora crassa, definite deletion mutants have yet to be confirmed. One possible alternative would be to use polarity mutants in which the gene product is drastically altered or even absent due to a nonsense mutation resulting in chain termination.

In addition or as an alternative, it might be possible to use an Escherichia coli strain which has the structural genes for the three DAHP synthases (Gibson and Pittard, 1968) deleted and transfer to it the N. crassa aro-6, aro-7 and aro-8 gene loci individually and in various combination. To do that, one can use the EcoRI restriction endonuclease (Hedgepeth et al., 1972) to generate DNA fragment having cohesive termini from the N. crassa chromosomes and then link these fragments to plasmids which can then be transferred to E.coli. There is evidence that eukaryotic DNA can replicate and transcribe in E.coli (Morrow et al., 1974). Provided translation can occur and the enzymes formed by individual products of aro-6, aro-7 and aro-8 are active, one can select for fragments that contain aro-6, aro-7 or aro-8. The properties and structures of the enzymes formed by the individual gene products can then be studied.

Another possible approach for the analysis of structural organisation is to treat the mycelia with compounds that can link together proteins which are located next to one another inside the cell. This will increase the chance of co-purification of these enzymes. The technique has been used successfully in the study of ribosomal proteins (Sommer and Traut, 1974) and should be applicable to the study of enzyme structure.

CHAPTER 7

MATERIALS AND METHODS

I. CULTURE METHODS

(i) Organism

St. Lawrence wild-type strain 74A *Neurospora crassa*, originally provided by N. H. Giles, was obtained from D. M. Halsall and P. J. Hoffmann. All strains were preserved on silica gel in order to provide a standard source for the cultures used in each experiment.

(ii) Preparation of slope cultures

Vogel's N. medium (Vogel, 1956) was used in culture media with additional supplements as required. Agar slopes were prepared in 75 x 10 mm test tubes containing 2.0 ml of Vogel's N. medium with 2% sucrose as carbon source and solidified with 1.5% Oxoid Ionagar. The medium was autoclaved for 20 min. The slopes were incubated at 30° C for 24 hours after inoculation and then kept at room temp for 2-5 days in daylight.

(iii) Small scale growth of organisms

Mycelium was grown in 2 l conical flasks containing 1 l, medium N with 2% sucrose. The medium was inoculated with a conidial suspension and grown (64 hours at 25°C) on a gyrotory shaker. Mycelium was harvested on a Buchner funnel, washed with 2x500 ml of distilled water, pressed dry between several layers of filter papers and stored frozen at -15°C until required.

(iv) Large scale growth of organisms

10 l bottles containing 8 l of medium N supplemented

with 2% sucrose were inoculated with a conidial suspension using one slope culture per bottle and the inoculated medium was incubated at 25°C with forced aeration for $2\frac{1}{2}$ to 3 days. The mycelium was harvested in an M.S.E. basket centrifuge, washed with 4 l of distilled water, and stored at -15°C until required.

II. ASSAY OF THE DAHP SYNTHASES

The enzyme, 3-deoxy-D-*arabino* heptulosonate 7-phosphate synthase (DAHP synthase) was classified as a lyase by the Commission on Enzymes of the International Union of Biochemistry and was given the systematic name 7-phospho-2-oxo-3-deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating) E.C.4.1.2.15 and the trivial name, phospho-2-oxo-3-deoxy-heptonate aldolase. This nomenclature was criticised (Jensen and Nester, 1966 and Doy, 1968b) as based on the reverse reaction of the enzyme which has not been shown to be reversible, and also pyruvate does not enter into the reaction. To avoid confusion with the synthetases (E.C. Class 6, ligases) the trivial name DAHP synthase is used in this thesis. DAHP synthase (*Phe*), DAHP synthase (*Tyr*) and DAHP synthase (*Trp*) are used to denote the phenylalanine-, tyrosine- and tryptophan-inhibited isoenzymes, respectively.

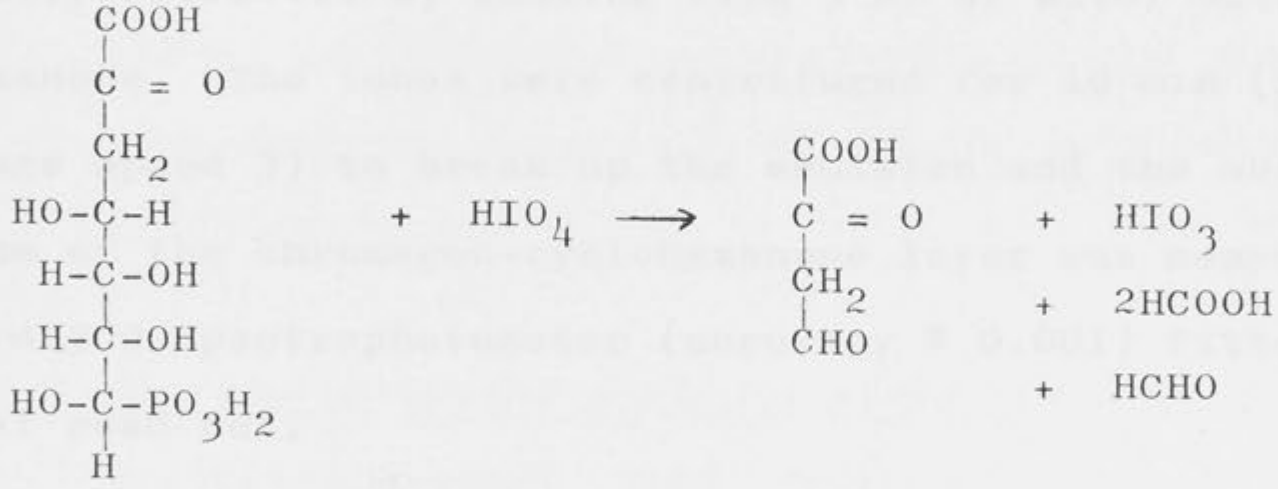
(i) The enzyme assay

Enzymic activity was determined by estimating the amount of DAHP formed from phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) during a ten min incubation period at 37°C, when the amount of enzyme was rate limiting.

The standard reaction mixture contained 0.02M tris-maleate buffer pH 6.4, 0.5 mM each of PEP and E4P and 0.1 mM of L-phenylalanine, L-tyrosine, or L-tryptophan when required in a total volume of 0.25 ml. Reaction mixtures were incubated at 37°C for 5 min. The reaction was initiated by the addition of enzyme and terminated by the addition of 0.1 ml of 10% trichloroacetic acid. The enzyme solution was added from a microlitre syringe (Hamilton Comp. Inc. California) or from a micropipette (H. E. Pedersen, Denmark).

(ii) Estimation of DAHP

Estimation of DAHP is based on the periodate oxidation of DAHP to yield formylpyruvic acid (Weissbach and Hurwitz, 1959).



Formylpyruvic acid is then condensed with 2-thiobarbituric acid on heating to yield a pink chromagen with an absorption maximum at 549 nm. Excess periodate is reduced to the iodide by sodium arsenite. Since periodate is also able to oxidise formylpyruvate the amount of colour formed is dependent on the time of reaction with periodate. The rate of oxidation of formylpyruvate exceeds the rate of oxidation of DAHP after 30 min thus lowering the amount of chromagen forming compound (Doy and Brown, 1965). Thus periodate oxidation of DAHP is never complete but gives a maximum colour yield. Of the compounds tested for specificity of

the assay (Srinivasan and Sprinson, 1959) only DAHP and malondialdehyde (the periodate oxidation product of 2-deoxygluconate) yielded a chromagen. The chromagen from the latter compound absorbed maximally at 532 nm.

The method of colour formation was basically that of Warren (1959) and Doy and Brown (1965). After enzyme reaction each assay mixture was incubated at 37°C for 30 min with 0.01M periodate ($NaIO_4$) in 0.03M H_2SO_4. Excess periodate was reduced by the addition of 0.5 ml of 2% (w/v) sodium arsenite in 0.5M HCl, mixed and left for 4 min. 2 ml of 0.3% (w/v) 2-thiobarbituric acid in 5 mM NaOH was added to each tube. The tubes were covered with foil and heated in a boiling water bath for 8 min. The tubes were cooled rapidly by plunginginto cold water, and the chromagen was immediately extracted by shaking with 3 ml of water saturated cyclohexanone. The tubes were centrifuged for 10 min (Heka bench centrifuge speed 3) to break up the emulsion and the absorbance at 549 nm of the chromagen-cyclohexanone layer was measured using a Gilford 300 Spectrophotometer (accuracy ± 0.001) fitted with a digital read out.

