THE ENZYME SYSTEMS EFFECTING RIBONUCLEOTIDE REDUCTION IN LACTOBACILLUS LEICHMANNII

A THESIS

submitted for the degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

MALCOLM DAVID ORR, M.B., B.S., B.Sc.

February, 1969.

This thesis embodies the results of research work carried out in the Department of Biochemistry, The John Curtin School of Medical Research, Australian National University from February, 1966 to February, 1969 during the tenure of an Australian National University Research Scholarship, for which I am indebted to the Council of the University.

STATEMENT

The results described in this thesis were obtained by myself under the supervision of Dr. R.L. Blakley and Dr. E. Vitols, with the exception of ultracentrifugation analysis of purified ribonucleotide reductase (Chapter III), which was performed by Dr. J.R. Dunstone, Department of Physical Biochemistry, John Curtin School of Medical Research, Australian National University. Amino-acid analysis and peptide mapping (Chapters III and IV) were performed by Dr. D.C. Shaw and Mr. L.B. James, as acknowledged in the text. Isotope studies on the alkylation of reduced ribonucleotide reductase were performed by Dr. R.L. Blakley.

M.D. Orr.

Candidate's Signature

ACKNOWLEDGEMENTS

I sincerely thank Dr. R.L. Blakley for his supervision, interest and encouragement during the course of this work. I am also indebted to Dr. E. Vitols for his guidance during the isolation and investigation of thioredoxin and thioredoxin reductase.

I thank Professor F.W.E. Gibson and members of his Department for useful discussions, and those members of other Departments of the University who have contributed to the production of this thesis.

In particular I express my appreciation to Dr. J.R. Dunstone of the Department of Physical Biochemistry; to Dr. D.C. Shaw and Mr. L.B. James of the Department of Biochemistry.

I am grateful to Mrs. Carole Withers for her skilled and rapid typing of this thesis.

PREFACE

The investigations reported in this thesis may be divided into two broad classifications, namely

1. Purification and properties of ribonucleoside triphosphate reductase.
2. Purification and properties of thioredoxin and thioredoxin reductase.

Chapter I contains background introductions to the above topics and includes a discussion on allosterism on the basis of currently proposed models which explain the phenomenon of multireactant kinetics. Methods are discussed whereby these various mechanisms may be differentiated. Chapter II describes the final purification of ribonucleotide reductase to electrophoretic homogeneity by the use of a new discontinuous buffer system developed for the purpose. The investigation of the properties of this electrophoretically homogeneous material is described in Chapter III. Chapter IV records the purification and properties of thioredoxin and thioredoxin reductase.

ABBREVIATIONS

5'-dA-cobalamin	5'-deoxyadenosylcobalamin
DMG	sodium dimethyl glutarate
DTNB	5,5'-dithiobis(nitrobenzoic acid)
G6P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
MMB	methyl mercuric bromide
MMI	methyl mercuric iodide
PMA	phenyl mercuric acetate
PMB	*para* mercuribenzoate
TEA	Triethanolamine
TEMED	N,N,N',N'-tetramethylene diamine
TES	N-tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid

TABLE OF CONTENTS

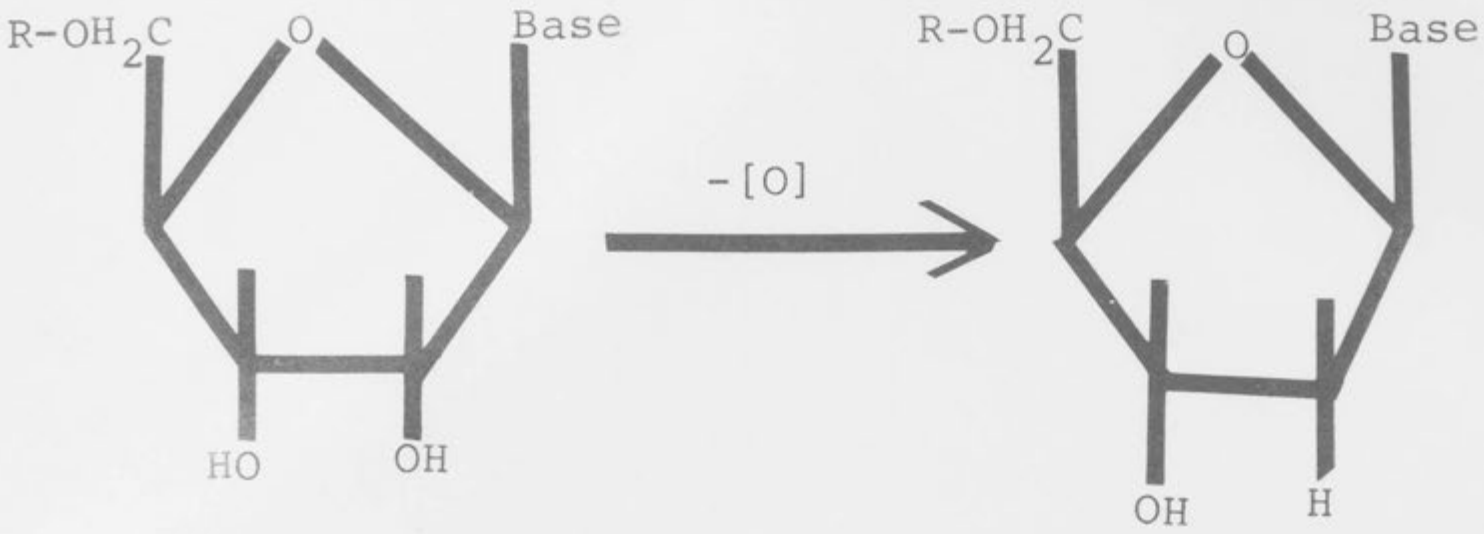


FIG. I.1. Ribonucleotide reduction.

The reaction proceeds without cleavage of the glycosidic bond or any of the phosphate linkages.

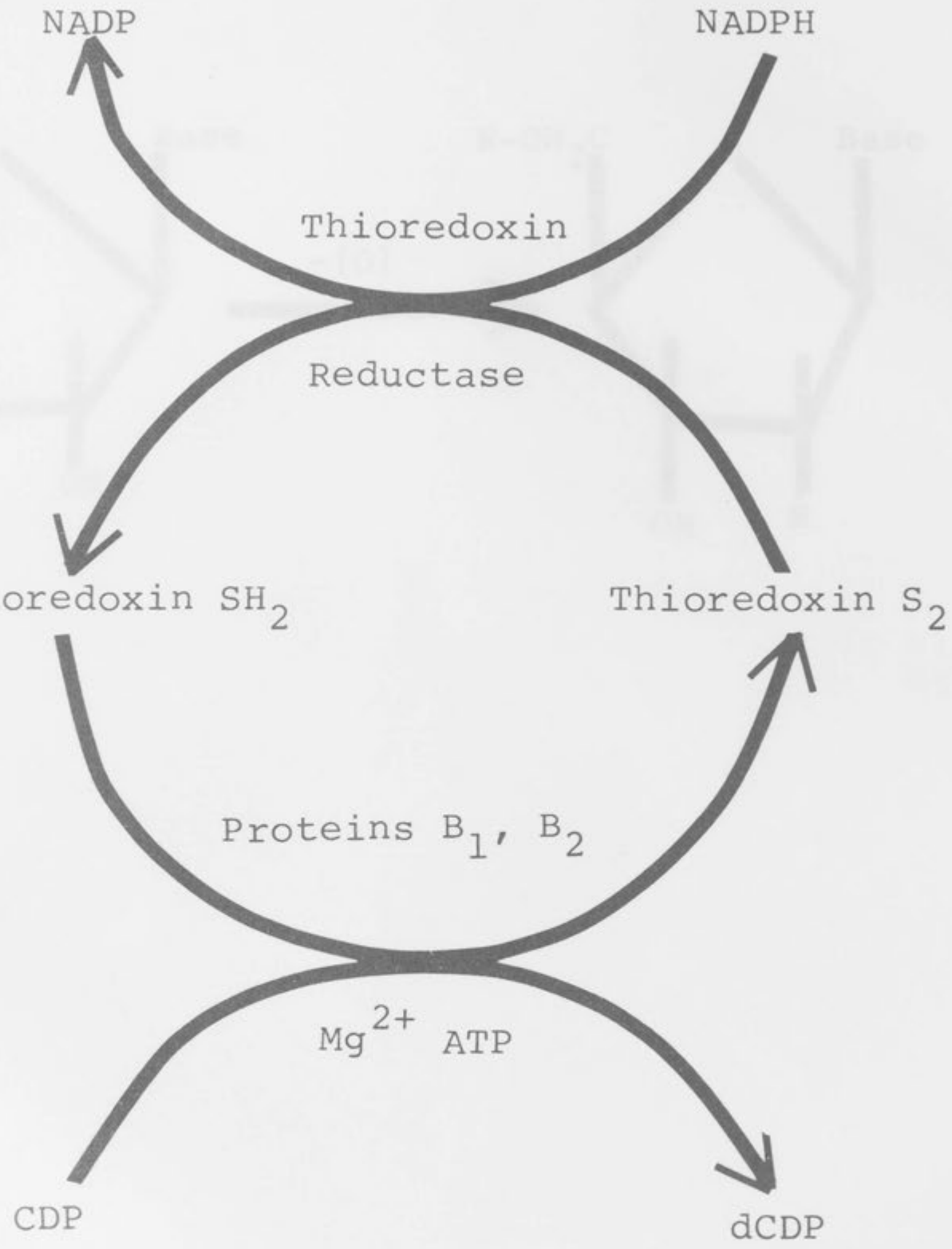


FIG. I.2. Ribonucleotide reduction in E. coli B.

CHAPTER I

INTRODUCTION

Ribonucleotide reduction. The synthesis of deoxynucleotides from ribonucleotides or ribonucleosides was demonstrated by the in vivo incorporation of labeled ribonucleosides into cellular DNA of rat tissues (Hammarsten et al., 1950). Rose and Schweigert (1953) were able to demonstrate such incorporation into the DNA of Lactobacillus leichmannii 313 and rat tissues without cleavage of the glycosidic bond. Subsequently, Larsson and Neilands (1966) showed that ribonucleotide reduction is the exclusive biosynthetic pathway for deoxyribonucleic acids in regenerating rat liver. These in vivo observations have been confirmed by the isolation of enzymes from several sources which catalyse the reaction shown in Fig. I.1.

One of the most extensively investigated ribonucleotide reductase systems is that isolated from Escherichia coli B by Reichard and his colleagues. The reaction coupling the reduction of CDP to dCDP with NADPH involves the participation of four proteins (Fig. I.2). These are thioredoxin (Laurent et al., 1964), thioredoxin reductase (Moore et al., 1964), and proteins B_1 and B_2, all of which have been highly

purified (Holmgren et al., 1965; Brown et al., 1967, 1968). The latter two proteins have values of $S_{20,w}$ of 7.8 S and 5.5 S, respectively.

The molecular weight of the B_2 subunit has been estimated as 82,000, whilst that of B_1 has not been so definitely established. Neither of these proteins is active alone, but together, in the presence of 0.01 M Mg^{2+}, they form the active complex which has been characterised in the ultracentrifuge with an $S_{20,w}$ of 8.8 S. Preliminary binding studies, performed on the separated subunits by the method of Hummel and Dreyer (1962) indicate that the nucleotide modifiers bind to the protein B_1. Brown et al. (1967) have concluded that the ribonucleotide reductase of E. coli is analogous to aspartate transcarbamylase from the same organism characterised by Gerhart and Schachman (1965) in that the reductase contains non identical regulatory and catalytic subunits. However, no definite measurements of binding of substrate to either subunit in the presence or absence of modifiers has been demonstrated for this enzyme.

Kinetic investigation of the purified reductase indicates that all ribonucleoside diphosphates can be reduced by the one system. Various nucleotides act as

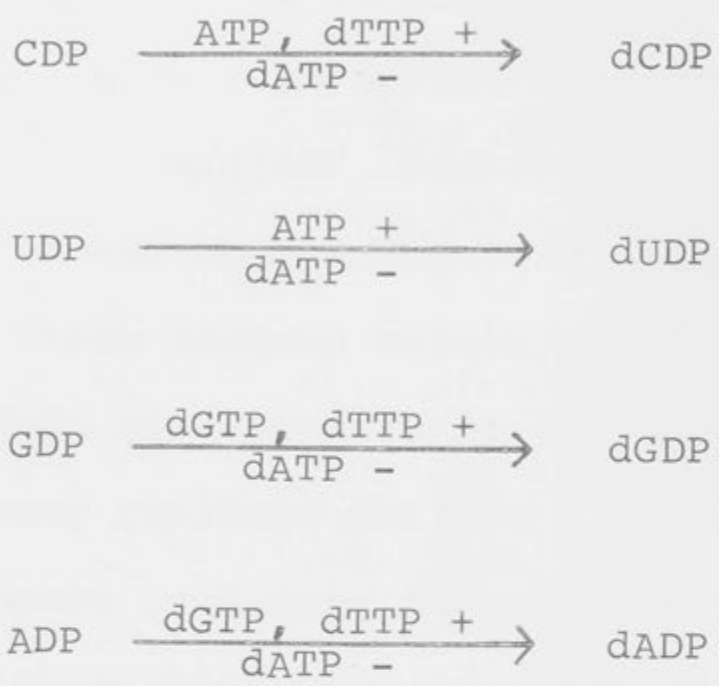


FIG. I.3. Activation (+) and inhibition (-) of E. coli ribonucleoside diphosphate reductase.

activators or inhibitors of the reduction. In view of the high concentration of Mg^{2+} present, the nucleotides presumably act as the magnesium complexes. dTTP and dATP activate and inhibit, respectively, the reduction of all four of the nucleoside diphosphates, dGTP stimulates the reduction of the purine diphosphates, while ATP activates for pyrimidine-diphosphate reduction. dCTP is slightly stimulatory for GTP reduction (see Fig. I.3).

It has been clearly demonstrated that the E. coli reductase is independent of 5'-dA-cobalamin or other cobalt-containing coenzymes. Brown et al. (1968) have shown that protein B_2 contains about 2 moles of non haeme iron per 82,000 molecular weight unit. The requirement for this prosthetic group, suggests that it may fill a similar position in the hydrogen transfer chain to that of 5'-dA-cobalamin, in the case of the enzyme system isolated from L. leichmannii.

Of the mammalian systems known to catalyse the reduction of ribonucleotides, the best characterised is the ribonucleoside diphosphate reductase found in cell-free extracts of the ascites form of Novikoff rat hepatoma (Moore and Reichard, 1964; Moore and Hurlbert, 1966). In general, this system bears many

similarities to that isolated from E. coli, both in substrate specificity and in metal requirements. It should be noted that the present knowledge of this enzyme is so far based only on a partly purified system. Although the requirement for Fe^{3+} was first demonstrated in this system (Moore and Reichard, 1964), the complexity and the low degree of purity rendered evaluation difficult. The hydrogen donor has been identified as NADPH mediated by thioredoxin and thioredoxin reductase (Moore, 1967).