The reproducibility of the DAHP synthase assay and DAHP estimation was tested on ten samples using the same enzyme preparation. The result was a ± 2.6% standard deviation from the mean absorbance at 549 nm. The extinction coefficient of DAHP is = $8.0 \times 10^4 M^{-1} cm^{-1}$ at 549 nm. The amount of DAHP in a unit of enzyme activity (0.1 umole) corresponds to $A_{549\ nm}=2.666$.

(iii) Unit of enzyme activity

The standard enzyme unit, U, as defined by the Commission on Enzymes in 1961, is that amount of enzyme which catalyses the transformation of one micromole of substrate per minute under defined conditions. The unit of

enzyme activity used in this report is one hundredth of a U, as that amount of enzyme required to catalyse the formation of 0.1 μmole DAHP in 10 min at 37°C. The activity of each isoenzyme was determined by subtracting the activity measured in the presence of the inhibitor concerned, from activity in the absence of inhibitors.

The concentrations of PEP and E4P solutions were determined enzymatically with DAHP synthase, by converting a small amount of each substrate to DAHP in the presence of a 10-fold excess of the other.

III. PROTEIN ESTIMATION

Protein was determined by the method of Lowry et al., (1951) using bovine serum albumin as the standard. Samples were dialysed against 0.02M KH_2PO_4-NaOH buffer pH 7.4 before the assay.

IV. PREPARATION OF CHROMATOGRAPHIC MATERIALS

(i) Diethylaminoethyl (DEAE) celluose

New anion exchanger was swollen in distilled water in a measuring cylinder. The time for the slurry to settle is calculated from:

$t = nh$ where t = time (min)

h = the total height of slurry in the measuring cylinder (cm)

n = a factor between 1.3 and 2.4.

For general usage, an n value of 1.8 is used. The supernatant water solution containing fines was immediately removed. New and used DEAE cellulose was regenerated by stirring the material in 0.1M NaOH for two hours, after

which it was washed with 0.02M potassium phosphate NaOH buffer until pH7.4 was attained.

(ii) Hydroxylapatite

Hydroxylapatite powder was suspended in buffer solution and allowed to settle. Half a volume of settled cellulose powder suspended in the same buffer, after removal of fines as described for DEAE cellulose, was added to the hydroxylapatite and the well mixed slurry used to pour the column. Addition of cellulose powder permits faster flow rates during column chromatography. Each column was re-used several times by passing a 0.5M phosphate buffer solution through it each time after use and re-equilibrating with 0.02M phosphate buffer solution pH 7.4. The capacity of the column was not significantly affected.

(iii) Sephadex G200

Sephadex G200 powder was swollen in buffer solution for 72 hours before pouring into columns. For urgent use, the suspension was boiled in a water bath for two hours to hasten swelling of gel particle.

(iv) Affinity chromatography

Gels for affinity chromatography were prepared according to the method of Cuatrecasus and Anfinsen (1971). Three kinds of affinity gels were made. 1. Trp residue was coupled through the amino group directly to the Sepharose 4B backbone. 2. 3,3'-Diamino-dipropylamine (Eastman Kodak) was coupled on to cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia Fine Chemicals) and Trp residue was then coupled through the carboxyl group on to the amino group of the substituted Sepharose by carbodiimide. The

idea is to leave a free -NH_2 group on the tryptophan residue. 3. Phe residue was coupled through the carboxyl group on to the amino group of the substituted Sepharose by carbodiimide.

To 50 ml packed volume of Sepharose 4B was added 50 ml of distilled water and the suspension was adjusted to pH 11 with 1M NaOH. The suspension was stirred in a well ventilated fume hood and 12.5 gm of finely divided CNBr was added, 10M NaOH was added drop by drop to maintain the suspension at pH 11. Pieces of ice were added to maintain the temperature at 20°C. The CNBr reacted in about 15-20 min and the pH no longer dropped indicating no more base uptake. 100 ml of iced water was added and the suspension immediately filtered by suction on a Buchner funnel. 250 ml of water at pH 10 was added and again filtered. The funnel was removed and the outlet was sealed with parafilm. The CNBr activated Sepharose was used to prepare the three type of gels.

(1) 20 mmoles of Trp dissolved in 20 ml of water at pH 10 were added to 20 ml of CNBr activated Sepharose and the suspension was transferred to a beaker and stirred at 4°C for 22 hours.

(2) 50 ml of 2M 3,3'-diamino-dipropylamine at pH 10 was added to 50 ml of activated Sepharose and the suspension was stirred with a glass rod. The suspension was transferred to a beaker and stirred at 4°C for 22 hours.

After stirring, the suspension was filtered, washed with 1 litre of water and resuspended in 50 ml of water. The extent of substitution was tested with sodium 2,4,6-trinitobenzenesulphonate. 1 ml of a saturated sodium tetra-

borate (borax) solution was added to 0.3 ml of the substituted Sepharose and three drops of a 3% solution of sodium 2,4,6-trinitrobenzenesulphonate (Sigma) were added. Orange colour on the gel indicates free aliphatic amine.

The tryptophan was then coupled to the substituted Sepharose by carbodiimide. 20 ml packed volume of substituted Sepharose was adjusted to pH 4.7 by 1M HCl. 1 gm of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide dissolved in 3 ml of water and adjusted to pH 4.7 by 1M HCl was added. The suspension was stirred and 20 mmoles of Trp dissolved in 20 ml of water was added drop by drop and the suspension was maintained at pH 4.7. The suspension was stirred at room temperature for 18 hours and then washed with 1 litre of 0.1M NaCl.

(3) Phe was used instead of Trp and the procedure was similar to (2) above.

For use, the gel was packed in a column and washed with 0.02M KH_2PO_4-NaOH buffer pH 6.4 plus 0.1% (w/v) mercaptoethanol. The enzyme solution in the same buffer was applied on to the column and 23 drop fractions were collected. The column was washed with the same buffer before eluting with 0.02M KH_2PO_4-NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol.

V. TEST FOR PROTEOLYTIC ACTIVITY

Trypsin-like proteolytic activity was tested by casein-yellow digestion. 2.8 ml of casein-yellow solution (1 mg/ml) was incubated with 0.2 to 0.5 ml of enzyme solution (0.1 mg/ml) or trypsin solution (1 mg/ml) in 0.02M KH_2PO_4-NaOH buffer pH 7.4 at 37°C for various times.

The undigested protein was precipitated by 0.5 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant was made alkaline by 0.5 ml of 10M NaOH. The absorbance at 422 nm was read against the corresponding blank with an equivalent amount of buffer solution in place of the enzyme solution.

VI. ANALYTICAL POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Non-denaturing gel

The apparatus and method used are essentially that of Davis (1964). For routine work, a 7% acrylamide, 0.1% N,N'-methylene bisacrylamide (Bis) (w/v, final concentration) resolving gel and a 3% acrylamide, 0.63% Bis (w/v, final concentration) stacking gel was used. Sample gel was omitted.

The Tris-glycine buffer system of Davis (1964) has a pH of 8.1 to 10 in the resolving gel and is unsuitable for DAHP synthase which is stable around pH 7.4. The triethanolamine (TEA) N-tris-(hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES) system of Orr (1969) was used which has a running pH of 6.8 to 8.0.

Gels were photopolymerised with 13.3 μM riboflavin. Ammonium persulphate was omitted because of reports of modification of protein by this compound on the gel (Fautes and Furminger, 1967; Bennick, 1968; Orr, 1969). N,N,N',N'-tetramethylethylenediamine(TEMED) concentration was the same as those described by Thelander (1967) (4.3 mM in the stacking gel and 6.5 mM in the resolving gel).

Gels were cast in glass tubes of internal diameter 0.57 cm at 4°C and electrophoresis was done at the

same temperature. Buffers were prepared with cold water and pH adjusted by addition of TEA at 4°C. Samples, each with 3 µl of bromophenol blue in water (50 mg/100 ml), 1 drop of glycerol and 100 µl of protein solution was applied directly on top of the stacking gel. The gel tubes were inserted into the apparatus and the upper resevoir buffer was layered carefully on top of each sample. Each of the buffer reservoirs was filled with 250 ml of buffer solution and a constant current of 3 milliamps per tube was used. Electrophoresis was terminated when the tracking dye moved to within 0.5 cm from the bottom of the resolving gel (approx. 2 hours). Gel were removed from the glass tubes by squirting water from a syringe between the gel and the tube wall.

The length of the gel and the distance moved by the dye were measured before staining. Two types of stains were used routinely. Gels were stained according to the method of Davis (1964) in a 9% glacial acetic acid (v/v) -45% methanol (v/v) solution in water containing 0.5% amido schwartz (w/v) for a minimum of 2 hours and destained in 7.5% acetic acid (v/v)-5% methanol (v/v) on a gyroshaker. In 1972, Diezel et al., reported a new type of stain, Coomassie brilliant blue G250, which gives instantaneous visualization of bands immediately after staining. G250 is less soluble in 12.5% (w/v) trichloroacetic acid than R250 so that the gel is not penetrated by the dye during staining in 12.5% (w/v) trichloroacetic acid, thus avoiding staining of background which would affect band detection immediately after staining.

The gel was immersed into 10 ml of 12.5% (w/v) trichloroacetic acid for 5 min to fix the protein. 0.2 ml

of an aqueous 0.25% (w/v) solution of Coomassie brilliant blue G250 was added and thoroughly mixed. After 15 min, the gel was transferred to 5% (v/v) acetic acid. In this solution, the stained bands intensify because the protein-bound dye becomes soluble, diffuses into the gel and binds to the interior protein zones. This method was used routinely for investigation requiring rapid visualization of protein bands.

(ii) Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Dodecyl sulphate polyacrylamide gel electrophoresis was performed according to the method of Weber et al., (1972) with slight modification by Hoffmann (1971).

Samples containing 1% (w/v) dodecyl sulphate and 1% mercaptoethanol were heated at 100°C for 3 min and were left standing at room temperature for 24 hours and heated for 2 hours at 37°C before applying on to the gel. The reason for this treatment is to allow the dodecyl sulphate molecules to penetrate the denatured protein and bind on to the individual subunits. These subunits will become negatively charged and by electrostatic repulsion the protein will separate into its individual subunits. The mercaptoethanol is to reduce any disulphide bonds if present. A 10% acrylamide, 0.14% Bis, (w/v), resolving gel was used. Gel buffer was 0.05M phosphate (2.2g $NaH_2PO_4.2H_2O$ + 5.1g Na_2HPO_4 anhydrous/litre) buffer pH 7.0 plus 0.2% (w/v) dodecyl sulphate. Electrophoresis was performed at a constant current of 3 milliamps per gel and increased to 5 milliamps per gel after the dye had entered about 1 cm into the gel. The run was terminated when the dye marker had reached to within 1.0 cm to 0.5 cm from the

end of the gel. Protein bands were stained by Coomassie brilliant blue R250 and destained as described by Weber and Osborn (1969). The molecular weights of unknowns were determined from a plot of log of molecular weight versus mobility (R_m) with respect to the dye marker bromophenol blue using known protein standards electrophoresed simultaneously in the same reservoir as the samples.

VII. Analytical ultracentrifugation

A Spinco Model E analytical ultracentrifuge equipped with schlieren optics and the photoelectric scanner was used. Samples were concentrated by ultrafiltration in a Diaflo cell (PM 10 membrane) and dialysed against 0.02M phosphate buffer pH 7.4 either with or without 0.1M KCl. Experiments were performed according to the method of Schachman & Edelstein (1972). The schlieren pattern was measured with a comparator (Gaertner Scientific Corporation, Chicago).

A two place analytical An-H Ti (titanium) and a four place An-F Ti rotor were used for speeds up to 60 000 r.p.m. The average speed was estimated by counting the number of revolutions indicated on the counter during a measured period of time. Aluminium-filled epon, 12 mm, single and double sector cells and synthetic boundary, capillary type, single and double sector cells were used according to situation.

(a) Sedimentation velocity

The observed sedimentation coefficients, S_{obs}, were calculated from the equation,

$$S_{obs} = \frac{1}{w^2 r} \frac{dr}{dt} = \frac{2.303}{60 w^2} \left(\frac{d(\log r)}{dt}\right) \qquad (7.1)$$

where w = angular velocity in radians per second

r = radial distance corrected for camera lens magnification in cm

t = time in second

t'= time in min

The result was divided by 10^{-13} to obtain the sedimentation coefficient in Svedberg units, S.

The d(logr)/dt' was determined from the slope of the plot of log r versus time and points were fitted by the method of linear least square. The S_{obs} was corrected to the standard conditions by the equation,

$$S_{20,w} = S_{obs}\left(\frac{n_t}{n_{20}}\right)\left(\frac{n_{sol}}{n_w}\right)\left(\frac{1-\bar{v}p_{20,w}}{1-\bar{v}p_{t,sol}}\right) \qquad (7.2)$$

where n_t = viscosity of water at t°C (temperature of centrifuge run)

n_{20} = viscosity of water at 20°C

n_{sol} = viscosity of solution at known temperature, t'°C

n_w = viscosity of water at t'°C

$\bar{v}$ = partial specific volume of protein

$p_{20,w}$ = density of water at 20°C

and $p_{t,sol}$ = density of sample at t°C

(b) Sedimentation equilibrium

Concentration gradient was monitored by the absorption at 280 nm using the photoelectric scanner. Molecular weights were calculated from the equation,

$$M = \frac{2RT}{(1-\bar{v}p)w^2} \times 2.303 \frac{d(\log c)}{d(r^2)} \qquad (7.3)$$

where R = Universal gas constant, 8.315×10^7 ergs per degree per mole

T = absolute temperature

$\bar{v}$ = partial specific volume of the protein

p = density of solution in gm/ml

w = angular velocity in radians per second

c = concentration

and r = radial distance in cm corrected for magnification.

The value of the partial specific volume of protein was taken to be 0.74. The log of concentration was plotted against the square of the radial distance. Points were fitted to a straight line by the method of linear least square using a PDP-8 digital computer (Digital Equipment Corp., Maynard, Mass., U.S.A.). Curves were plotted and the slope at any point along the curve was determined and substituted into equation 7.3 for the molecular weight at any point along the curve.

VIII. DETERMINATION OF MOLECULAR WEIGHT BY GEL FILTRATION

Gel filtration is a liquid column chromatographic method of separating solute molecules according to differences in molecular size. The fractionation range of a gel represents the molecular weight spectrum over which separation can be expected to occur. It corresponds to the more or less linear region of a sigmoid curve which shows the relationship between the log of the molecular weight and one of several functions of the elution volume.

At the molecular level, gel filtration is envisaged as liquid-liquid partition in which the accessible volume of stationary phase is a variable quantity dependent upon

the extent to which a particular solute may penetrate the gel. Two distribution coefficients, viz., K_D (Gelotte, 1960) and K_{av} (Laurent and Killander, 1964) have been used. The distribution coefficients are related to the elution volume V_e in a column experiment by Eqs. 7.4 and 7.5.

$$V_e = V_o + K_D V_i \qquad (7.4)$$

$$V_e = V_o + K_{av}(V_b - V_o) \qquad (7.5)$$

V_o is the void volume, the volume of the space between the gel particles. V_i is the inner volume, the volume of the liquid contained within the gel. V_b is the total bed volume. Both K_D and K_{av} are independent of the geometry of the columns.

For the determination of molecular weight, both the zonal method (Andrews, 1964) and the frontal method (Winzor and Scheraga, 1963) were used.

(a) Zonal method

The procedure used was bascially that of Andrews (1964). Samples were applied on to a G200 column (2.5 x 100 cm) and the elution volume was measured from the start of sample application to the half-height point of the leading edge of the effluent peak. The void volume was determined by the blue dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden).

The concentration of blue dextran in the eluate was measured by the absorbance in an EEL colourimeter using a red filter (OR 1). The elution of protein was monitored by the absorbance at 280 nm and the elution of enzyme was followed by assaying for enzymic activity.

The plot of log molecular weight versus K_{av}, $(V_e-V_o)/(V_b-V_o)$, was used to determine molecular weights of unknowns after calibration with known protein standards.