Cobamide-dependent ribonucleotide reduction in Lactobacillus leichmannii. The first indications that cyanocobalamin, or a derivative, was involved in ribonucleotide reduction in this organism were provided by the nutritional studies of Kitay et al. (1950) and Shive et al. (1951), who replaced vitamin B_{12} with deoxyribonucleotides as an essential growth factor. Downing and Schweigert (1956) demonstrated that addition of the vitamin to growing cultures of L. leichmannii supplied with uniformly labeled thymidine-^{14}C caused significant dilution of the label incorporated into the bacterial DNA. Guanosine-^{14}C could be incorporated into the DNA of the cell only in the presence of vitamin B_{12} (Manson, 1960), and the preliminary studies of Dinning and Spell

(1959) on the vitamin dependence for the incorporation of ribose-1-^{14}C into the DNA was confirmed by Blakley and Barker (1964), who definitely established the presence of a cobamide-dependent ribonucleotide reductase in cell-free extracts of this organism. Subsequent purification of this enzyme has been followed by examination of its specific requirements with regard to hydrogen donor, cobamide coenzymes, nucleotide substrates and modifiers. As discussed later, the physiological hydrogen donor for the system is reduced thioredoxin (Orr and Vitols, 1966), but the thioredoxin system isolated from E. coli B (Laurent et al., 1964; Vitols and Blakley, 1965) and the model hydrogen donor, reduced lipoic acid (Blakley, 1965) have been used in many of the in vitro studies.

5'-dA-Cobalamin has been used for the majority of the in vitro studies, but with 2 mM GTP as substrate, α-(benzimidazolyl)-5'-deoxyadenosylcobamide has the same apparent K_m (8×10^{-7} M; Vitols et al., 1967a). α-(Adenosyl)-5'-deoxyadenosylcobamide is less active (apparent K_m 2.8×10^{-6} M). 2',5'-Dideoxyadenosylcobalamin, although slightly active itself, acts as a competitive inhibitor in the presence of 5'-dA-cobalamin. The absolute requirement of a cobamide-coenzyme for ribonucleotide reduction cannot be re-

placed by cyano-, hydroxo- or methylcobalamin.

Nucleoside triphosphates are the substrates of the reaction (Blakley et al., 1965). The rate of reduction of the various nucleotides is a function of the hydrogen donor and ionic strength used. With E. coli thioredoxin as the hydrogen donor and 0.2 M potassium phosphate, pH 7.5, decreasing activity is observed, GTP > CTP > ATP > UTP > ITP (Vitols et al., 1967a). However, when assayed with reduced lipoate in the presence of 1 M sodium acetate, maximum activity is shown by GTP followed by ITP, ATP, CTP and UTP (Blakley, 1966a). The effect of various deoxynucleoside triphosphates on the reduction of ribonucleotides by reduced thioredoxin is shown in Table I.1.

Hydrogen donor specificity of ribonucleotide reductases. Purification of the E. coli ribonucleoside diphosphate reductase by Reichard and his colleagues lead to the discovery of the two protein system which provides the coupling of NADPH to ribonucleotide reduction. This system comprises thioredoxin (Laurent et al., 1964), a small protein (mol. wt. 12,000), which contains only two half-cystine residues. These residues are capable of reduction by NADPH in the presence of a specific flavoprotein, thioredoxin reductase (Moore et al., 1964). Reduced thioredoxin is the physiological

TABLE I.1. Ribonucleotide reduction by the enzyme isolated from *L. leichmannii* in presence and absence of deoxyribonucleotides

Results are expressed as percentages of the rate of GTP reduction under assay conditions with 4 μM DBC coenzyme, 7 μM thioredoxin, 18 μg thioredoxin reductase and 20 μg of ribonucleotide reductase in 0.2 M. Phosphate buffer, pH 7.5.

Substrate 1 mM	Deoxyribonucleotide added (1 mM)				
	None	dGTP	dATP	dCTP	dTTP
GTP	100	86	104	92	96
ATP	20	103	15	15	17
CTP	36	41	102	31	32
UTP	14	23	14	37	17
ITP	6	5	6	7	38

Adopted from Vitols *et al.* (1967a).

hydrogen donor for ribonucleotide reduction. Both of these proteins have been extensively studied (c.f. Holmgren, 1968; Thelander, 1968).

Dihydrolipoate and other dithiols capable of cyclic oxidation are effective as the hydrogen donor for the reaction. The demonstration that reduced thioredoxin isolated from E. coli could be used in the reaction with an apparent K_m of 4×10^{-6} M (c.f. K_m 1.0×10^{-2} M for reduced lipoate), suggested that a similar system was present in L. leichmannii (Vitols and Blakley, 1965). This prediction has been verified by the isolation of thioredoxin and thioredoxin reductase from this organism (Orr and Vitols, 1966) and the preparation of these components will be described.

Other cobamide-coenzyme dependent ribonucleotide reductase. Recently Cowles and Evans (1968) have described the isolation and partial purification of ribonucleotide reductase from Rhizobium meliloti and have characterised some of its properties. No data is yet available on the structure of the enzyme, but it appears to bear some similarities to the system isolated from L. leichmannii. The preferred cobamide is 5'-dA-cobalamin, but although direct comparison of the conditions is not possible, the apparent K_m for

this enzyme (K_m = 5.5 x 10^{-6} M) appears to be higher than that reported for the L. leichmannii reductase (8.8 x 10^{-7} M). Substrate inhibition occurs at high levels but 2 mM GTP provided maximal reduction. The rates of reduction of other nucleoside triphosphates is, however, lower than for the corresponding diphosphate. Further clarification of the substrate specificity awaits greater purity of the isolated protein. Cowles and Evans (1968) have reported the unpublished observations of Wong, that a thioredoxin system has been isolated from this organism.

Table I.2 provides a summary of the ribonucleotide reductases which have been isolated and partially purified.

Allosterism. Regulation of enzymic reactions by the specific action of end products of a reaction sequence were clearly demonstrated by Umbarger (1956) and Yates and Pardee (1956). This feedback control phenomenon was often accompanied by sigmoidal or "S" shaped plots of initial velocity against substrate concentration, which differed from the hyperbolic plots and linear double reciprocal plots observed for enzymes fulfulling the requirements of the Michaelis-Menten (1913) hypothesis.

TABLE I.2. Properties of ribonucleotide reductase isolated from various sources

	E. coli	Novikoff Hepatoma	*L. leichmannii*	*R. meliloti*
Best substrate	CDP	CDP	GTP	(GTP)
Hydrogen transfer system	NADPH → FADH → $\begin{matrix}SH\\ \vert\\ SH\end{matrix}$ → (Fe^{3+})	NADPH → FADH → $\begin{matrix}SH\\ \vert\\ SH\end{matrix}$ → (Fe^{3+})	(NADPH → FADH → $\begin{matrix}SH\\ \vert\\ SH\end{matrix}$ → 5'-dA-cobalamin)	(NADPH → FADH → $\begin{matrix}SH\\ \vert\\ SH\end{matrix}$ → 5'-dA-cobalamin)
Metal requirement	Mg^{2+}	Mg^{2+} Fe^{3+}	Nil	Unknown
Activator	ATP dTTP	ATP dTTP	dGTP dATP dCTP	Unknown
Inhibitor	dATP	dATP	-	-
Subunit structure	B_1 Mg^{2+} B_2	Unknown	Unknown	Unknown

Features shown in parenthesis are results from preliminary investigations.

Desensitisation to feedback control was observed in some enzymes following treatments with heat or organic mercurials, and dissociation of the protein molecule under these conditions lead to the proposal by Monod and Jacob (1961) and Gerhart and Pardee (1962) that feedback control was mediated at a site which was interacting with but distinct from, the substrate site. Monod and Jacob used the term "allosteric" to define a modifier which bore little steric resemblance to the substrate. This original, concise definition has been expanded to embrace several other concepts which have been summarised by Koshland and Neet (1968). Enzymes have been classified as allosteric, if they demonstrated one or more of the following characteristics : the possession of a modifier site distinct from the catalytic site; if a sigmoidal relationship existed between initial velocity and/or substrate binding as a function of reactant concentration, be it substrate or modifier; if the reaction sequence is at a branch point in a metabolic pathway; or if the kinetics or other properties of the enzyme conformed to one of the models which have been proposed to explain the kinetic and thermodynamic effects observed. Following the interpretation of Koshland and Neet, the term "allosteric effect"

will be used to define the change in binding or activity at the catalytic site caused by the binding of a molecule at a different site. No direct effect is assumed between modifier and substrate, and the effect of modifier on the catalytic site is assumed to result from a structural modification of the enzyme.

Biological function of allosteric enzymes. The differences in initial velocity observed over small changes in substrate concentration, as well as causing the sigmoidal kinetics, provide the organism with a wide range of catalytic activity. This range of activity may be extended in either direction by the effect of allosteric modifiers. The advantage to the cell lies in the fact that these effects offer mechanisms for immediate control of biological syntheses. Induction and repression of enzymes, while constituting important factors in the overall regulation of cell growth, are subject to a long lag phase and cannot participate in the fine regulation necessary in the actively growing cell.

Care must be exercised however, in the extrapolation of in vitro data to the conditions present in the cell. Allosteric effects observed with ribonucleotide reductase require relatively high concentrations of

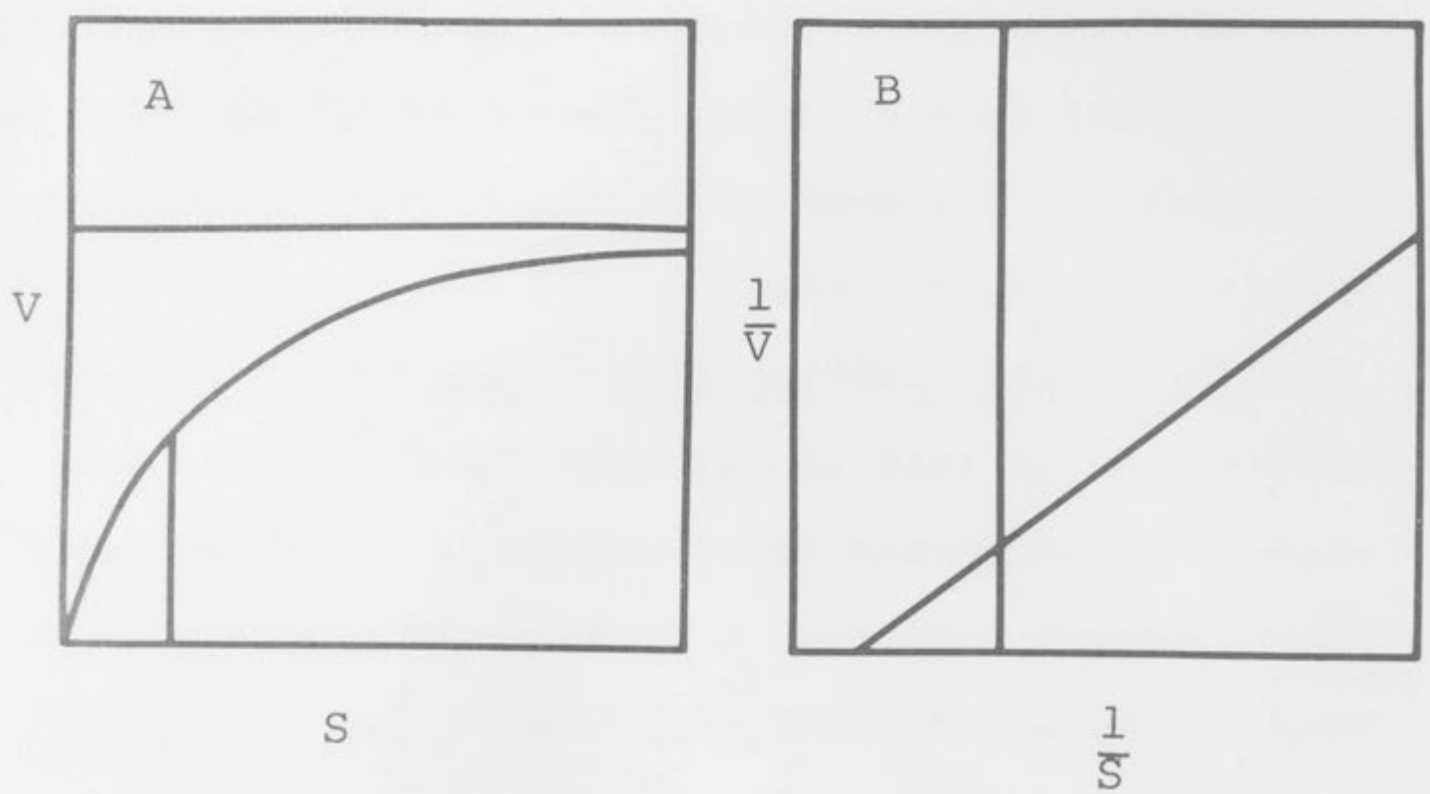


FIG. I.4. A. Plot of initial velocity as a function of substrate concentration for an enzyme which conforms to the Michaelis-Menten (1913) formulation.

B. Double reciprocal form.

modifier, far in excess of the levels normally attributed to tissues. However, only limited information is available with regard to intracellular compartmentalisation, both with regard to membrane barriers and kinetic association of enzymes which may facilitate the maintenance of higher concentrations of reactants in the vicinity of the active sites than in the general cell sap (c.f. Atkinson, 1968). Repression and derepression by deoxynucleotide concentration probably contributes substantially to the regulation of deoxynucleotide synthesis in L. leichmannii (Blakley and Vitols, 1968).

Kinetic representation of an allosteric enzyme.

For an enzyme which conforms to Michaelis-Menten (1913) kinetics, the plot of initial velocity as a function of substrate concentration is a rectangular hyperbola, the double reciprocal plot of which is linear. The intercepts of this line on the ordinate and abscissa provide, respectively, the reciprocal of the apparent maximum velocity (V_{max}) and the (negative) reciprocal of the apparent Michaelis constant ($K_{m\ app}$) shown in Fig. I.4. In terms of such a kinetic model, inhibitors combine at the catalytic site with resultant alteration of either the V_{max},

the $K_{m\ app}$ or both, but usually without loss of linearity of the double reciprocal plot.

In the case of some, but not all, allosteric inhibitors, the double reciprocal plots are also linear. However, even when linear double reciprocal plots are obtained with an allosteric inhibitor, it is sometimes possible to differentiate such an inhibitor from one which combines at the catalytic site. In order to differentiate the two types of inhibitor, the initial velocity is studied as a function of substrate concentration at various concentrations of the inhibitor, and a family of linear double reciprocal plots is obtained. The secondary plot of the slopes of these lines as a function of inhibitor concentration is usually linear (or in some cases, parabolic) for an inhibitor binding at the catalytic site whether the inhibition is competitive, non-competitive or uncompetitive for a particular variable substrate. On the other hand, the secondary replot is usually hyperbolic for an allosteric inhibitor.