(b) Frontal method

The frontal elution procedure (Winzor, 1969) was used with a column of Sephadex G200, 13.5 ml bed volume. Sample volumes used were at least equal to orgreater than the bed volume. Sample in 0.02M KH_2PO_4-NaOH buffer pH 7.4 plus 0.1% (w/v) mercaptoethanol was applied directly to the gel column by a pipette and collection was started immediately. 20 drop fractions were collected. The volumes of fractions were determined by weighing individual collection tubes before and after collection.

The elution volume was taken as that volume which corresponded to the position of half the height of the plateau which in turn corresponded to the concentration of the applied sample. Two values of the elution volume can be estimated from one experiment, one from the advancing profile and the other from the trailing profile. The origin for the trailing profile corresponded to the effluent volume at which elution with buffer was recommenced. The data were treated as in the zonal method.

IX. ENZYME KINETICS

For general information, publications of Cleland (1963a,b; 1967) and Plowman (1972) were used. For the identification of reaction mechanisms, initial velocity and inhibition studies were used.

In general, two basic patterns are observed when reciprocal velocities are plotted against reciprocal

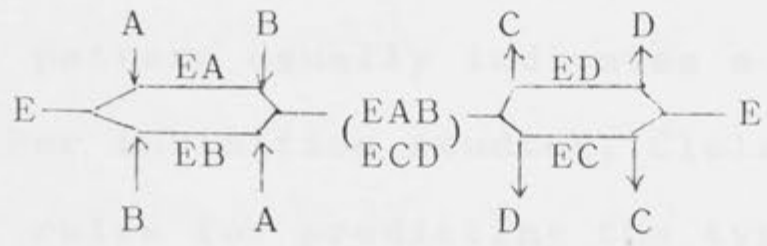
substrate concentrations. If no irreversible step intervenes between the points of combination of the two substrates in the reaction sequence, the reciprocal plots will intersect to the left of the vertical axis; but if an irreversible step occurs in the reaction sequence between the times of combination of the two substrates, the reciprocal plots are parallel. Irreversible steps are release of a product not present initially, addition of a substrate present in infinite concentration, or any step involving a very large negative free energy change in a reaction with a large equilibrium constant.

For a bireactant reaction, the following mechanistic types may be recognised.

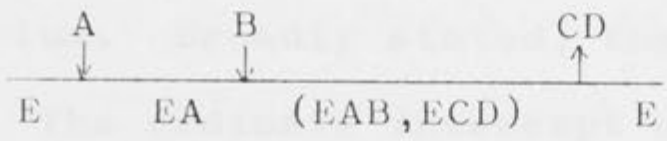
(a) Sequential Mechanisms

1. Random-order systems. The enzyme is assumed to have two binding sites, one for each substrate or product. The substrates may be bound independently of each other and in any order.

In the notation of Cleland,



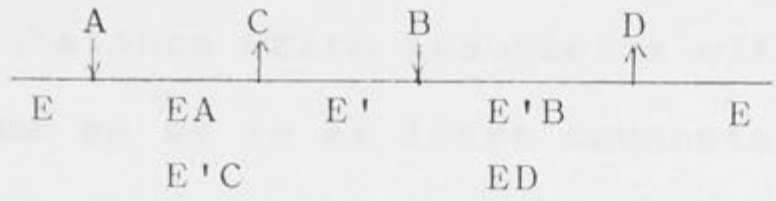
2. Ordered systems. The substrates are bound to the enzyme in a mandatory order.



(b) Non-Sequential Mechanisms

"Ping-pong bi bi " mechanisms. One product is formed and released before the second substrate is bound by the

enzyme.



When treated according to equilibrium (Michaelis-Menten) theory the random-order model gives a rate equation of the type,

$$v = \frac{V}{1 + \frac{K_{(A)}}{[A]} + \frac{K_{(B)}}{[B]} + \frac{K_A K_{(B)}}{[A][B]}} \qquad (7.6)$$

The ordered model treated by steady-state theory gives an equation of the same form as equation 7.6.

In the ping-pong system, the rate equation derived from steady-state theory is,

$$v = \frac{V}{1 + \frac{K_{(A)}}{[A]} + \frac{K_{(B)}}{[B]}} \qquad (7.7)$$

The double-reciprocal plot for the sequential mechanisms will give an intersecting pattern whereas the parallel pattern usually indicates a ping-pong mechanism.

For inhibition studies, Cleland (1963b) formulated a set of rules for predicting the type of inhibition to be expected from an examination of steady-state mechanisms containing no random sequences unless they are in rapid equilibrium. Broadly stated, these rules are as follows:

Rule 1. The ordinate intercept of a double-reciprocal plot is affected by a substance which associates reversibly with an enzyme form other than the one with which the variable substrate combines.

Rule 2. The slope of a double-reciprocal plot is affected by a substance which associates with an enzyme form that is the same as or is at least connected by a series of reversible steps to the enzyme form with which the variable substrate combines.

If only Rule 1 applies, uncompetitive inhibition is observed. If only Rule 2 applies, competitive inhibition is observed. Noncompetitive inhibition results if both rules prove to be applicable.

X. RADIOACTIVE BINDING STUDIES

The method and apparatus of Paulus (1969) were used. The apparatus was manufactured by Metaloglass, Inc., Boston, Mass., and PM 10 (7 mm diameter) ultrafiltration membrane (Amicon Corp.) was used. Samples, 0.2 ml, were pipetted into the eight channels of the upper block and the sample pots were sealed by polycarbonate screw plugs. Pressure (40 psi) was applied to the apparatus through the adaptor at the top.

After filtration, 5 ml of ethylene glycol was injected through the rinse channels in the lower block to wash the bottom side of the Diaflo membranes. The membrane was removed and transferred to a vial containing 1 ml of water. 10 ml of scintillation fluid (Bray, 1960) was added and the sample was counted in a liquid scintillation counter (Beckman, Model LS-250). Blank values were determined by filtering a sample without protein to correct for a small volume of solution which was retained by the Diaflo membrane.

XI. PEPTIDE MAPPING

Reduced and alkylated protein (Crestfield et al., 1963) (1 mg) was dialysed against 0.1M NH_4HCO_3 pH 8.5. Trypsin (20 ug) was added and the solution was incubated at 37° C for four hours. The solution was dried under vacuum in the presence of conc H_2SO_4. Peptides were redissolved in 0.2 ml 0.1 M $NaHCO_3$ pH 8.5 and the solution was added to 0.2 ml dansyl chloride (2mg per ml) in acetone. The pH was check (pH 10). Dansylation was allowed to proceed for overnight during which time all the dansyl chloride would have been hydrolysed to the hydroxyl. The solution was dried in vacuum. Acetone was added to the dried sample and the solution was applied on to the polyamide sheet (20 x 20 cm) using a micropeptide. The solvent systems used for developing the peptide map were: first dimension, water-90% formic acid (200:3, v/v); second dimension, benzene-acetic acid (9:1, v/v). Other details were described in the figures showing the peptides pattern.

XII ASSAYS OF OTHER ENZYMES

(a) Lactate dehydrogenase (Rabbit muscle, Sigma)

Lactate dehydrogenase was assayed with a Gilford 2400 recording spectrophotometer at 340 nm. The reaction mixture contained 5 mM sodium pyruvate, 0.1 mM reduced nicotinamide adenine dinucleotide ($NADH^+$), the enzyme solution and 50 mM sodium phosphate buffer, pH 7.4 in a total volume of 3 ml. All assays were carried out at 23°C.

(b) Pyruvate kinase (Rabbit muscle, Calbiochem)

Pyruvate kinase activity was assayed by the coupled method with a Gilford 2400 recording spectrophotometer at 340 nm. The reaction mixture contained 1 mM ADP, 1 mM PEP, 0.15 mM $NADH^+$, 10 mM $MgCl_2$, 0.1M KCl, 2 µl lactate dehydrogenase (rabbit muscle, Sigma), 0.1M Tris-HCl buffer pH 7.4 plus pyruvate kinase solution in a total volume of 3 ml.

(c) Glucose-6-phosphate dehydrogenase (Yeast, Sigma)

Glucose-6-phosphate dehydrogenase activty was assayed as for lactate dehydrogenase. The reaction mixture contained 0.6 mM glucose-6-phosphate, 0.2 mM reduced nicotinamide adenine dinucleotide phosphate ($NADPH^+$), 10 mM $MgCl_2$, 0.1M Tris-HCl pH 8.0 and enzyme to a total volume of 3 ml.