Departure from Michaelis-Menten kinetics with non-linear double reciprocal plots may occur also in the absence of a modifier. In the best known cases of this kind, the plot of initial velocity versus

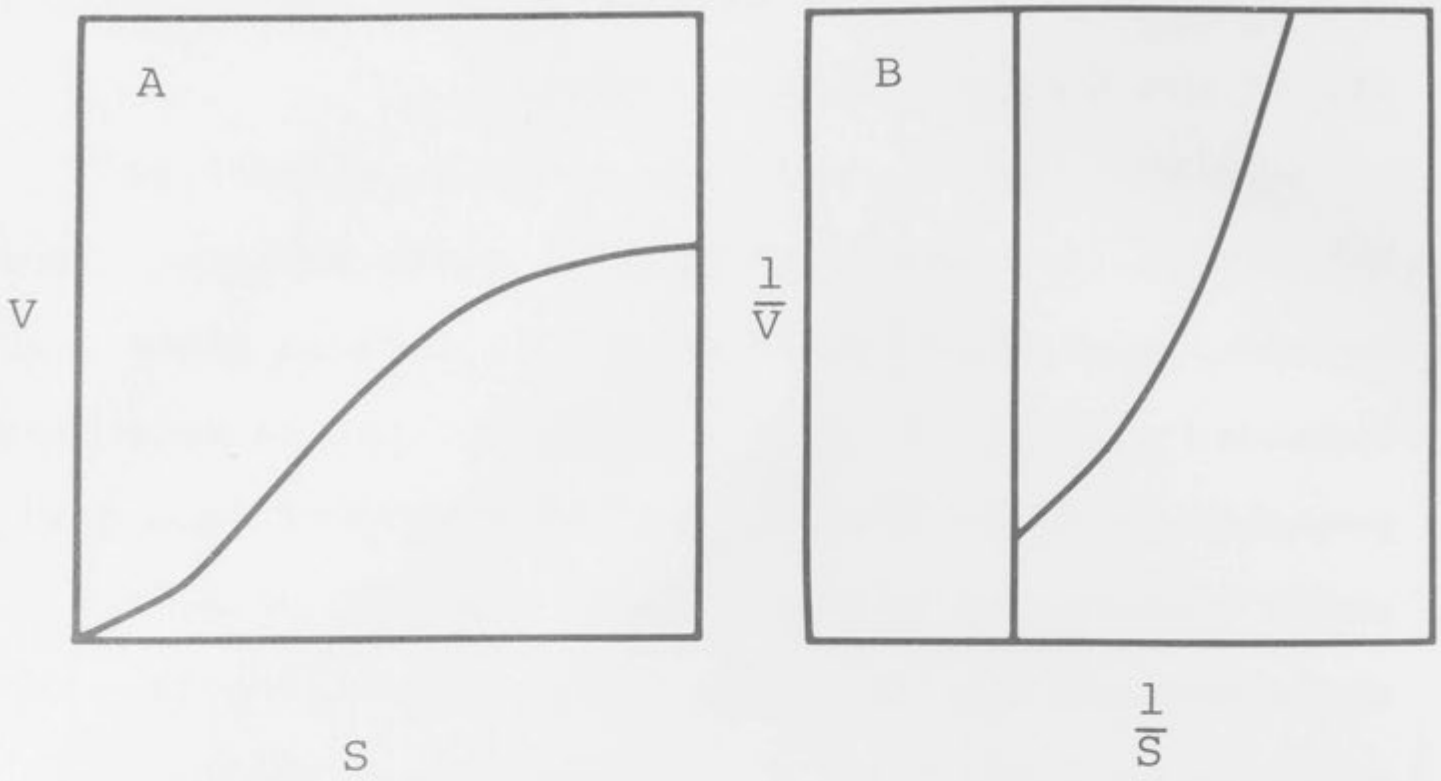
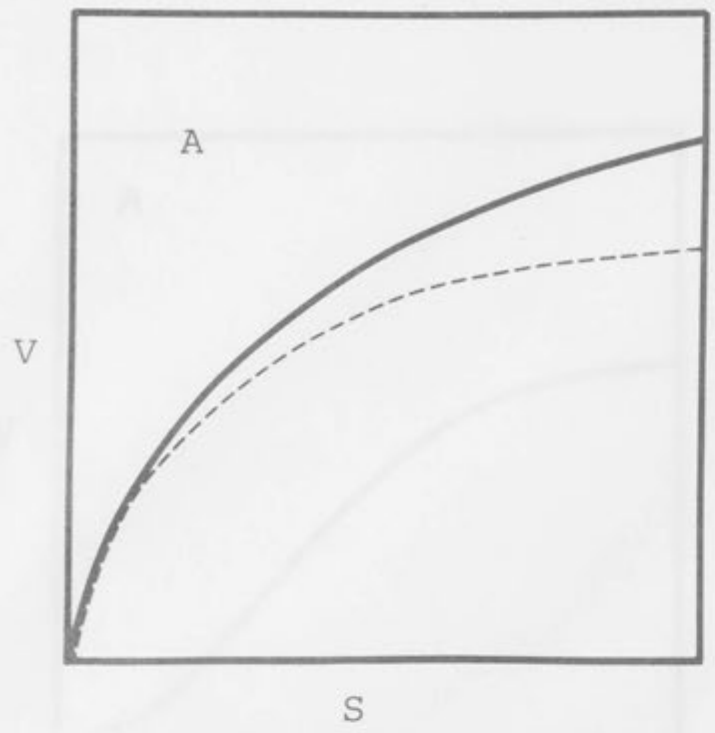


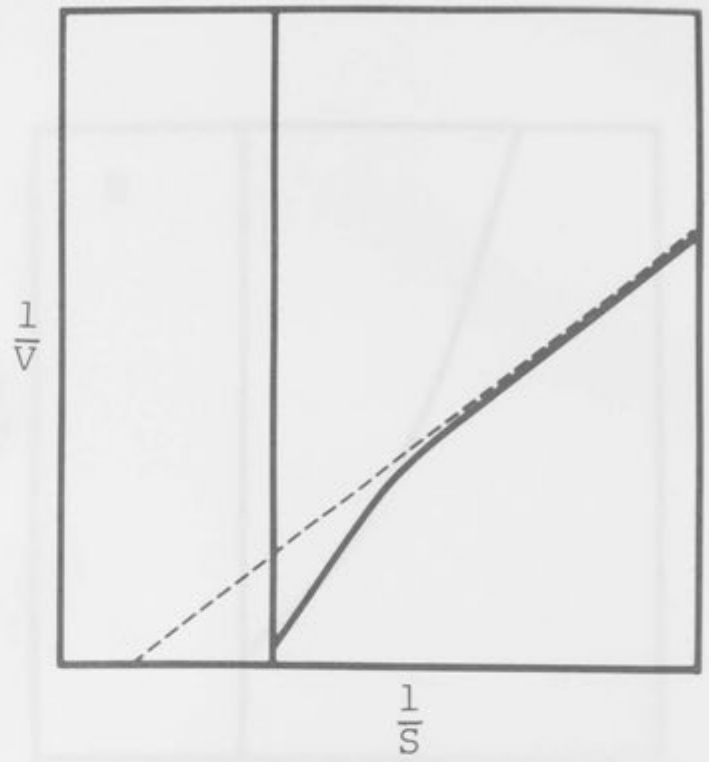
FIG. I.5. Multireactant kinetics.

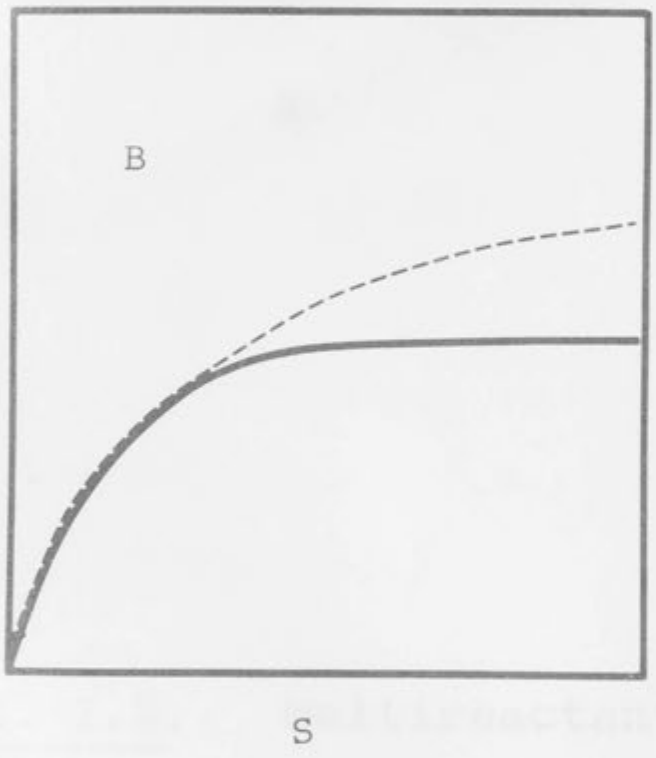
A. Plot of initial velocity as a function of substrate concentration.

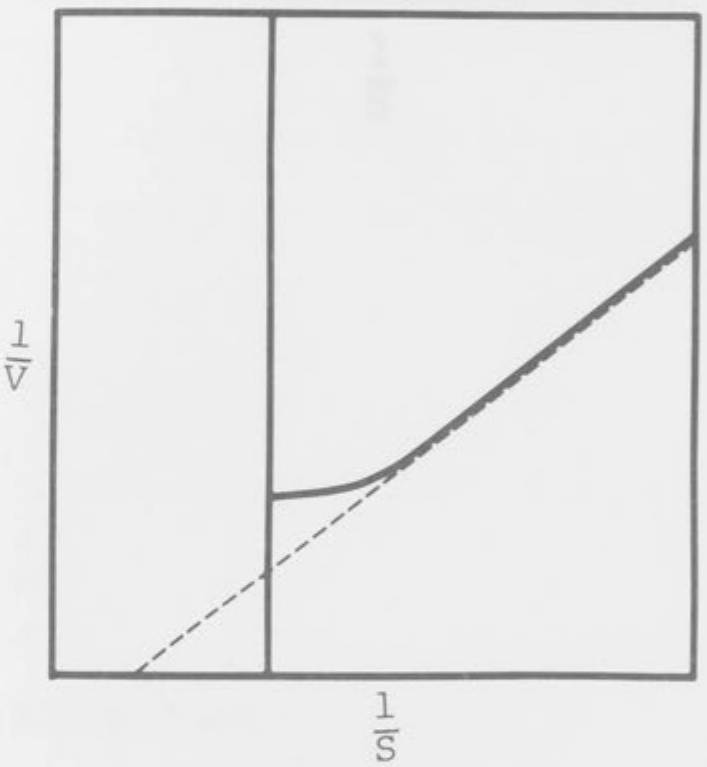
B. Double reciprocal plot.

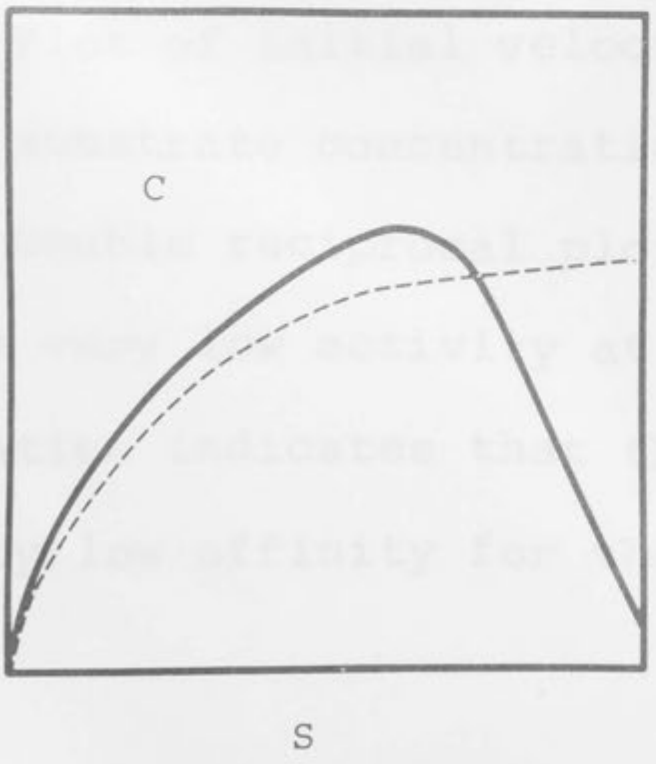
The very low activity at low substrate concentration indicates that the free enzyme has a very low affinity for the substrate.


A
V
S


1/V
1/S


B
V
S


1/V
1/S


C
V
S

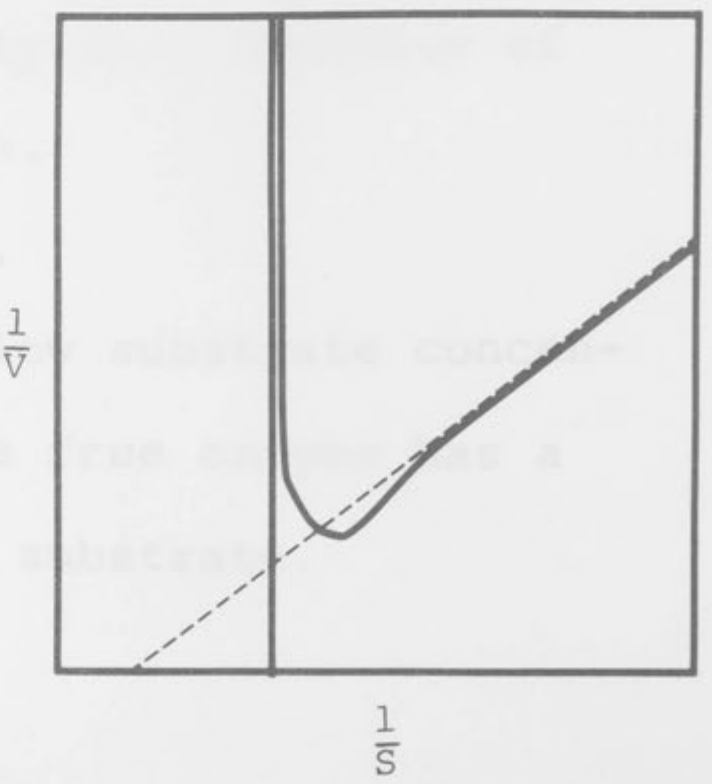

1/V
1/S

FIG. I.6. Multireactant kinetics.