XIII. CHEMICALS

Phosphoenolpyruvate, trisodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo. Erythrose-4-phosphate dimethylacetal dicyclohexylammonium salt was purchased from Fine Chemicals of Australia, Melbourne and was converted to the free phosphate by the method of Ballou (1963).

Protamine sulphate was obtained from Scharz/Mann, Orangeburg, N.Y., U.S.A. 2-Mercaptoethanol, acrylamide, N,N'-methylene bisacrylamide (Bis), N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were purchased from Eastman Organic Chemicals, Rochester, N.Y., and sodium dodecyl sulphate was obtained from Matheson, Coleman and Bell, Los Angeles, California. Coomassie brilliant blue G250 was purchased from Serva FeinbiochemicaHeidelberg and Coomassie brilliant blue R250 was obtained from Sigma, St. Louis, Mo. Gradiopore gel (2.5% to 27%), was from Isolab Inc.

DEAE-cellulose (Cellex-D), agarose (Bio-Gel, A0.5M) and hydroxylapatite (Bio-Gel HTP) were purchased from BioRad Laboratories, Richmond, California, and Sephadex G25, G200 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsalla, Sweden. The cellulose powder was Whatman column chromedia CF-11.

Bovine serum albumin, trypsin and pyruvate kinase (rabbit muscle) were purchased from Calbiochem, San Diego, Calif. Ovalbumin (chicken egg) was a gift from the Biochemistry Department, John Curtin School of Medical Research, Australian National University, Canberra. Lactate dehydrogenase (rabbit muscle) was obtained from Sigma, St. Louis, Mo. Casein-yellow was obtained from Calbiochem, San Diego, Calif., and phenylmethylsulphonyl fluoride was purchased from Calbiochem, Los Angeles, Calif.

3,3'-Diamino-dipropylamine was purchased from Eastman Kodak, Rochester, N.Y., U.S.A. 1-Ethyl-3 (3-di-methyl-aminopropyl)-carbodiimide HCl was obtained from Sigma. ^{14}C-labelled-L-Tryptophan was purchased from

Radiochemical centre, Amercham, England.

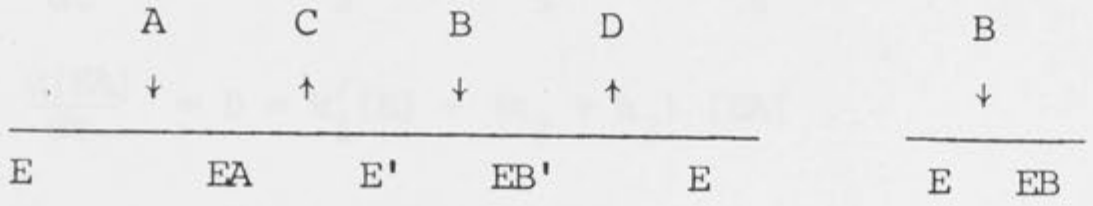
Dansyl chloride (1-Dimethylaminonaphthalene-5-sulpho-chloride; 5-Dimethylamino-1-naphthalene sulphonyl chloride; N,N-Dimethyl-1-naphthylamine-5-sulphonic acid chloride; DNS) was purchased from Sigma, St. Louis, Mo. Polyamide sheets were purchased from Eastman Kodak, France.

All other chemicals used were reagent grade and solvents were used without further purification.

APPENDIX I

DERIVATION OF THE RATE EQUATION FOR A PING-PONG MECHANISM WITH INHIBITION BY THE SECOND SUBSTRATE

Consider the reaction sequence:



with B, the second substrate also acting as a dead end inhibitor by binding to E. This is a basic ping-pong bi bi reaction with substrate inhibition by B. These reactions can be represented by the following equations,

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E' + C \qquad (1)$$

$$E' + B \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E'B \xrightarrow{k_6} E + D \qquad (2)$$

$$E + B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EB \qquad (3)$$

where k_i is the velocity constant.

For ease of calculation, let

$$k_1' = k_1 [A] \qquad (4)$$

$$k_4' = k_4 [B] \qquad (5)$$

$$k_7' = k_7 [B] \qquad (6)$$

In the steady state,

$$\upsilon = \frac{d[C]}{dt} = \frac{d[D]}{dt} = k_3 [EA] = k_6 [E'B] \qquad (7)$$

Also, the conservation of mass gives the equation

$$[E_o] = [E] + [E'] + [EA] + [E'B] + [EB] \quad (8)$$

where $[E_o]$ is the initial enzyme concentration.

There are five steady-state relationships,

$$\frac{d[E]}{dt} = 0 = k_2[EA] + k_6[E'B] - k_1'[E] + k_8[EB] - k_7'[E] \quad (9)$$

$$\frac{d[E']}{dt} = 0 = k_3[EA] + k_5[E'B] - k_4'[E'] \quad (10)$$

$$\frac{d[EA]}{dt} = 0 = k_1'[E] - (k_2 + k_3)[EA] \quad (11)$$

$$\frac{d[E'B]}{dt} = 0 = k_4'[E'] - (k_5 + k_6)[E'B] \quad (12)$$

$$\frac{d[EB]}{dt} = 0 = k_7'[E] - k_8[EB] \quad (13)$$

From (11), $$[E] = \left(\frac{k_2 + k_3}{k_1'}\right)[EA] \quad (14)$$

From (7), $$[E'B] = \left(\frac{k_3}{k_6}\right)[EA] \quad (15)$$

Combining equations (10) and (15) and rearranging,

$$[E'] = \left(\frac{k_3}{k_6}\right)\left(\frac{k_5 + k_6}{k_4'}\right)[EA] \quad (16)$$

From (13) $$[EB] = \left(\frac{k_7'}{k_8}\right)[E] \quad (17)$$

Substituting equation (14) for [E] into equation (17)

$$[EB] = \left(\frac{k_7'}{k_8}\right)\left(\frac{k_2 + k_3}{k_1'}\right)[EA] \quad (18)$$

Substituting these values into the conservation equation (8),

$$[E_o] = \left(\frac{k_2 + k_3}{k_1'}\right)[EA] + \left(\frac{k_3}{k_6}\right)\left(\frac{k_5 + k_6}{k_4'}\right)[EA] + [EA] + \left(\frac{k_3}{k_6}\right)[EA]$$

$$+ \left(\frac{k_7'}{k_8}\right)\left(\frac{k_2 + k_3}{k_1'}\right)[EA] \quad (19)$$

and rearranging,

$$[EA] = \frac{[E_o]}{1 + \frac{k_3}{k_6} + \left(\frac{k_2 + k_3}{k_1'}\right) + \left(\frac{k_3}{k_6}\right)\left(\frac{k_5 + k_6}{k_4'}\right) + \left(\frac{k_7'}{k_8}\right)\left(\frac{k_2 + k_3}{k_1'}\right)} \tag{20}$$

The rate equation $\upsilon = k_3[EA]$ becomes

$$\upsilon = \frac{k_3\,[E_o]}{\left(\frac{k_6 + k_3}{k_6}\right) + \left(\frac{k_2 + k_3}{k_1'}\right) + \left(\frac{k_3}{k_6}\right)\left(\frac{k_5 + k_6}{k_4'}\right) + \left(\frac{k_7'}{k_8}\right)\left(\frac{k_2 + k_3}{k_1'}\right)} \tag{21}$$

Divide both denominator and numerator by

$$\left(\frac{k_6 + k_3}{k_6}\right),$$

$$\upsilon = \frac{\left(\frac{k_3 \quad k_6}{k_6 + k_3}\right)[E_o]}{1 + \left(\frac{k_2 + k_3}{k_1'}\right)\left(\frac{k_6}{k_6 + k_3}\right) + \left(\frac{k_3}{k_6 + k_3}\right)\left(\frac{k_6 + k_5}{k_4'}\right) + \left(\frac{k_7'}{k_8}\right)\left(\frac{k_2 + k_3}{k_1'}\right)\left(\frac{k_6}{k_3 + k_6}\right)}$$

Substituting $k_1' = k_1[A], k_4' = k_4[B]$ and $k_7' = k_7[B]$,

$$\upsilon = \frac{\left(\frac{k_3 \quad k_6}{k_3 + k_6}\right)[E_o]}{1 + \left(\frac{k_2 + k_3}{k_1}\right)\left(\frac{k_6}{k_6 + k_3}\right)\frac{1}{[A]} + \left(\frac{k_6 + k_5}{k_4}\right)\left(\frac{k_3}{k_6 + k_3}\right)\frac{1}{[B]} + \left(\frac{k_6}{k_3 + k_6}\right)\left(\frac{k_2 + k_3}{k_1}\right)\left(\frac{k_7}{k_8}\right)\frac{[B]}{[A]}} \tag{22}$$

which reduces to the form

$$\upsilon = \frac{C_1 [E_o]}{1 + \frac{C_2}{[A]} + \frac{C_3}{[B]} + \frac{C_2}{[A]} \frac{[B]}{C_4}} \tag{23}$$

Equation (23) represents the general form of the ping-pong bi bi equation with substrate inhibition by B.

$$\upsilon = \frac{V}{1 + \frac{K_{(A)}}{[A]} + \frac{K_{(B)}}{[B]} + \frac{K_{(A)}}{[A]} \frac{[B]}{K_{s(B)}}} \tag{24}$$

where V = maximum velocity,

$K_{(A)}$ = Michaelis constant for A,

$K_{(B)}$ = Michaelis constant for B

and $K_{s(B)}$ = Dissociation constant for EB.

The reciprocal form of equation (24) is,

$$\frac{1}{\upsilon} = \frac{1}{V} + \frac{K_{(A)}}{V} \frac{1}{[A]} + \frac{K_{(B)}}{V} \frac{1}{[B]} + \frac{K_{(A)}}{V} \frac{[B]}{[A]} \frac{}{K_{s(B)}} \tag{25}$$

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ADDENDUM

PH.D. THESIS ENTITLED

"THE TRYPTOPHAN INHIBITED

3-DEOXY-D-*ARABINO* HEPTULOSONATE 7-PHOSPHATE SYNTHASE

FROM *NEUROSPORA CRASSA*"

Submitted January 1976

Ip Kam Ming

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CONTENTS

Fig. 4.1, 49-50 means figure 1 of chapter 4 and can be found between pages 49 and 50.

Because there are three DAHP synthase isoenzymes in Neurospora crassa, it is important to remember that the terms DAHP synthase (Trp) activity refer to DAHP synthase activity that is inhibited by tryptophan and DAHP synthase (Phe) activity refer to DAHP synthase activity that is inhibited by phenylalanine.

I. INTRODUCTION

This chapter is an addendum to chapters 3 and 4. In this chapter, the relevant facts from the studies presented in chapters 3 and 4 are drawn together in a detailed wholistic analysis. However, the readers will have to refer to the two chapters mentioned above for figures, tables and details of the experimental analyses.

Unlike data obtained from kinetic studies which are quantitative and have a firm mathematical basis, most of the data obtained in the studies of the structure of the enzyme are qualitative and require extensive follow up work. However, many of these if thoroughly followed through to the end, will undoubtedly be of fundamental importance to the understanding of protein interaction, biosynthetic pathway organisation and possibly proteolytic degradation.

Apart from this Introduction, there are three more sections. The section under Aim will spell out the main objectives of the work described in chapters 3 and 4. The Evidence section will describe the relevant results obtained and discuss their significance. Alternative interpretations for experimental results are presented where appropriate. In some cases, a choice between alternatives is possible but in others, a firm decision will require further works. The last section outlines the conclusion obtained from these studies and reasonable speculation as the basis for future work will also be presented.

To facilitate reference to figures and tables in the main body of the thesis, the following method is used.

II. AIMS

To purify the DAHP synthase (Trp), identify the aro-8 gene product and study its properties.

III. EVIDENCE

(a) Analysis of the active band 1 (Rm 0.1) and band 2 (Rm 0.15) and identification of the aro8 gene product.

The purification of DAHP synthase (Trp) is summarised in Table 3.1 (35-36). The purified DAHP synthase (Trp) from the Sephadex G200 column shows two bands on non-denaturing polyacrylamide gels (Fig. 4.1a, 49-50). Only band 1 (Rm 0.1) has DAHP synthase activity and is inhibited by tryptophan. The protein in this band has a molecular weight of 235 000 as determined on gradient gel (Table 4.2, 68-69). (The result from gradient gels is preferred over the other two methods for reasons explained on page 60 and also because it is a direct comparison of molecular weight at the point where the proteins have stopped migrating, and therefore less subject to effect of charge differences). Dodecyl sulphate gel electrophoresis of whole sample shows two bands of molecular weights 59 000 and 48 000 (Fig. 4.1a, 49-50). A third band of 44 000 occurs only occasionally (Fig. 4.1b, 49-50). Performic acid oxidation does not affect the banding pattern.

Protein 1 of molecular weight 235 000 contains only the 59 000 polypeptide (Fig. 4.3a, 51-52) and is therefore a homo-tetramer. Since protein 1 has DAHP synthase (Trp) activity, the 59 000 polypeptide is responsible for both DAHP synthase activity and inhibition by tryptophan. This

is consistent with the genetic findings that one genetic locus codes for both activity and inhibition (Halsall and Doy, 1969). The 59 000 polypeptide is therefore a product of the genetic locus aro-8 which codes for DAHP synthase (Trp) activity.

Band 2, Rm 0.15, (Fig. 4.1b, 49-50) has DAHP synthase (Trp) activity (it can appear instead of band 1 for some samples) and contains, in addition to the 59 000 and 48 000, a 44 000 component (Fig. 4.3b, 51-52). Band 2, however, is not always present. In view of the occurrence of proteolytic enzymes in Neurospora crassa (Siepen, Yu and Kula, 1975), the occasional occurrence of the 44 000 molecule in association with band 2 means that it could be a degradation product.

(b) Is the 44 000 polypeptide a degradation product of the 59 000 polypeptide?

If one considers that both the 44 000 and the 48 000 components are degradation products of the 59 000 component, one would also have to consider whether they are produced by the same or different proteolytic enzymes. If different proteolytic enzymes are involved, one is always present and digests the 59 000 molecule to a 48 000 molecule, whereas the other is active only occasionally to form a 44 000 molecule. This 44 000 molecule interacts with the 59 000 molecule to form band 2 on non-denaturing gel. Band 2 is active, whereas band 3, with the 48 000 molecule, is not. If one considers that the 44 000 molecule is degraded from the 48 000 molecule, one would have to argue that the further digestion of the 48 000 molecule to the

44 000 molecule renders the enzyme that contains these molecules in addition to the 59 000 molecule from inactive to active. This argument is possible but unattractive. Thus, the 44 000 molecule is likely to be degraded from the 59 000 molecule but not from the 48 000 molecule. However, the 44 000 molecule has not been obtained in sufficient amount to do a tryptic peptide analysis. Presumably, some of the 59 000 molecules in the native tetrameric enzyme are degraded by a proteolytic enzyme to a 44 000 component. Since the tetramer is still active, one has to conclude that the degradation does not inactivate the activity and inhibition sites of the enzyme molecule.

(c) Analysis of the inactive band 3 (Rm 0.20).

Band 3 (Fig. 4.1a, 49-50), of molecular weight 210 000 as determined on gradient gels (Table 4.2, 68-69), has no DAHP synthase (Trp) activity and contains the 48 000 component (Fig. 4, 3c, 51-52). For some samples, the 59 000 component is also present (Fig. 4.3c, 51-52). However, the components of bands 1 and 2 can sometimes migrate in the same band 3 position and cannot be separated on 7% and 10% polyacrylamide gels (Fig. 4.1b, c, 49-50). The dodecyl sulphate gel patterns remain unchanged even though the non-denaturing gels have changed showing that the same components are present before and after the change observed on non-denaturing gels. Two possible explanations for this phenomenon are discussed below.

i. Proteins from band 1 and band 2 become associated with proteins from band 3, ie the 59 000 molecule becomes

associated with the 48 000 molecule to form heteropolymers. The migration rate of this heteropolymer is nearly the same as the 4 x 48 000 tetramer.

ii. An alternative explanation is that the 59 000 and the 44 000 components change in conformation and now migrate at a faster rate equivalent to that of band 3. This alternative does not depend on a physical association between the 59 000 and the 48 000 molecules.

Alternative i. implies that the 59 000 molecule can associate with the 48 000 molecule, at least *in vitro* if not *in vivo*. The question then arises. Is the 48 000 molecule a degraded product of the 59 000 molecule?