A. Substrate activation

B. Substrate inhibition

C. Substrate activation and inhibition

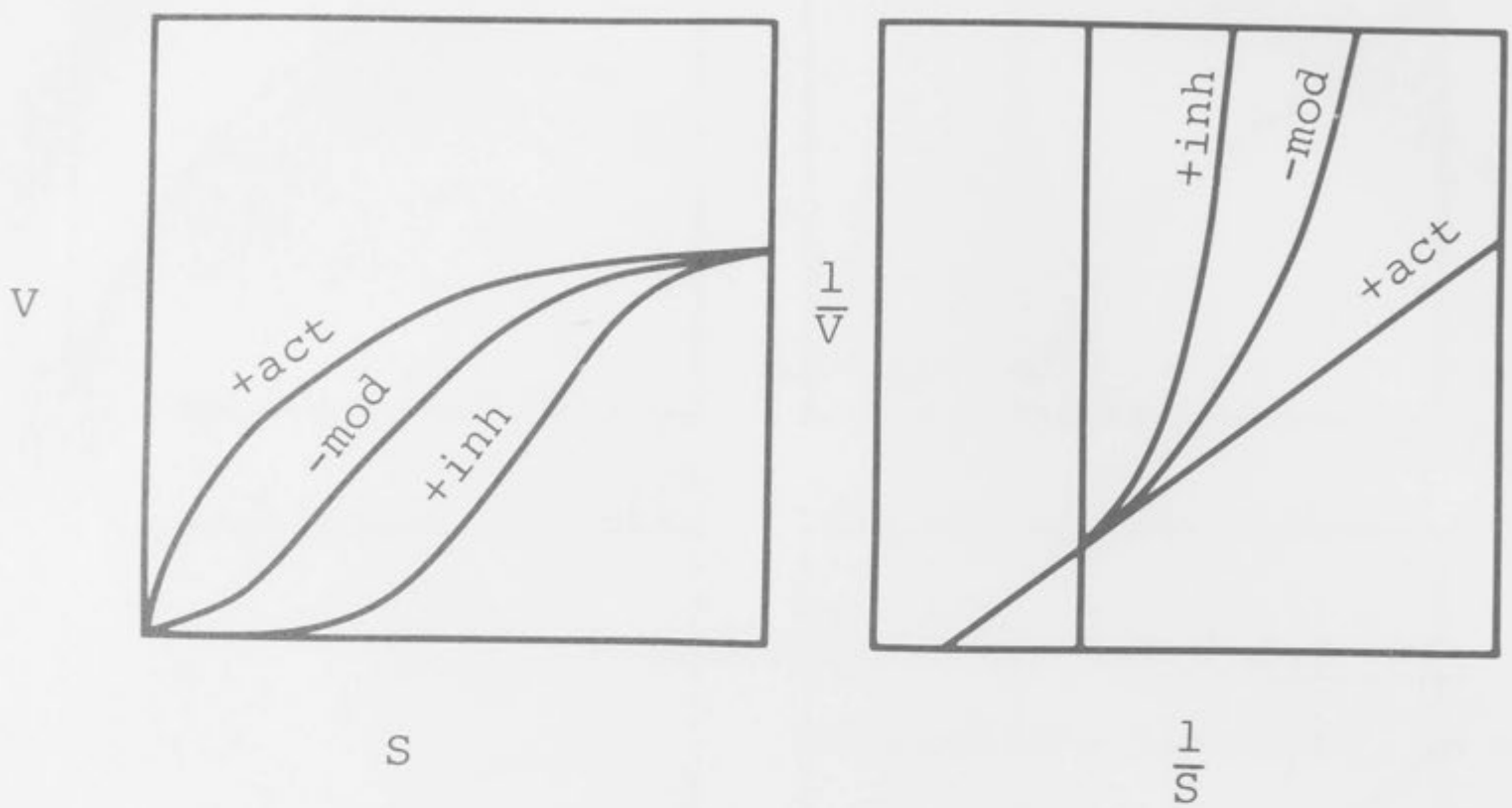


FIG. I.7. Activator and inhibitor effect on sigmoidal multireactant kinetics.

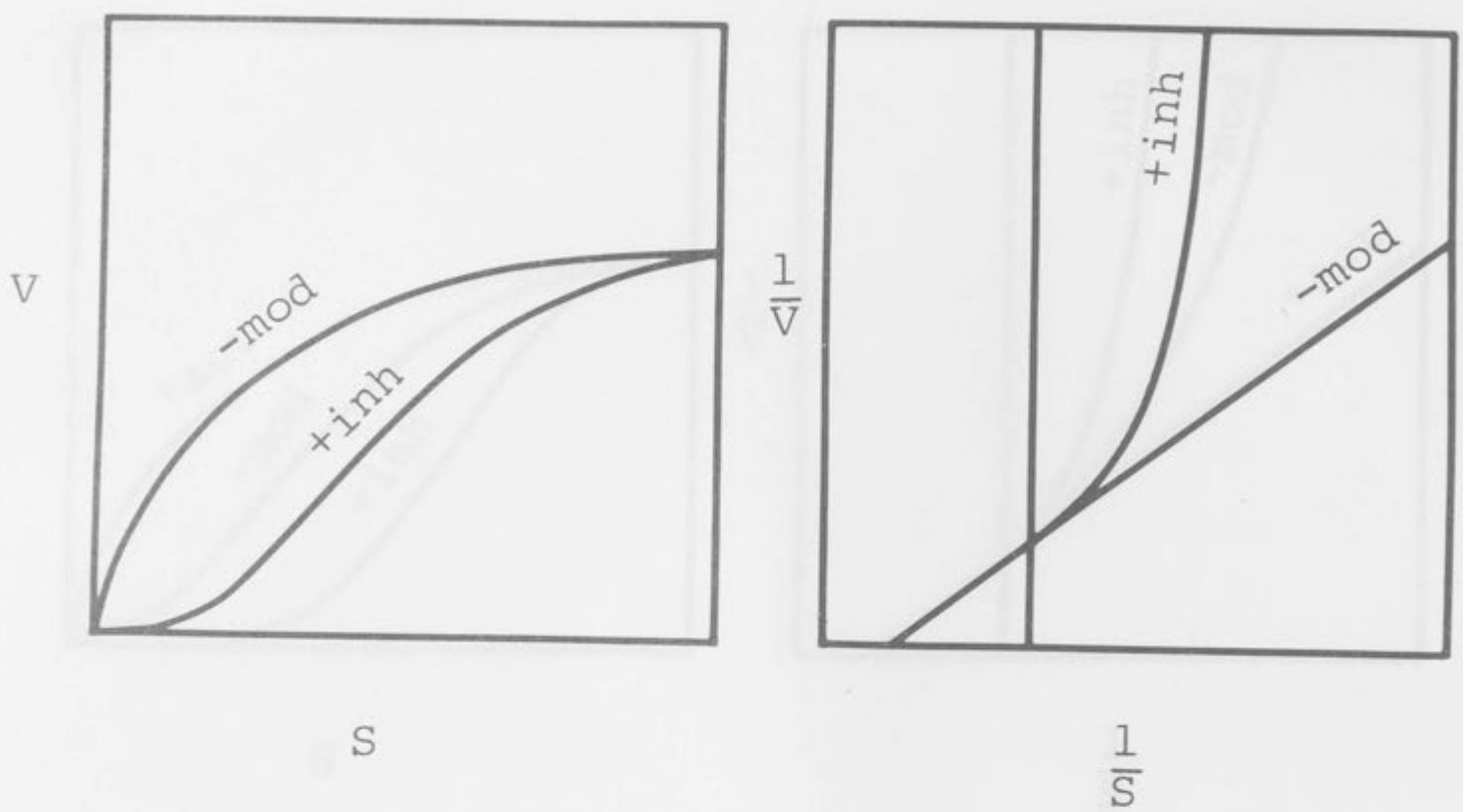


FIG. I.8. Inhibitor effect on a special case of multireactant kinetics shown in Fig. I.7.

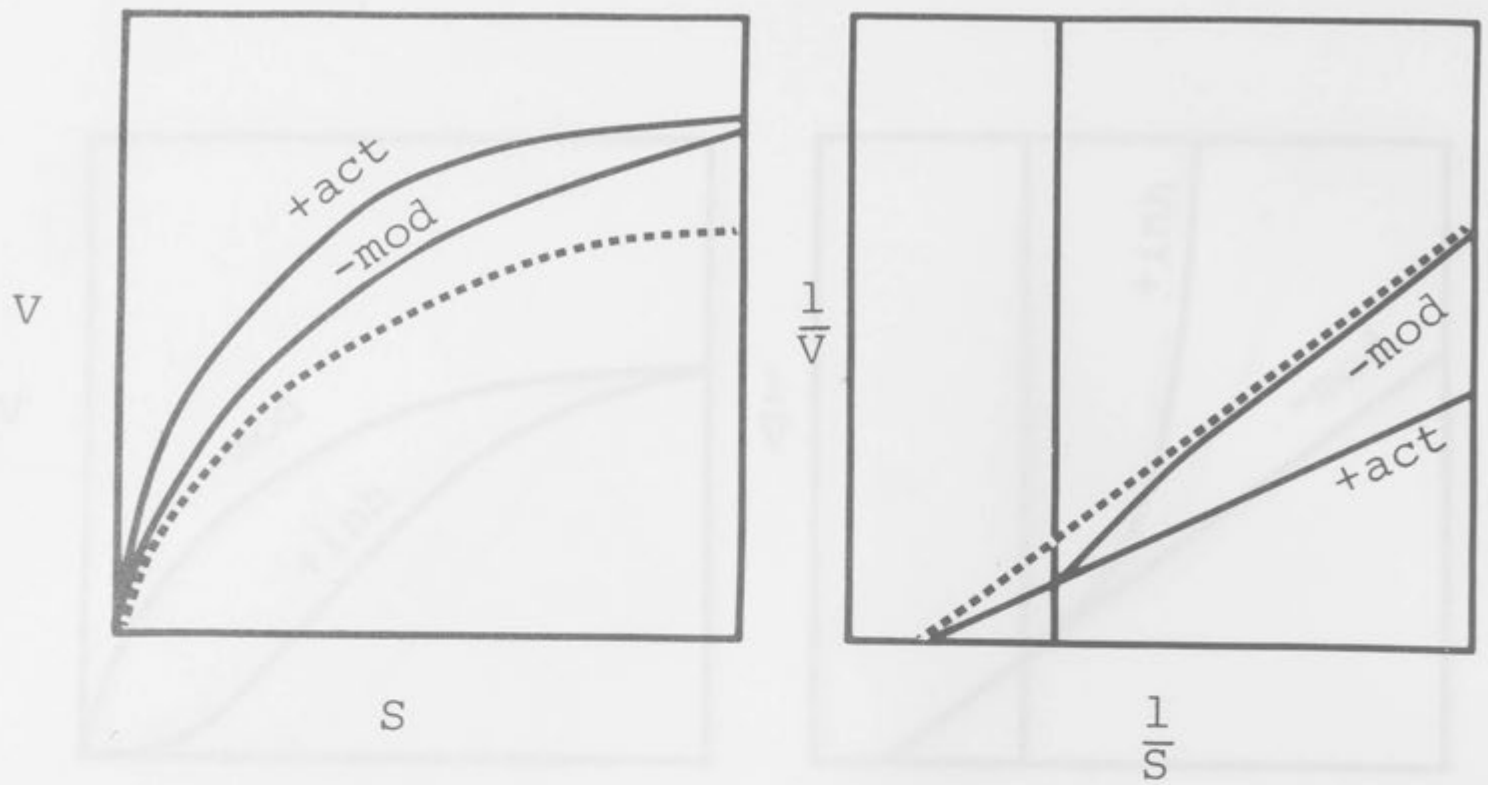


FIG. I.9. Activation of multireactant kinetics. The unmodified reaction depicts the presence of multiple forms of the enzyme which can bind substrate. The effect of activator is to promote the formation of the modifier-enzyme species with the higher affinity for substrate. The unireactant form of the kinetics is indicated by the dashed lines.

substrate concentration is sigmoidal, and the double reciprocal plot hyperbolic or parabolic, as shown in Fig. I.5. Other forms of multireactant kinetics are shown in Fig. I.6. In these cases, product formation occurs at low substrate concentrations, but increasing concentrations either activate the system (Fig. I.6A) or inhibit product formation (Fig. I.6B). Fig. I.6C indicates the plots observed where inhibition at high substrate concentration is preceded by activation at intermediate levels.

The kinetic observations described above may be modified in the presence of allosteric activators or inhibitors, as shown in Figs. I.7, 8 and 9. Generally speaking, allosteric activators tend to establish kinetics conforming to the Michaelis-Menten hypothesis, while allosteric inhibitors increase the multireactant features of the relationship. Examples of these reactions are aspartate transcarbamylase where aspartate and carbamyl phosphate both exhibit sigmoidal kinetics (Fig. I.7) accentuated in the presence of the allosteric inhibitor, CTP. Sigmoidal kinetics are lost in the presence of the allosteric activator, ATP. Fig. I.8 shows a special case of the previous mechanism, exhibited by phosphoribosyl pyrophosphate amidotransfer-

ase of Aerobacter aerogenes (Nierlich and Magasanik, 1965). The double reciprocal plot of initial velocity as a function of PRPP concentration is linear, but becomes parabolic in the presence of AMP or GMP without change in the V_{max}. Multireactant kinetics of the type shown in Fig. I.6C and Fig. I.9 occur with ribonucleoside triphosphate reductase. Frieden (1964) has developed initial velocity equations which predict the behaviour exemplified in Figs. I.5-9.

Enzyme structures proposed to explain allosteric phenomena. Various models have been described in an attempt to elucidate a physical basis for the effects encountered with allosteric enzymes. These include the proposals of Sweeny and Fisher (1968), Nichol et al. (1967) and Frieden (1964), who propose a single catalytic site for each functional unit. Those of Monod et al. (1965) and Koshland et al. (1966) propose multiple catalytic sites on the functional protein (oligomer) which consists of several identical subunits (protomers). The single catalytic site models will be discussed first.

Proposals of Sweeny and Fisher (1968). This model explains sigmoidal kinetics on the assumption that the liberation of product at the single catalytic

site proceeds *via* several reaction mechanisms which function simultaneously. The authors consider only substrate effects, and although they do not offer any explanation for the action of allosteric modifiers exemplified in Figs. I.7-9, these can be rationalised in terms of the model if it is assumed that activators and inhibitors may promote a more favourable or less favourable reaction pathway, respectively. A possible criticism of the hypothesis lies in the feasibility of multiple reactions occurring at a stereospecific site.