(d) Is the 48 000 polypeptide a degradation product of the 59 000 polypeptide?

If the 48 000 molecule is degraded from the 59 000 molecule, one would expect to find the amino acid sequence of the 48 000 molecule in the 59 000 molecule. This is assuming that proteolytic enzymes do not cleave in the middle of a polypeptide, digest away some amino acids, and then rejoin the broken ends. This is a reasonable assumption since no such enzymes have yet been isolated from *N. crassa*. In the tryptic peptide map of the 59 000 molecule, one should be able to find at least 80% of the peptides from the 48 000 molecule. The fact that the two peptide maps (Fig. 4.6a, b, 57-58) only show about 30% similarity means that the 48 000 differs significantly from the 59 000 molecule. This conclusion is valid provided that the sample containing the 48 000 molecules

is not contaminated by a protease that degrades the tryptic peptides but not the original proteins. If so, this protease is present in the sample from band 3 but not band 1. The present investigation cannot discount this possibility so that one can only conclude that the 48 000 molecule is not likely to be a degraded product of the 59 000 molecule.

Thus discussion in (b) and (d) suggests that the 44 000 and the 48 000 molecules arise from different processes. The fact that the 44 000 component is a degraded product of the 59 000 molecule does not imply that the 48 000 component is also a degraded product of the 59 000 molecule.

(e) Is there any physical association between the 48 000 and the 59 000 polypeptide?

The two alternatives proposed above to explain the formation of one band from three bands on polyacrylamide gel are not distinguishable by the available experimental evidence. Additional evidence from the studies of bands 4, 5 and 6 (Fig. 4.25, 71-72) does suggest some association.

(1) Evidence from band 4 (Rm 0.25)

Band 4 (Fig. 4.25, 71-72) proteins can be isolated by gel electrophoresis. On re-running on non-denaturing gel, only one band is present. Dodecyl sulphate gels show more than five bands including the 59 000, 48 000 and 44 000 molecules (Fig. 4.28c, 73-74). Although likely that these are the same molecules that are found in bands 1, 2 and 3, it has not been established unequivocally that they are the same components. However, with some

samples isolated from band 4 by electrophoresis, DAHP synthase (Trp) activity can be demonstrated. When these samples were re-electrophoresed, band 1 could be found (Fig. 4.28b, 73-74, and discussed on page 73). Since band 1 has been shown to have DAHP synthase (Trp) activity and contains only the 59 000 component, the concomitant appearance of band 1 and activity shows that the 59 000 molecule observed from band 4 is the same 59 000 molecule that is responsible for DAHP synthase (Trp) activity. There is, however, no direct evidence to show that the 48 000 molecules as found in bands 1 and 4 are the same. To prove that would involve isolating the individual component and compare the amino acid sequences of the two polypeptides and this has not been done.

However, one thing seems certain. Band 1 protein, DAHP synthase (Trp), can be derived from band 4 proteins. Yet band 4 is inactive. Therefore, the 59 000 component in band 4 is inactivated, perhaps, by combination with other polypeptides. The fact that band 4 migrates as one band on gradient gel (pg. 73) suggests, but does not prove, that it consists of one molecule. Thus, the 59 000 polypeptide is capable of giving DAHP synthase (Trp) activity only when it is separated from the other polypeptides and forms a homo-tetramer as in band 1. This is evidence that the 59 000 polypeptide is

associated with the 48 000 and the 44 000 polypeptides. However, it remains possible that polypeptides of lower molecular weights are formed by degradation of the 59 000 molecule. Yet, these degraded products must still be able to interact with the 59 000 molecule as the evidence suggests.

(2) Evidence from the G200 column (Fig. 3.3, 35-36

Analyses of samples from the G200 column in the purification procedure also suggest protein interaction. Fractions from the slowest eluting peak (Fig. 3.3, 35-36) when concentrated were found to have DAHP synthase (Trp) activity (pg 71). On non-denaturing gel (Fig. 4.25, 71-72), band 1 is found. Since band 1 has been identified with DAHP synthase (Trp) activity, this band 1 as found in the slowest eluting peak is the same band 1 as found in the fastest eluting peak. However, band 1 is not found in the middle peak which has no DAHP synthase (Trp) activity. This means that the enzyme has dissociated and then re-associated to form the active enzyme after the fractions are concentrated. The dissociation might have occurred on the column or before applying to the column. Band 3, however, is different. One cannot be sure whether the band 3 in the slowest eluting peak is the same band 3 as found in the fastest peak.

The slowest eluting peak has a weight-average molecular weight of 48 000 (pg 71). This would mean that the protein which is active and has a molecular weight of 240 000 on the G200 column (Fig. 3.3, 35-36, Table 4.2, 68-69), has dissociated into monomers. The 59 000 monomer then can recombine to form an active tetramer. During this process, it is quite possible that the 59 000 molecule, instead of recombining with one another combine with other molecules, e.g. the 48 000, 44 000 and 33 000 molecules to form band 4 and band 5. In fact, during the whole of this investigation, all the evidence seems to suggest that this process is going on all the time. For example, band 4 after isolation from the polyacrylamide gel has an $S_{20,w}$ of 11 S (molecular weight of 300 000) yet band 4 can be found in the middle (molecular weight of 110 000) and slowest (molecular weight of 48 000) eluting peaks from the sieving column and has a molecular weight of 140 000 on gradient gel. This apparent discrepancy in molecular weight as determined by different methods, is consistent with the idea of polypeptides interaction. It is unlikely that this kind of interaction can occur between totally unrelated polypeptides. It is therefore likely that the interacting polypeptides are related. They may be products of proteolytic enzymes or independent molecules, the present evidence does not allow distinction between the two alternatives. In fact, the 59 000 molecule itself may be a degraded product of a larger polypeptide!

(f) What is the effect of the 48 000 polypeptide on DAHP synthase (Trp) activity?

When the components of band 1 are found to migrate in the position of band 3, the enzyme is inactive. Therefore, if there is any association between the 59 000 and the 48 000 molecules, the result would be an inactive protein. Similarly, as discussed above for band 4, the 59 000 molecule is inactive when found together with other molecules. Consider the activity peak from the G200 column (Fig. 3.3, 35-36), fractions in the leading edge contain less of the 59 000 molecule than those in the trailing edge (Fig. 4.5, 53-54) but the activity profile is fairly symmetrical. If all forms of DAHP synthase (Trp) activity are determined solely by the 59 000 molecule, activity should follow the distribution of the 59 000 molecule (in the form of tetramers). The fact that this does not happen indicates that the 48 000 molecule is affecting the DAHP synthase (Trp) activity. Again the effect is a reduction in enzyme activity.

(g) Why study protein interaction?

Why is it necessary to study any protein interaction if a pure DAHP synthase (Trp) can be isolated that contains only the 59 000 molecules?

As discussed in Chapter 2 (pg. 30), the criterion of purity of an enzyme preparation varies according to whether the enzyme is a single independent protein or one that has other proteins associated with it in biological function. Some of these associated proteins may not contribute to the activity of the enzyme but may be necessary for structural integration into the overall framework of the cell organisation.

Throughout the work up for the purification procedure, significant information about the structure of the enzyme was obtained. There are evidence for conformational change of the enzyme molecules and possible interaction between the aro-7 (coding for DAHP synthase (Phe)) and the aro-8 gene products. This evidence will be discussed below. Knowing this information means one cannot simply isolate a protein that has DAHP synthase (Trp) activity and claim that that is the native enzyme. To do so would be a gross simplification of a much more complex system. We have to be aware that that is likely to be only one form of the enzyme and ask again if there is any other form of the enzyme that we might have missed.

(h) Evidence for conformational change during purification

It was found that the activity and inhibition of DAHP synthase (Trp) can change during storage (Table 3.1, 35-36). The 55% $(NH_4)_2SO_4$ fraction was initially uninhibitable by either one of the three aromatic amino acids, phenylalanine, tyrosine and tryptophan (pg. 38). However, after storage at 4°C for 12 hours, 40% of the total activity was lost and whatever DAHP synthase activity that could be detected then, more than 90% of it was inhibited by tryptophan. This indicates that a change has occurred. The conformation of the enzyme may be changed so that the enzyme becomes less active but inhibitable by tryptophan. Alternatively, a previously inactive enzyme may become active and the original active enzyme has become inactive. The new active enzyme is

inhibited by tryptophan. The present evidence does not distinguish between the two alternatives. The important thing is, the enzyme can change and is changing during purification. It is of interest to remember also that with DAHP synthase (Tyr) (Hoffmann, et al., 1972), the purified enzyme slowly loses its activity even in the presence of phosphoenolpyruvate but as long as there is a bit of activity left, addition of fresh phosphoenolpyruvate can restore the preparation back to full activity. Clearly a change in conformation is occurring. Change of this kind therefore seems to be a common phenomenon for the DAHP synthases in N. crassa. This is also consistent with the occurrence of dissociation and association on the G200 column as discussed previously.