Polymerisation (disaggregation) theory of Nichol *et al.* (1967). This theory takes into account the significant changes in size occasionally encountered with enzymes on dilution or in the presence of substrate. The hypothesis explains multireactant kinetics on the basis of an equilibrium between monomer and oligomer states of the enzyme, where both types of molecule can bind the substrate. The equilibrium may be represented

$$nA \rightleftharpoons C$$

If the binding on A and C is considered to be equivalent and if the number of binding sites on A (np) equals the number of binding sites on C (q), classical hyperbolic substrate binding is observed. If either of

these conditions is not met, i.e. if the sites are not equivalent or np does not equal q, the binding curves become sigmoidal, especially if one of the forms (oligomer) does not bind the reactant. Although not stated by the authors, sigmoidal binding would also be obtained if the polymeric species had a small but finite affinity for the substrate. The model assumes that each monomer possesses a catalytic site and a polymerisation site. In the oligomer, access of the substrate to the catalytic site is restricted and in the monomer-substrate complex, polymerisation is retarded. The hypothesis explains multireactant kinetics as described in Fig. I.5.

Nichol et al. (1967) do not consider allosteric activation or inhibition by non-substrate ligands. A simple extension, namely that activators and inhibitors stabilise the monomer or oligomer, respectively, may be used to explain the phenomena depicted in Figs. I.7 and I.8.

Single catalytic site, single modifier site (Frieden, 1964). This model proposes a single protein with one catalytic site and one modifier site. Multireactant kinetics occur if the substrate (at high concentration) can occupy the modifier site to provide

the activated or inhibited form of the enzyme. This effect is presumed to take place via a conformational change in the protein subsequent to occupation of the modifier site. This model can explain all examples of multireactant kinetics and the effects of allosteric activators and inhibitors described in Figs. I.5-9.

Cooperativity (symmetry) hypothesis of Monod et al. (1965). The present formulation extends the earlier concepts of allosterism by postulating that allosteric proteins are composed of a number of identical protomers which constitute the complete oligomer. The model is described by the following statements :

1. Allosteric proteins are oligomers, the protomers of which occupy equivalent positions, implying at least one axis of symmetry.
2. For each ligand (i.e. substrate or modifier) binding stereospecifically to the protein, there is only one site on each protomer.
3. The conformation of each protomer is constrained by its association with other protomers.
4. Two (at least) states designated R and T are reversibly accessible to allosteric oligomers.

5. The affinity of one (or several) of the stereospecific sites towards the corresponding ligand is altered when a transition occurs from one to the other state.
6. When the protein transforms from one state to another, its molecular symmetry (including the symmetry of the conformational constraints imposed upon each protomer) is conserved.

In addition to these features, the model implies that the binding of substrate to one state of the enzyme displaces the equilibrium between the two states of the enzyme. L is the equilibrium constant for this transition $R \rightleftharpoons T$ and

$$L = \frac{[T]}{[R]} .$$

The dissociation constants for a substrate bound to the R and T forms are designated K_R and K_T, respectively. The term c is defined as the ratio between the dissociation constants $\frac{K_R}{K_T}$. As shown by Monod *et al.* (1965), the degree of "cooperativity" (departure from Michaelis-Menten kinetics in the direction of sigmoidal plots of velocity versus substrate concentration) depends on the value of L, c and the number of protomers (n). This effect is more marked when L is large, when c is

small and when n is greater than unity. This type of behaviour is referred to as a homotropic effect. In terms of the model, parabolic double reciprocal plots indicate that equilibrium exists between the states R and T of an oligomeric enzyme and favours T which has much the lower affinity for the substrate. When this is not the case (e.g. when L = 0, c = 1 or n = 1) the double reciprocal plots are linear.

Modifiers, that is allosteric activators or inhibitors, act by displacing the equilibrium between T and R, activators promoting the formation of R, the species of high substrate affinity and inhibitors promoting the formation of T, the species of low substrate affinity. Monod et al. (1965) assume for simplicity that the substrate has a significant affinity only for R (i.e. does not bind to T) and that the inhibitor and the activator bind only to T or R state, respectively. These restrictions are not essential for the treatment of the model, and Monod et al. (1965) are aware that in a practical situation, substrate and modifiers would show significant binding to all states of the enzyme. The effect of allosteric modifiers on the sigmoidal phenomena have been termed heterotropic. The model explains kinetic plots as shown in Fig. I.7 and I.8. The model predicts positive homo-

tropic effects and both positive and negative heterotropic effects.

Frieden (1967) has indicated that as the equation derived by Monod et al. (1965) deals with ligand binding rather than initial velocity data, the use of the modified relationship to apply to kinetic investigations is only valid if three assumptions are met : (i) that conversion of enzyme from one form to the other is not a rate limiting step in the overall reaction; (ii) that the modifier affects only the equilibrium between the two forms and does not affect their intrinsic kinetic parameters; and (iii) the derived equation can define substrate effects, but is not valid for heterotropic effects. Therefore, while the equations derived by Monod et al. (1965) may interpret thermodynamic data, the form modified for use with initial velocity data must be interpreted with caution.

Induced fit (sequential) model of Koshland et al. (1966), Kirtley and Koshland (1967) and Haber and Koshland (1967). This theory provides for a larger number of hybrid states than does that of Monod et al. (1965). The model has general application and Haber and Koshland (1967) have shown that the simultaneously occurring changes in subunit conformation postulated

in the Monod *et al.* (1965) theory provides one of the limiting cases for the sequential hypothesis. The model proposes that a flexible interaction exists between the ligand (substrate or modifier) and the protomer which induces a conformational change in the latter with an alteration in the substrate binding. The effect of this conformational change on adjacent protomers will depend on both the magnitude of the induced change and the strength of the inter-protomer bonds. This theory proposes subunit structure and equilibrium between at least two conformational states. The equilibrium may be perturbed by the binding of substrate or modifier.

Various arrangements of the model have been proposed for four protomers including tetrahedral, square and linear. In the tetrahedral arrangement, one protomer may directly affect all three others; in the square, one protomer is close to two with the third protomer more removed; in the linear arrangements, the central protomers may interact with the two adjacent subunits, whilst the terminal protomers may act directly with only one other unit. Koshland *et al.* (1966) have shown that these arrangements of the protomers cannot be distinguished by thermodynamic treatments, and all arrangements are equivalent for the purpose of this discussion. The essential differ-

ence from the Monod *et al*. (1965) hypothesis is the relatively large number of possible hybrid oligomer configurations. The model predicts both positive and negative homotropic and heterotropic effects.

The multireactant kinetics shown in Fig. I.7 and I.8 can be interpreted in terms of this model. As the hypothesis incorporates the concept that substrate may bind at the modifier site, the multireactant kinetics described in Figs. I.6 and I.9 may also be explained. The generality of the treatment permits its application to almost any allosteric reaction, which, of course, is catalysed by a protein with the necessary subunit structure. However, as the initial concept was developed on the basis of binding data, similar considerations to those discussed for the Monod *et al*. (1965) hypothesis should be used when applying the theory to initial velocity studies.

Methods of investigating regulatory mechanisms. Although some types of kinetic behaviour of allosteric enzymes (c.f. Fig. I.6) are difficult to explain on the basis of many of the proposed models, all of the theoretical treatments described in the previous section are compatible with the commonly encountered types of multireactant kinetic behaviour. Generally,

chemical and other physical properties of the enzyme must be investigated in order to determine which of the various models applies to a particular system.

Binding studies. The use of equilibrium dialysis and gel filtration techniques can provide important information for differentiation of the models. If the number of binding sites for substrate and/or modifier is large, e.g. four, this result may suggest one of the cooperative mechanisms. Conversely, the presence of only a single binding site for substrate or modifier excludes these possibilities, but is consistent with the Sweeny and Fisher (1968) theory.

The plots of binding as a function of substrate concentration will be sigmoidal in the case of the cooperative or aggregation models, but will be hyperbolic when the predominant effect causing the multireactant kinetics is a change in a rate limiting step with consequent alteration in V_{max}. A mechanism involving alterations in V_{max} can also be inferred from the observation of unchanged binding of substrate in the presence of a modifier. Binding studies may be used to determined whether a ligand exhibits two or more widely differing dissociation constants with a particular enzyme. Where more than one value is ob-

tained, the mechanisms involving cooperativity are excluded as these models do not predict more than one detectable dissociation constant. The cooperative model of Monod et al. (1965) would also be excluded where competition existed between substrate and modifier for a binding site on the protein.

This technique appears to be a useful tool in determining which model applies to the situation under study. However, the method requires relatively large amounts of pure enzyme of known molecular weight and methods must be available for the accurate quantitative determination of low substrate concentrations and of small relative changes in substrate concentration. Limitations in analytical methods usually cause relatively large errors which render interpretation difficult.

Disaggregation of the protein. The theory of Nichol et al. (1967) postulates that disaggregation occurs under physiological conditions and that the disaggregation equilibrium is influenced by substrate. Therefore, it should be possible by polyacrylamide gel electrophoresis, gel filtration or ultracentrifugal analysis to detect the presence of low molecular weight species in the presence of substrate if the

enzyme behaves as described by this model. From the extension of the theory previously described, the addition of an allosteric modifier should affect such disaggregation.

Determination of subunit structure. Tryptic digestion followed by peptide mapping in conjunction with the results of amino acid analysis and physical determination of the molecular weight gives a value for the maximum chemical molecular weight. In the absence of identical subunit structure the chemical and physical molecular weight are the same, a result which would exclude the cooperativity models. Where the physical molecular weight is a multiple of the chemical molecular weight, the data are consistent with the presence of identical subunits. End-group analysis may be used to provide supporting evidence regarding the possible presence of subunits but quantitation is difficult. Complete amino-acid sequence data provides the most unequivocal evidence of subunit structure.

Subunit structure may be indicated by a change in molecular weight after treatment with high concentrations of urea, guanidine hydrochloride or in the presence of mercurial compounds. While these treat-

ments should disrupt only non-covalent bonding, care should be exercised against rupture of peptide bonds during the investigation.

Conformational changes associated with substrate and modifier binding. As all of the theories described postulate, or are compatible with, a conformational change induced in the enzyme by the binding of ligands, the demonstration of such a change does not discriminate between the proposed mechanisms of allosteric action. However, distinctions can be made by relating the degree of saturation of the enzyme with substrate to the degree of conversion of the enzyme to the changed state. For this purpose, any property of the enzyme which reflects the change in state may be used. Such properties which have been used include sedimentation velocity, reaction rate with organic mercurials (Gerhart and Schachman, 1968), rate of tryptic digestion (McClintock and Markus, 1968, 1969) and compliment fixation in the presence of specific antisera (Bethell et al., 1968b). McClintock and Markus (1969) have proposed that if the substrate concentration required to convert a given percent of enzyme to the changed state is plotted against the substrate concentration required to give the same

percent maximum activity, a linear relationship is obtained where the conformational change is sequential as in the Koshland et al. (1966) model, but is non-linear for a concerted mechanism as proposed by Monod et al. (1965).

Analysis of an enzyme along the lines proposed above may provide sufficient data to determine which proposal best describes the properties of a particular enzyme.

Aspartate transcarbamylase and allosterism. The development by Gerhart and Holoubek (1967) of a specific strain of E. coli diploid in the cistrons of aspartate transcarbamylase synthesis and especially selected for a limited ability to synthesise pyrimidines has permitted the production of gram quantities of the crystalline enzyme. Largely because of consequent availability of large amounts of this protein in pure form it is the best characterised allosteric enzyme at the present time. The fact that the structural and functional properties of this enzyme appear to conform to the postulates of Monod et al. (1965) and Koshland et al. (1966) has stimulated investigation of the mechanism of regulation of this enzyme. Reported physical parameters are shown in

Table I.3.

Aspartate transcarbamylase catalyses the first step in the synthesis of pyrimidines and is subject to allosteric inhibition by CTP, the end product of the reaction sequence, and allosteric activation by ATP. These effects serve to regulate the synthesis of pyrimidines according to physiological requirements. Initial velocity plots as a function of substrate concentration are sigmoidal in the case of aspartate (Gerhart and Pardee, 1962; Gerhart, 1964) and also with the other substrate carbamyl phosphate (Bethell et al., 1968a). Allosteric modifiers produce a change in the kinetics as shown in Fig. I.7.

Mercuribenzoate (PMB) causes dissociation of the enzyme into two species of different molecular weights which may be separated by ion exchange chromatography or zone ultracentrifugation. The subunits which have molecular weight 1.0×10^5 and 2.7×10^4 bind substrates and modifiers, respectively (Changeux et al., 1968). The isolated subunits that bind substrate have catalytic activity greater than that of the native enzyme at low substrate concentrations and double reciprocal plots obtained with these subunits are linear. The subunits that bind modifiers

TABLE I.3. Aspartate transcarbamylase

Author	Year	No. of Binding Sites		Method used	Molecular Weight			Method
		Catalytic	Regulatory		ATC	Catalytic	Regulatory	
Gerhart Schachman	1965	-	8	Ultracentrifuge	3.1×10^5	1.0×10^5	3×10^4	Ultracentrifuge
Herve Stark	1967				3.1×10^5	1.0×10^5	2.7×10^4	N Terminal amino acid analysis Fingerprint
Changeux								
Gerhart Schachman	1968	4	4	Binding studies e.g. dialysis	3.1×10^5	1.0×10^5	3×10^4	Ultracentrifuge
Weber	1968a				3.1×10^5	4.2×10^4	2.3×10^4	Ultracentrifug-ation 8 M urea
Weber	1968b				3.1×10^5	3.3×10^4	1.7×10^4	Amino acid sequence
Wiley Lipscomb	1968				(3.1×10^5)	3.3×10^4*	1.7×10^4*	Crystallography

*These values for the molecular weight of the subunits were calculated from the model postulated from crystallographic studies namely six catalytic and six regulatory subunits.

(regulatory subunits) are completely devoid of catalytic activity. Preliminary studies of sedimentation in the presence of 3 M guanidine hydrochloride suggested that the catalytic subunit (mol. wt. 1.0 x 10^5) comprises "two or more" polypeptide chains (Gerhart and Schachman, 1965) and led to the assumption that the enzyme is comprised of four regulatory subunits and four catalytic subunits. Studies by Hérve and Stark (1967) using end group analysis and peptide mapping substantiated this hypothesis. Earlier binding studies (Gerhart and Schachman, 1965) indicated the binding of 8 moles of modifier analogue per mole of enzyme, and it was assumed that each regulatory subunit contained two modifier binding sites. Subsequent binding studies by Changeux et al. (1968) have been interpreted as only one binding site per regulatory subunit and one substrate site for succinate (analogue of aspartate) on each of the catalytic units. The structure of this enzyme appears to provide the first evidence for the regulatory system described by Monod et al. (1965) with an oligomeric structure composed of four catalytic-regulatory unit monomers. The binding of substrate in conjunction with the physical measurements of sedimentation

velocity and rate of reaction with PMB have been interpreted by Changeux and Rubin (1968) in terms of this concerted cooperative model. Evidence is submitted that with only 15% of the protein bound with substrate analogue, 50% of the enzyme exists as the loose configuration (i.e. the R state of the Monod et al. (1965) hypothesis).

McClintock and Markus (1968) present data on the tryptic digestion of aspartate transcarbamylase in the presence of substrate (aspartate) and allosteric modifiers (CTP and ATP). In contrast to the studies described above, these authors found that the variation of the state of the enzyme as measured by its susceptibility to proteolytic digestion in the presence of various concentrations of substrate, exactly correlated with the curves of activity as a function of the substrate concentration. They consider that the sequential model of Koshland et al. (1966) offers the more consistent explanation for these phenomena. McClintock and Markus (1969) conclude that binding of substrate and analogue may differ in their effect on the intersubunit binding.

Fellenberg et al. (1968) have studied aspartate transcarbamylase using compliment fixation with

specific antisera as a measure of the conformational change in aspartate transcarbamylase and its subunits. Their findings suggest that the maximum effect observed on the complete molecule after the binding of ligand is in the particular subunit bound, be it catalytic or regulatory. In the apparent absence of the concerted effect predicted by Monod et al. (1965), these results also seem to favour the sequential theory of Koshland et al. (1966).

Ultracentrifugal analysis of the PMB dissociated subunits (Weber, 1968a) in 8 M urea appear to confirm the proposed structure of four regulatory and four catalytic subunits, although the published molecular weights are somewhat lower than those of Gerhart and Schachman (1965). Weber (1968b) has now suggested, on the basis of amino acid sequence studies on the regulatory subunit, that the molecular weight of this polypeptide is 1.7×10^4 and although the sequence studies on the catalytic subunit are incomplete, he has postulated that the substrate structure of the enzyme is of the form comprising six catalytic units and six regulatory subunits. The crystallographic studies of Wiley and Lipscombe (1968) in suggesting at least two axes of symmetry appear to be compatible with this conclusion.

The history of the investigation of this enzyme illustrates that data are still imperfect and that interpretation is difficult, especially with the binding studies. Molecular weights derived from ultracentrifugal analysis in 8 M urea may be subject to large errors (c.f. Weber, 1968b) which make interpretation difficult. The results of the amino-acid sequence studies are potentially the most accurate and in view of the present discrepancies, final assessment of structure may require complete elucidation of the amino-acid sequence.

Ribonucleotide reductase and allosterism. Kinetic investigation of the two most highly purified ribonucleotide reductases from E. coli and L. leichmannii indicate that both enzymes are subject to allosteric modification in the presence of specific nucleoside triphosphates. While both activation and inhibition have been demonstrated with ribonucleoside diphosphate reductase, no allosteric inhibitors have been found for ribonucleoside triphosphate reductase (Fig. I.2). Beck (1967) has shown in a reaction mixture in the absence of alkali-metal ions, that reduction of a nucleoside triphosphate in the presence of the specific deoxynucleoside triphosphate activator may be inhibited to a certain extent in the presence of a

non-specific deoxynucleotide, presumably through competition for the allosteric site.

Vitols et al. (1967) propose a single catalytic site-single modifier site model to explain the observed kinetic behaviour of ribonucleoside triphosphate reductase. Substrate activation occurs when the modifier site is occupied by substrate at high substrate concentrations.

Goulian and Beck (1966) concluded that reported multiple forms of the enzyme observed on polyacrylamide gel electrophoresis and sedimentation analysis were due to aggregation of the enzyme. The only evidence for cooperative effects was supplied by Beck et al. (1966), who published sigmoidal plots of initial velocity as a function of E. coli thioredoxin-$(SH)_2$ concentration. However, these plots could not be observed in this laboratory with E. coli thioredoxin (Vitols, unpublished results) nor with L. leichmannii thioredoxin (Orr and Vitols, 1966).

The subunit structure of ribonucleoside diphosphate reductase has been well characterised. Goulian and Beck (1966) have suggested that aggregation of the molecule may explain the multiple bands seen on polyacrylamide gel electrophoresis. Apart from

hydrogen donor specificity, however, the reductases isolated from E. coli and L. leichmannii bear few similarities (Table I.2). The work reported in this thesis describes the isolation in pure form of the three proteins involved in ribonucleotide reduction in L. leichmannii and attempts to obtain evidence for the mechanism of allosteric regulation of the reductase.

CHAPTER II

THE PURIFICATION OF COBAMIDE-DEPENDENT RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE FROM LACTOBACILLUS LEICHMANNII

Introduction

Ribonucleotide reductase of Lactobacillus leichmannii (Blakley et al., 1964; Blakley, 1965) has been extensively purified in this laboratory (Vitols et al., 1967a; Hogenkamp, 1967). Highly purified enzyme preparations reduce between 50 and 180 μmoles of ATP per hour per milligram of protein under standard conditions (Blakley, 1966a) as assayed by the diphenylamine method (Blakley, 1966b). Chromatography of such enzyme preparations under various conditions on DEAE-Sephadex, hydroxyapatite, calcium phosphate or alumina C_γ gel and fractional precipitation with ethanol in the presence of Mg^{2+} ions did not produce significant further increase in specific activity (Brownson and Blakley, unpublished results). When the purified enzyme was examined in the ultracentrifuge under conditions for the determination of sedimentation velocity, a single, symmetrical protein boundary was observed (Vitols et al., 1967b).

An alternative purification procedure for the

isolation of this enzyme from L. leichmannii has been described by Goulian and Beck (1966), who have demonstrated a specific activity of 35 µmoles of dCTP produced per hour per mg of this preparation. As this rate of CTP reduction under conditions of activation by ATP is approximately 2.2 times the rate of reduction of ATP under the assay conditions used in this laboratory (Vitols et al., 1967a; Blakley, 1966a), the enzyme reported by Goulian and Beck would have an activity under the latter conditions of 16 µmoles of ATP reduced per hour per mg of protein. They have shown that this enzyme migrated as a single band during electrophoresis on cellulose acetate at three different pH values and that it appeared to be homogeneous in the ultracentrifuge. However, when the preparation was subjected to electrophoresis on polyacrylamide gel, two major and two minor bands were observed.

The object of the present work was to investigate whether the enzyme prepared by the method of Vitols et al. (1967a) was indeed homogeneous as suggested by the initial data obtained with the ultracentrifuge, or whether electrophoretic examination would indicate

apparent heterogeneity as in the preparation by Goulian and Beck (1966). In fact, the results of electrophoresis on polyacrylamide gel appeared to indicate the presence of proteins in various amounts that migrated at several different velocities (Vitols et al., 1967b). In view of this discrepancy between the results with electrophoresis on polyacrylamide gel and the apparent homogeneity as indicated by the behaviour in the ultracentrifuge, both in the work of Goulian and Beck and the present study, we have investigated the origin of the multiple bands observed in electrophoresis experiments. It has been shown that although bands of more slowly migrating protein are produced during electrophoresis in buffer systems which operate at pH 9 to 10, systems which resolve in the pH range 5.8-8.0 separate the enzyme from other inactive contaminating proteins, without the production of any slowly migrating material. By the use of such systems it has been possible to demonstrate the heterogeneity of the ribonucleotide reductase purified by conventional methods. The application of this new buffer system to preparative electrophoresis has enabled the isolation of active enzyme which will migrate as a

single band during electrophoresis on polyacrylamide gel.

Materials and Methods

Chemicals. Acrylamide, N,N'-methylene-bis-acrylamide and N,N,N',N'-tetramethylene diamine (TEMED) were purchased from Eastman Organic Chemicals, Rochester; the last reagent was redistilled under reduced pressure prior to use. N-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES) and 2-(N-morpholino)ethane sulphonic acid (MES) (Good et al., 1966) were obtained from Calbiochem., Los Angeles.

Enzyme preparation. Reductase was purified from extracts of cells grown as previously described (Ghambeer, 1966; Blakley, 1965). Unwashed, wet cell-paste (100-140 g) was suspended in seven volumes of 0.1 M potassium phosphate buffer pH 7.3 containing 1 mM EDTA; cells were disrupted and debris removed as previously described (Vitols et al., 1967a) (Step 1). After removal of some impurities from the cell-free extract with protamine sulphate as reported (Vitols et al., 1967a) (Step 2), reductase was precipitated at pH 7.3 by the slow, mechanical addition of crystalline ammonium sulphate (0.36 g/ml of protein

solution from Step 2). Stirring was continued for 30 minutes after the addition of the ammonium sulphate, and the precipitated protein was collected by centrifugation. The precipitate was dissolved in a minimal volume (about 30 ml) of 0.01 M Tris-acetate buffer, pH 7.3, containing 1 mM EDTA, and was dialysed against 20 volumes of the same buffer for at least four hours (Step 3). The reductase solution was applied to a 110 x 4 cm column of Sephadex G100 previously equilibrated with the Tris-acetate buffer described in Step 3; this buffer was also used to elute the proteins. Active fractions were combined (total volume 100-200 ml) (Step 4). Reductase was precipitated by the slow addition of crystalline ammonium sulphate (0.43 g/ml of solution from Step 4). The suspension was stirred for 30 minutes after the addition of all the ammonium sulphate and the precipitate collected by centrifugation. The precipitated protein was dissolved in a minimal volume (about 30 ml) of 0.01 M imidazole-acetate buffer, pH 5.0, containing 1 mM EDTA and dialysed against 20 volumes of the same buffer for at least four hours (Step 5). The reductase solution was then applied to a 110 x 4 cm column of Sephadex G100 previously equilibrated with the imidazole-

acetate buffer described in Step 5; this buffer was also used to elute the proteins. Active fractions were combined (total volume 100-200 ml) adjusted to pH 7.0 and made 10 mM with respect to EDTA (Step 6). Protein was fractionally precipitated by the slow mechanical addition of crystalline ammonium sulphate. After the addition of 0.29 g of ammonium sulphate per ml of solution from Step 6, the suspension was stirred for a further 30 minutes and centrifuged. To the supernatant solution was added 0.07 g of ammonium sulphate per ml of solution from Step 6 and the protein suspension was centrifuged after 30 minutes stirring. A further 0.07 g of ammonium sulphate per ml of solution from Step 6 was added to the supernatant and the suspension centrifuged after 30 minutes stirring. The three fractions of precipitated protein obtained after the additions of ammonium sulphate were dissolved separately in minimal volumes of 0.1 M DMG buffer, pH 7.3 (volumes 5-20 ml) and were dialysed against this buffer (25 volumes) for at least four hours (Step 7). These reductase preparations had a specific activity in the range 50-110 µmoles of ATP reduced per hour per mg of protein. Total yield was 100-200 mg. Reductase from Step 7 was used for most of the experi-

ments described. For some purposes, less pure material from Steps 3 or 5 was used.

Activity determinations on ribonucleotide reductase

Colorimetric assay. Mixtures were prepared as described by Blakley (1966a) and contained 1 M sodium acetate. Reaction time was ten minutes. The amount of dATP produced was determined by the diphenylamine method (Blakley, 1966b) with the modification that the mixture was made 1 mM with respect to copper acetate prior to the addition of the diphenylamine reagent. Deoxyadenosine was used as a standard. Absorbance at 595 mμ was measured after 16 hours incubation at 37°C. Specific activity is expressed as the number of μmoles of ATP reduced per hour per mg of protein.

Spectrophotometric assay. This was performed with a Cary 14 recording spectrophotometer as previously described (Orr et al., 1966). Thioredoxin and thioredoxin reductase were isolated from L. leichmannii by a method described in Chapters IV and V, or from Escherichia coli as described by Laurent et al. (1964).

Protein determinations. These were performed by the biuret method of Gornall et al. (1949), or spectrophotometrically from the absorbance at 280 mμ.

A solution containing 1 mg of ribonucleotide reductase per ml was shown to have an absorbance at 280 mμ of 1.09. The reductase used for this determination was homogeneous when electrophoresed in polyacrylamide gel at pH 6.8 at 3°. A solution of this material of known absorbance at 280 mμ was extensively dialysed against distilled water and the dry weight determined after lyophilization.

Analytical polyacrylamide gel electrophoresis

Apparatus and gels. Samples were electrophoresed in an apparatus manufactured to the design of Davis (1964). Gels were cast in uniform bore glass tubes, 0.5 cm internal diameter and 7.5 cm long. Early experiments were performed with a polyacrylamide gel polymerised from a solution of cyanogum (Raymond et al., 1959), but later, gels were prepared from solutions containing 9% acrylamide and 0.1% bis acrylamide. The above mixture was used routinely for all experiments with ribonucleotide reductase, except where stated otherwise.

Buffers. The 0.02 M Tris-glycine buffer pH 8.4, used throughout the apparatus in the initial experiments was later replaced with the discontinuous buffer system of Ornstein (1964) as modified for preparative

electrophoresis by Thelander (1967) (Table II.1). As will be shown, the latter system was unsatisfactory for use even in analytical work and other buffer systems were investigated. The TEA-TES-chloride system also shown in Table II.1 was used for the majority of the present work.

Catalyst system for gel polymerisation. Riboflavin (13.3 μM) was added to the gel solution and photopolymerisation was effected with a Buchler light source. TEMED concentrations used were the same as those described by Thelander (1967) (Table II.1).

Temperature. Electrophoreses were performed at 0-3°C unless otherwise stated.

Destaining of gels. Gels stained with amido-black were decolourised in 500 volumes of 7% acetic acid at 50°C in a New Brunswick gyrotary shaker. Although not completely destained, most protein bands could be discerned easily after 8-12 hours. The protein free portions of the gels were completely destained after a further 8 hours shaking in more 7% acetic acid. Electrophoretic destaining as described by Davis (1964) was of no use because it produced further migration of the stained protein.