(i) Effect of Zn^{2+} and the occurrence of two kinds of DAHP synthase (Phe) activity

With the 55% $(NH_4)_2SO_4$ fraction that eluted unretarded through an Trp-affinity column, subsequent chromatography on a DEAE-cellulose column gave two separate peaks of DAHP synthase activity (Fig. 3.4, 41-42 and Discussion on pg. 41). One of these peaks is inhibited 95% by Trp and 7% by Phe and the other peak is inhibited 83% by Phe and 22% by Trp. Furthermore, the Phe-sensitive DAHP synthase activity peak is very sensitive to Zn^{2+} inhibition (86% inhibition by 1 mM Zn^{2+}), whereas the Trp-sensitive DAHP synthase activity peak is only inhibited 34% by Zn^{2+}.

The fact that phenylalanine inhibitable DAHP synthase activity can be obtained from a sample which previously is not inhibited by phenylalanine, shows that the polypeptide that is responsible for inhibition by Phe must be present in the preparation. What is more, this separated DAHP synthase (Phe) activity is very sensitive to inhibition by Zn^{2+}. Therefore, in the presence of Zn^{2+}, DAHP synthase (Phe) activity should be very low even if the necessary components are present. However, samples from the DEAE-cellulose column (Table 3.1, 35-36), when concentrated, is inhibited 20% by Phe in the presence of Zn^{2+} (Table 3.4, 40-41). There is no inhibition by Phe in the absence of Zn^{2+}. It is therefore unlikely that this portion of the Phe-sensitive DAHP synthase activity has the same conformation as the one that can be separated out from the $(NH_4)_2SO_4$ fraction as discussed previously. However, it is known from genetic studies that the genetic locus aro-7 is responsible for DAHP synthase (Phe) activity (Halsall and Doy, 1969). Mutations in aro-7 that affect the activity site will result in no detectable phenylalanine inhibitable activity. The presence of DAHP synthase (Phe) activity is indicative of the product of aro-7 being present. Therefore, this is direct evidence that the product of aro-7 is still present in the preparation after the anion exchange column in the purification procedure (Table 3.1, 35-36). Furthermore, this product of aro-7 is likely to be arranged in a different conformation or composition from that present in a sample in which the majority of the

DAHP synthase activity is inhibited by Phe. To go a little further, it is possible that the product of aro-7 can exist as homopolymer or heteropolymer in combination with other polypeptides. There is already evidence (Halsall, et al., 1971; Hoffmann, 1971) that the aro-7 gene product can form heteropolymer with the aro-6 gene product. Since this minor DAHP synthase (Phe) activity is detectable from an almost 100% tryptophan inhibitable DAHP synthase activity only in the presence of Zn^{2+} and that in the presence of Zn^{2+}, the amount of activity inhibitable by Trp is reduced, it is possible that there is some interaction between the aro-8 product and the aro-7 product. However, the available evidence is insufficient to arrive at a definite conclusion so that the above inference can only remain as a reasonable probability.

(j) What is the implication of the evidence presented in (h) and (i)

As discussed in section (i), in the early stages of purification, in the presence of Zn^{2+}, activity inhibitable by tryptophan is reduced and a phenylalanine inhibitable activity has appeared. The implication of this result is that the addition of Zn^{2+} leads to polypeptide interaction resulting in reduction in DAHP synthase (Trp) activity and appearance of DAHP synthase (Phe) activity. However, this appearance of DAHP synthase (Phe) activity is detectable only in the early stages of purification and DAHP synthase (Phe) activity is known to be very

unstable (from previous work in this laboratory, and my own experience with the isoenzyme). If in the later stages of the purification, the polypeptide responsible for DAHP synthase (Phe) activity has lost its potential for activity, either through change in conformation or composition, any change in polypeptide interaction which would have resulted in the appearance of DAHP synthase (Phe) activity would not be detectable. And the result would be interpreted as a reduction in DAHP synthase (Trp) activity. The interaction with the 48 000 molecule may just be what is happening as discussed above. This would imply that the 48 000 molecule is a product of aro-7. This suggestion is possible, since the 48 000 molecule does have a certain degree of similarity with the 59 000 molecule as determined by peptide mapping. This would be expected for two proteins both having the same enzyme activity. However, there is no direct evidence to prove that the 48 000 polypeptide is a product of aro-7 so that the above discussion is only a suggestion.

There is one point, however, which must be made very clear. Any evidence and inference for protein interaction is from in vitro experimentation. Any interaction that occurs may only be a result of experimental manipulation and condition. What is happening in vivo may be entirely different.

IV. CONCLUSION

The following conclusions can be made from the evidence discussed above.

(1) DAHP synthase (Trp) as isolated by the present purification procedure has a molecular weight of 235 000. It contains four subunits of a 59 000 polypeptide.

(2) The monomer of molecular weight 59 000 is the product of the genetic locus aro-8 which codes for DAHP synthase (Trp) activity.

(3) A 44 000 polypeptide that is present in the active band 2 (Rm 0.15) may be a degradation product of the 59 000 polypeptide.

(4) A 48 000 polypeptide that is present in the inactive band 3 is unlikely to be a degradation product of the 59 000 molecule. The origin of this polypeptide is not known. The effect of the presence of this molecule, however, is a reduction in DAHP synthase (Trp) activity.

(5) There is evidence from molecular sievings that the active tetramer dissociates into its monomers and these monomers later, when concentrated, reassociate to give the active tetramer.

(6) It is speculated that during the process of reassociation, the 59 000 monomer, in addition to combining with one another, combine with other molecules, e.g. the 48 000 and the 44 000 molecules, to form bands 3, 4 and 5. These bands are inactive. The 48 000, 44 000 and 33 000 molecules

in bands 4 and 5 may be degradation products of the 59 000 molecules or they may be independent molecules. The available evidence cannot distinguish between the two possibilities.

(7) Evidence based on the analysis of an inactive band 4 (Rm 0.25) suggests association between the 59 000, 48 000 and 44 000 polypeptides. An active enzyme can be generated from the inactive band 4 proteins. The implication is that the association of the 59 000 molecule with the other molecules results in loss of DAHP synthase (Trp) activity. When the 59 000 molecule is able to form a homo-tetramer, enzyme activity is regenerated.

(8) The activity of DAHP synthase (Trp) and the degree of inhibition by tryptophan change during purification. This observation is consistent with the molecular complexity postulated for the enzyme.

(9) A phenylalanine inhibitable DAHP synthase activity can be isolated from a totally tryptophan sensitive sample by elution from a Trp-affinity column followed by anion exchange on DEAE-cellulose column. This DAHP synthase (Phe) activity is very sensitive to inhibition by Zn^{2+}.

(10) Phenylalanine inhibition can also be demonstrated in the presence of Zn^{2+} from samples after the DEAE-cellulose column. This is taken as an indication of the presence of the product of aro-7 which codes for DAHP synthase (Phe) activity. It is further speculated that the product of aro-7 is associated with the product of

aro-8 to give this DAHP synthase (Phe) activity which is detectable only in the presence of Zn^{2+}. This minor DAHP synthase (Phe) activity is different from a sample with a majority of DAHP synthase (Phe) activity, which, in turn, could be made up of a homopolymer of the aro-7 gene product.

(11) There are reasons to speculate that the 48 000 molecule is the product of aro-7. To substantiate this speculation, the DAHP synthase (Phe) will have to be purified and analysed.

(12) In terms of biochemical purity, the preparation from Step 8 of the purification procedure is only 70%-50% pure in the sense that it contains components that have no DAHP synthase (Trp) activity. The contaminants, however, may be of significance in our understanding of protein interaction, enzyme organisation and/or proteolytic degradation.

A pure enzyme can be isolated, if desired, by polyacrylamide gel electrophoresis.

REFERENCE

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