TABLE II.1. Details of the Tris-glycine-chloride buffer system and the TEA-TES-chloride buffer system used for analytical and preparative electrophoresis of ribonucleotide reductase

References	Thelander (1967)	Standard system used for electrophoresis of ribonucleotide reductase
Buffers used	Tris-glycine-chloride	TEA-TES-chloride
Operating pH of resolving gel	8.9-10.0	6.8-8.0
Constituents of upper electrode buffer		
Molarity of slow anion	0.0373 M glycine	0.0384 M TES
NaEDTA	0.001 M	0.001 M
Cationic buffer	Tris	TEA
Final pH	8.3 (25°)	6.3 (0°)
Constituents of upper gel buffer (Final concentrations)		
Fast anion	.032 M Phosphate	.06 M Cl^-
Catalyst (hydrogen donor)	.0043 M TEMED	.0043 M TEMED
NaEDTA	.001 M	.001 M
Cationic buffer	Tris	TEA
Final pH	6.9 (25°)	5.8 (0°)
Constituents of resolving gel buffer (Final concentrations)		
Fast anion	.06 M Cl^-	.06 M Cl^-
Catalyst (hydrogen donor)	.0065 M TEMED	.0065 M TEMED
NaEDTA	.001 M	.001 M
Cationic buffer	Tris	TEA
Initial pH	8.8-8.9 (25°)	6.8 (0°)
Constituents of lower electrode buffer and elution buffer		
Anion	.06 M Cl^-	.032 M Phosphate
NaEDTA	.001 M	.001 M
Cationic buffer	Tris	TEA
Final pH	7.0 (25°)	5.8 (0°)

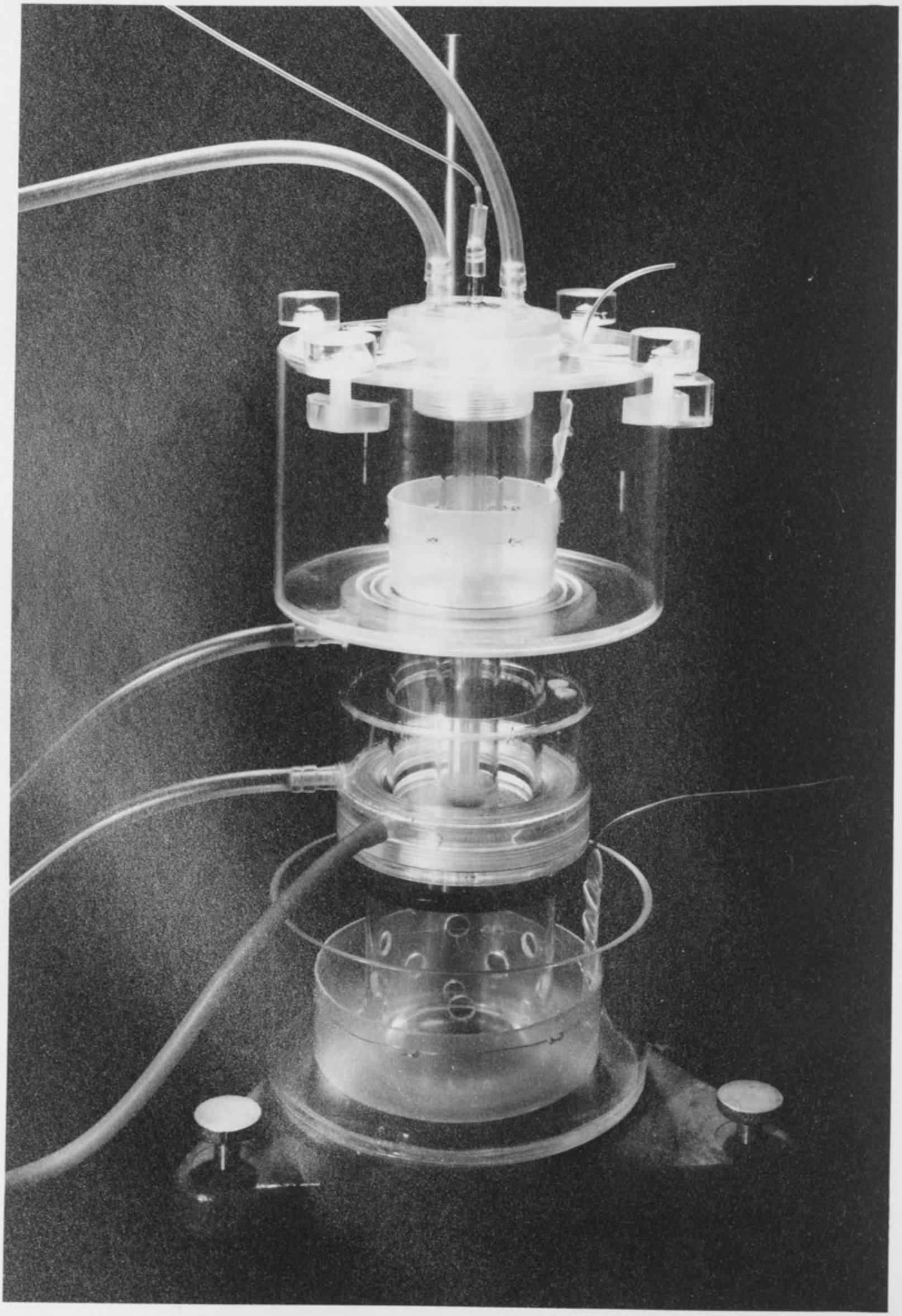


FIG. II.1. Apparatus used for preparative polyacrylamide-gel electrophoresis.

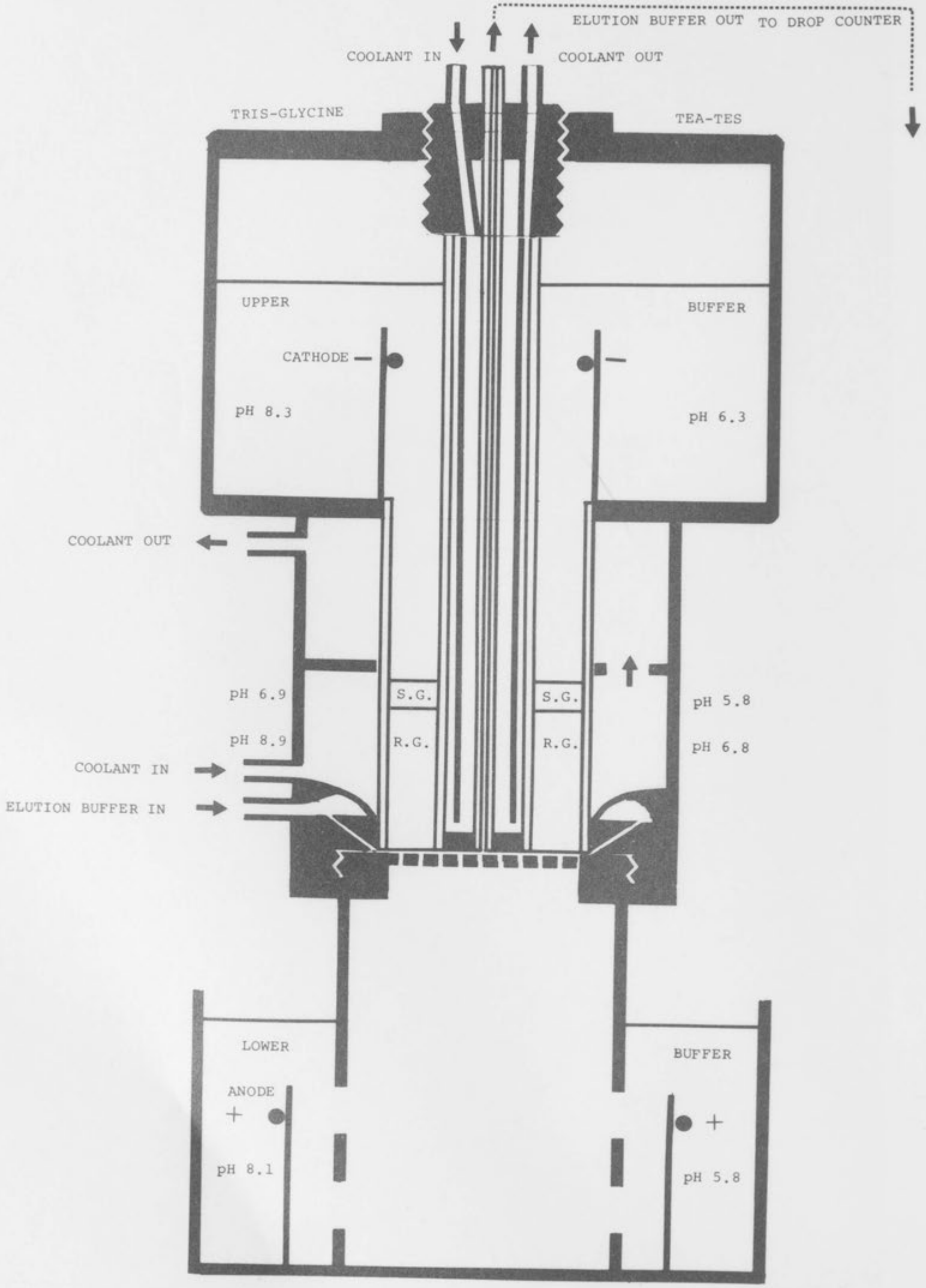

ELUTION BUFFER OUT TO DROP COUNTER
COOLANT IN
COOLANT OUT
TRIS-GLYCINE
TEA-TES
UPPER
BUFFER
CATHODE
pH 8.3
pH 6.3
COOLANT OUT
pH 6.9
pH 8.9
S.G.
R.G.
S.G.
R.G.
pH 5.8
pH 6.8
COOLANT IN
ELUTION BUFFER IN
LOWER
BUFFER
ANODE
pH 8.1
pH 5.8

FIG. II.2. Vertical cross sectional view of the assembled apparatus used for preparative electrophoresis.

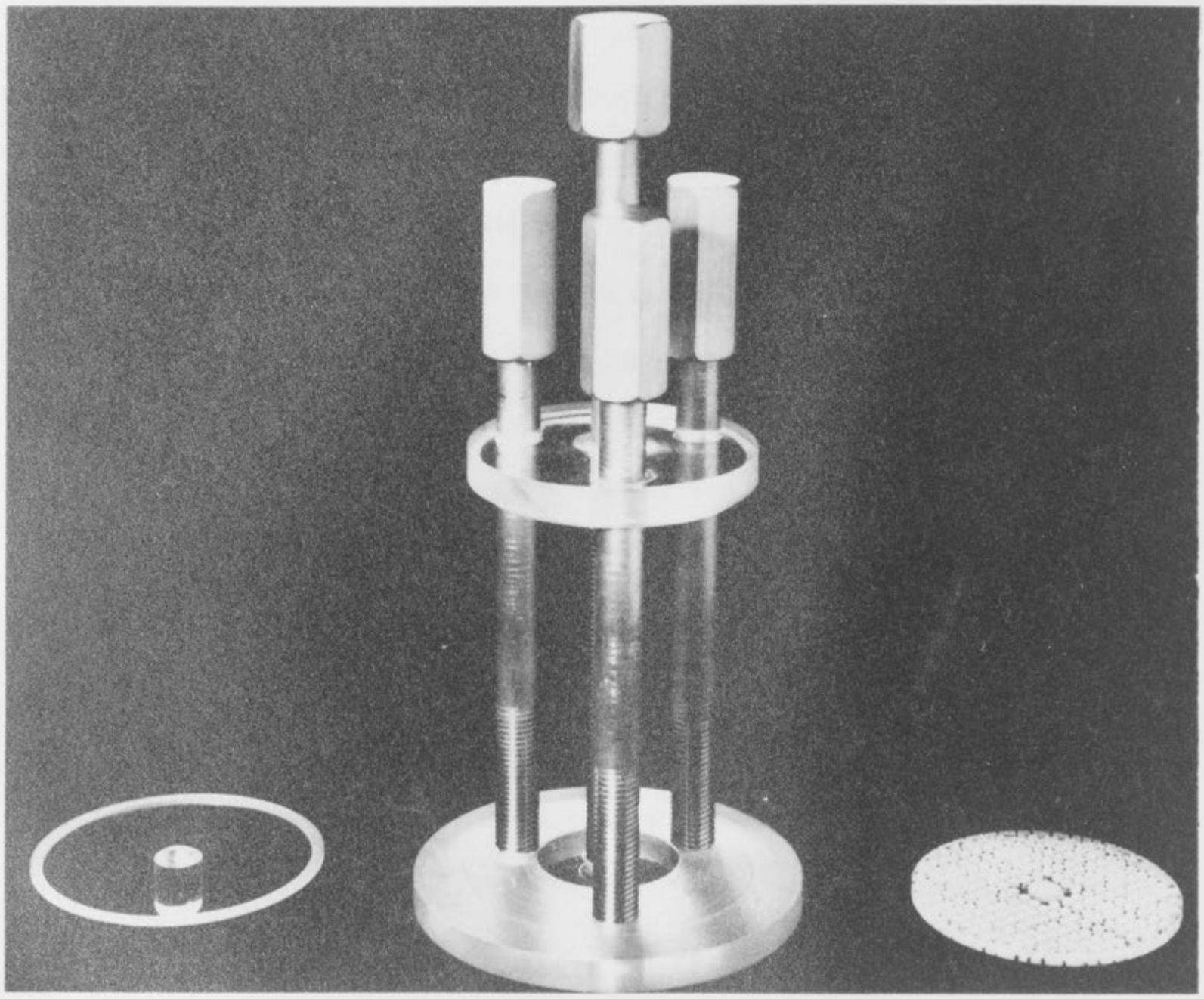
Annular bands migrate down through the gel into the space between the gel and the semipermeable membrane, and are eluted through the central capillary by the centripital flow of buffer.

Preparative polyacrylamide gel electrophoresis

(a) Apparatus. Some initial recovery determinations were performed using the apparatus and buffers described by Shuster and Schrier (1967). A commercial apparatus, purchased from I.W. Lowe of Adelaide, was used for most of the preparative work described. The design incorporates the essential features of the system described by Jovin et al. (1964). The basic unit (Fig. II.1) is relatively inexpensive because it is constructed of perspex which permits greater ease of precision manufacture than glass. Critical dimensions of the gel cross sectional area and the distance between the electrodes are very similar to those of the Buchler apparatus. A schematic vertical cross sectional view of the apparatus is shown in Figure II.2.

In experiments in which the elution chamber was filled with dye, it was found that when buffer entered the chamber through a single entry port (as in the apparatus originally purchased), this entering buffer swept the chamber asymmetrically. Symmetrical clearing was obtained by increasing the number of entry points to five.

The volume of the elution chamber determines the extent of remixing of fractions successively emerging



A B C

FIG. II.3. A. Solid perspex spacer disc used for the casting of the lower surface of the resolving gel.
B. Press developed for the rapid adjustment of the height of the external glass sleeve.
C. Perforated perspex support for the semipermeable membrane.

from the gel. This chamber is bounded below by the semipermeable membrane and above by the lower surface of the gel, the position of which is determined by the level of the glass sleeves of the water jackets. A press (Fig. II.3b) was developed to facilitate the adjustment of the position of the outer glass sleeve. Satisfactory results were obtained with an elution chamber 0.2-0.5 mM in height with a volume of 0.33 to 0.83 ml. An elution buffer flow rate of 0.715 ml per minute was used with an elution chamber of these dimensions.

The semipermeable membrane is held in position by an "O" ring at its periphery and supported underneath by a rigid, perforated perspex disc (Fig. II.3c). An elution cell with some similarities has been described by Gordon et al. (1967). The use of a rigid support for the flaccid membrane eliminates the necessity for hydrostatic equilibrium between the lower and upper buffer compartments as required in the apparatus of Jovin et al. (1964). The interstices of the perforated disc and the hollow columnar support were filled with gel to prevent the accumulation of gaseous electrolysis products under the membrane.

(b) Casting of gels. Gels were cast after the method of Jovin et al. (1964). The lower surface of resolving gel was formed by sealing the lower surfaces of the gel chamber with the spacer disc (Fig. II.3a) covered by a "Visking" membrane which had been soaked in water. A Buchler light source provided illumination for polymerisation at 13.3 μM riboflavin. A stacking gel 0.5 cm thick and a resolving gel 3 cm thick (vertical dimensions) were used in most experiments.

(c) Preparation of samples. The sample, containing 10-50 mg of purified ribonucleotide reductase in 1-5 ml of 0.1 M DMG pH 7.3, was mixed with sufficient upper gel buffer to give a total volume of 8 ml. This volume of buffer contained sufficient sucrose to produce a sucrose concentration of 20% after mixture with the enzyme solution. The sample was pipetted through the upper buffer so as to form a layer directly above the stacking gel. After application of the sample, the apparatus was covered with a dark cloth.

(d) Cooling. Coolant fluid (20 litres of water containing 1 litre of ethylene glycol) was circulated from a refrigerated water bath thermostated at -1°C. Individual pumps were used for the external jacket and internal cold finger. In addition, preparative

electrophoresis was performed in a room at 3-5°C unless otherwise stated.

(e) Elution buffer flow. This was maintained with a DCL reduction geared micropump (F.A. Hughes) at a rate of 43 ml per hour. Fractions of 150 drops (5 ml) were collected from a 1 mM I.D. polythene tube on a Gilson fraction collector. Pressures above and below the resolving gel were equalised by adjusting the effective level of the buffer in the elution buffer reservoir and the height of the discharge point at the drop counter to that of the surface of the upper electrode buffer.

(f) Power supply. Potential gradient was derived from a precision regulated D.C. Power Supply of Universal Electronics. Typical running potentials were 70 volts (50 milliamps) during the migration of the sample through the stacking gel, and 190-240 volts (85 milliamps) for the resolution of the species. Total running time was 8-12 hours.

(g) Assay and concentration of fractions. In early experiments, reductase activity was assayed on all fractions that contained protein as determined by absorbance. Later, fractions known from experience to contain the same protein or mixture of proteins

were combined and concentrated 20-40 fold prior to activity determinations. Samples were concentrated by pressure dialysis against 0.1 M DMG, pH 7.3. Pressure outside the dialysis tube was reduced to 30 mm Hg. After concentration, sac contents were dialysed for at least 4 hours against 0.1 M DMG, pH 7.3, and the volume of the dialysed solution calculated from its weight. Protein concentration was determined spectrophotometrically.

Determination of the stability of ribonucleotide reductase under the conditions of electrophoresis

The stability of ribonucleotide reductase was examined under conditions as similar as possible to those of gel electrophoresis with respect to pH value, buffer, catalyst used for gel polymerisation and temperature. The pH of the gel during electrophoresis was determined on sections of the gel after 3 hours electrophoresis by immersion of the pieces in small quantities of indicator solutions or by maceration onto moistened indicator papers.

Buffer solutions were prepared with pH values extending over the useful pH range for the buffer, or over that portion of the range corresponding to gel pH, as experimentally determined above. Solutions

FIG. II.4. Electrophoresis of ribonucleotide reductase at 25° in Tris-glycine (Thelander, 1967). The sample was 100 µg of reductase purified to step 7, as described in Methods.

0.038 M with respect to TES or glycine, or 0.06 M with respect to chloride, acetate or phosphate were adjusted to the starting pH of the resolving gel which was used in the system under investigation. Cationic buffers used for this adjustment were Tris, imidazole and TEA. Samples of these solutions were then adjusted to the various pH values in the range investigated by the addition of cationic buffer (for the higher pH values) or the acidic form of the anion (for the lower pH values). Samples of enzyme at a concentration of 25-40 mg per ml were diluted ten to fifty fold by the buffer or were dialysed against 100 volumes of the buffer. The final pH of all mixtures was determined with a glass electrode. Enzyme was kept in the buffer for 6 to 20 hours at 0° or 25°C, the two temperatures used for electrophoresis, before assay.

Results

Gel electrophoresis of ribonucleotide reductase in Tris-glycine buffer. As seen in Fig. II.4, the reductase preparation, purified as described in Methods, appears to be a mixture of proteins when electrophoresed at 3° or 25°C in Tris-glycine buffer (Table II.1). This result contrasts with the observ-

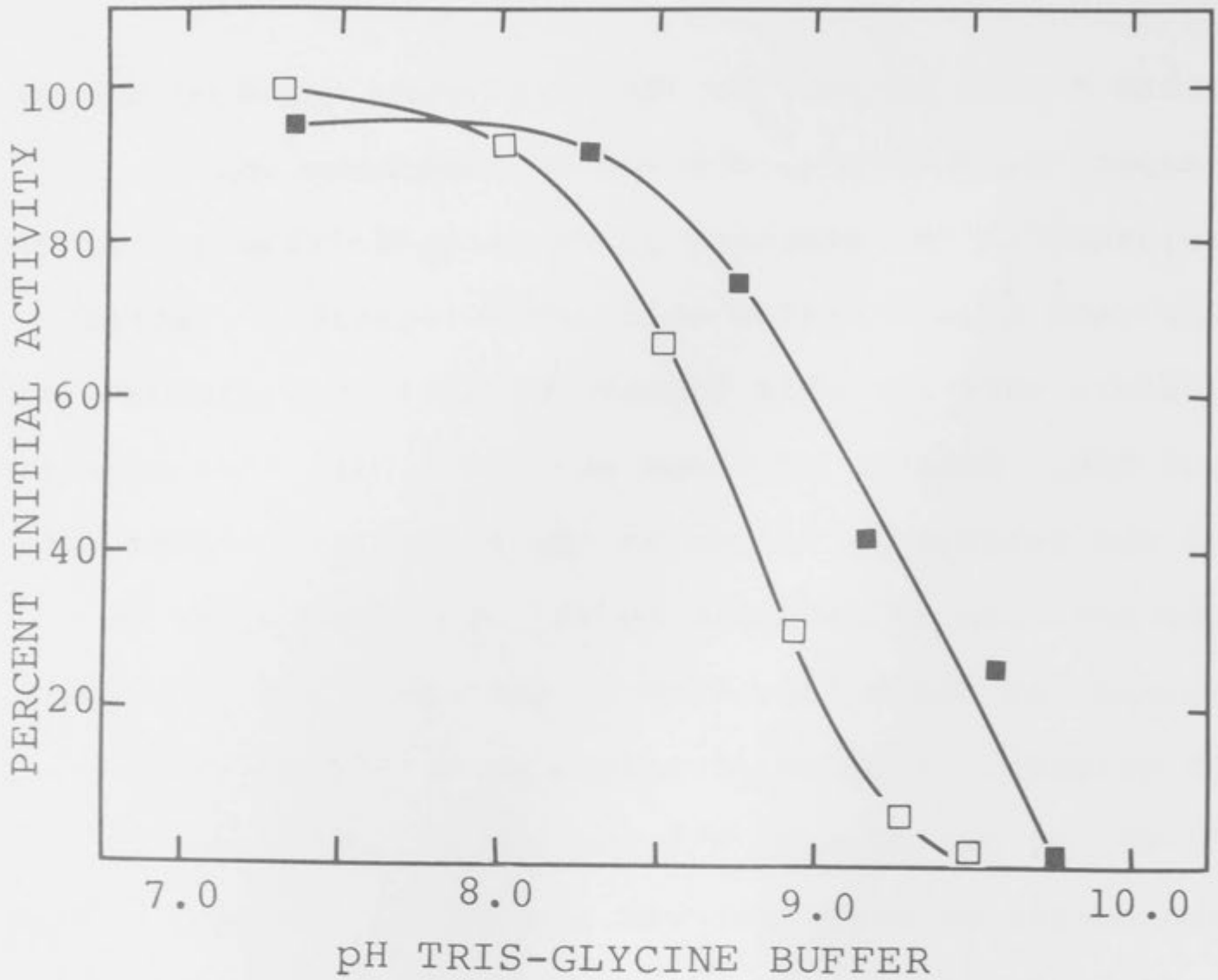


FIG. II.5. The stability of ribonucleotide reductase in Tris-glycine buffer.
Buffer solutions were prepared as described in Methods. Reductase was kept in this buffer at 0°C (■—■) or 25°C (□—□) for 16 hours prior to assay.

ance of a single symmetrical peak in the Schlieren pattern during sedimentation velocity determinations on such preparations in the ultracentrifuge. The different proportions of protein of low mobility seen in gels electrophoresed at different temperatures indicates instability of some component in the mixture.

When a similar reductase preparation was electrophoresed in Tris-glycine buffer in the preparative apparatus of Shuster et al. (1967) only 30-45% of the applied activity could be recovered in fractions eluted after 5 hours electrophoresis. This poor recovery indicated inactivation and suggested that at least some of the proteins seen in analytical gels might be inactivated enzymes.

Stability of ribonucleotide reductase at various pH values. In view of this evidence for inactivation of reductase during gel electrophoresis, the stability of the reductase was investigated in Tris-glycine buffer at the pH values encountered in this technique. The pH of an analytical gel, determined as described in Methods, ranged from pH 9 to 10, and Tris-glycine buffers including this range were prepared as described. The stability of the reductase during 16 hours exposure to these buffers is shown in Fig. II.5. The loss of

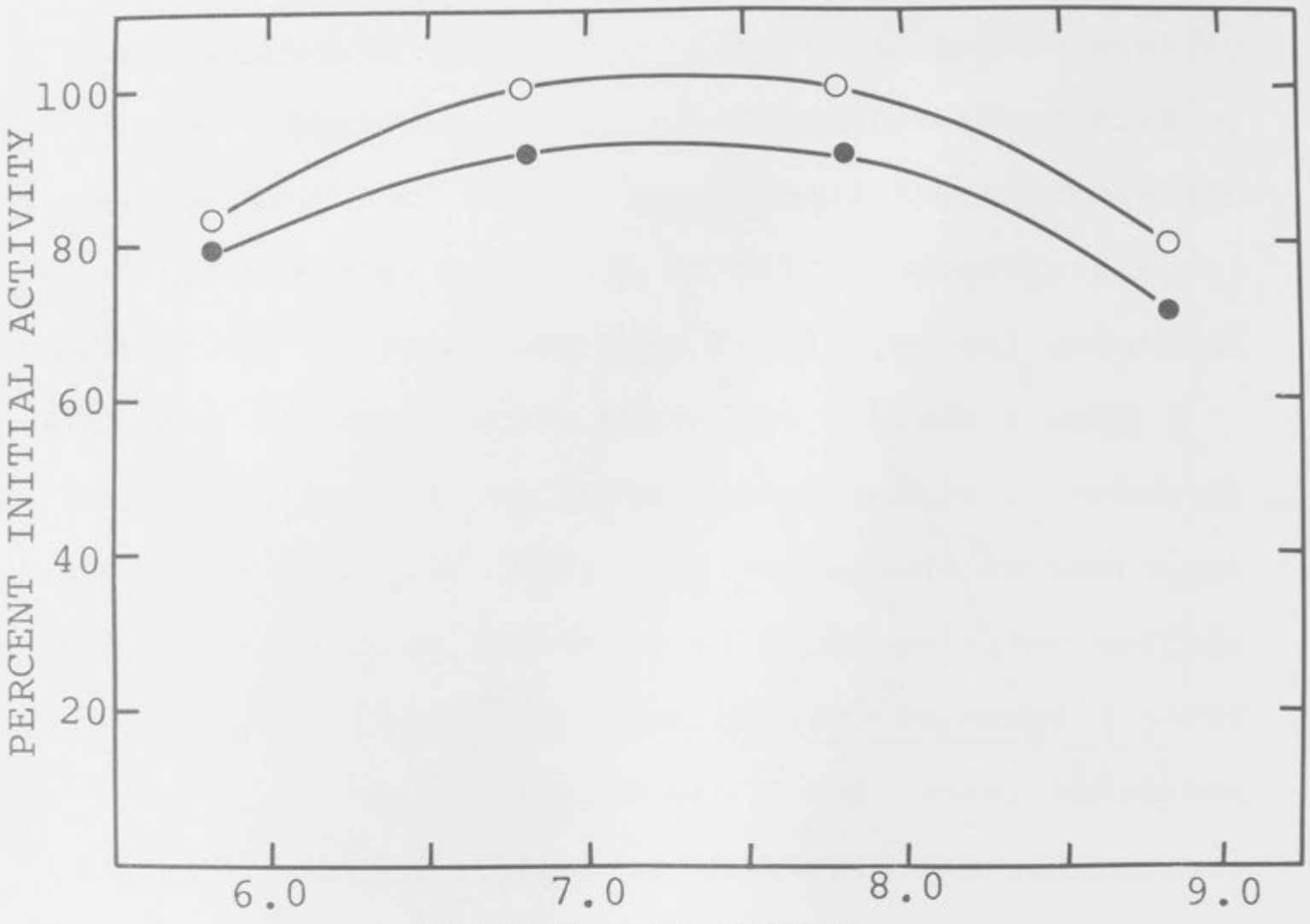


FIG. II.6. The stability of ribonucleotide reductase in TEA-TES buffer.
Buffer solutions were prepared as described in Methods. Reductase was kept in this buffer at 0°C (O—O) or 25°C (●—●) for 16 hours prior to assay.

activity above pH 8.0 and especially above pH 8.8 is consistent with the poor recovery of activity demonstrated during preparative electrophoresis.

The stability of the reductase in the presence of other buffers was examined by the same technique. Imidazole buffers containing various anions gave good recovery below pH 7.0 (greater than 90%), but the reductase was completely inactivated in 0.16 M imidazole-acetate pH 8.0 for 16 hours at 0°C. Imidazole-phosphate produced 50% inactivation under the same conditions. The stability of the reductase during 7 hours dialysis against imidazole buffers is shown in Table II.2. Inactivation was greatest with imidazole-acetate and least with imidazole-phosphate. Removal of the imidazole buffer by dialysis against imidazole-free buffer did not increase activity and suggests that the inactivation in imidazole buffers is irreversible. Addition of ATP (10 mM) to the reductase prior to exposure to imidazole-acetate pH 8 did not prevent the inactivation.

The stability of the reductase in TEA-TES buffer is shown in Fig. II.6. The results indicate that at 0°C this system offers stable conditions for ribonucleotide reductase between pH 6.8 and 8.0. Even at

TABLE II.2. Stability of ribonucleotide reductase during dialysis against imidazole-containing buffers

Buffers were prepared as described in Methods. In experiment I, the reductase was dialysed for 7 hours at 3°C against imidazole-containing buffers. Experiment II shows the activity after a further 16 hours dialysis against 1000 volumes of 1×10^{-3} M EDTA pH 7.3. Activity was measured by the spectrophotometric assay, and is expressed as a percentage of the initial activity.

Buffer system pH 8.0	Percent initial activity	
	I	II
Imidazole-chloride	39	38
Imidazole-acetate	11	8
Imidazole-phosphate	87	70

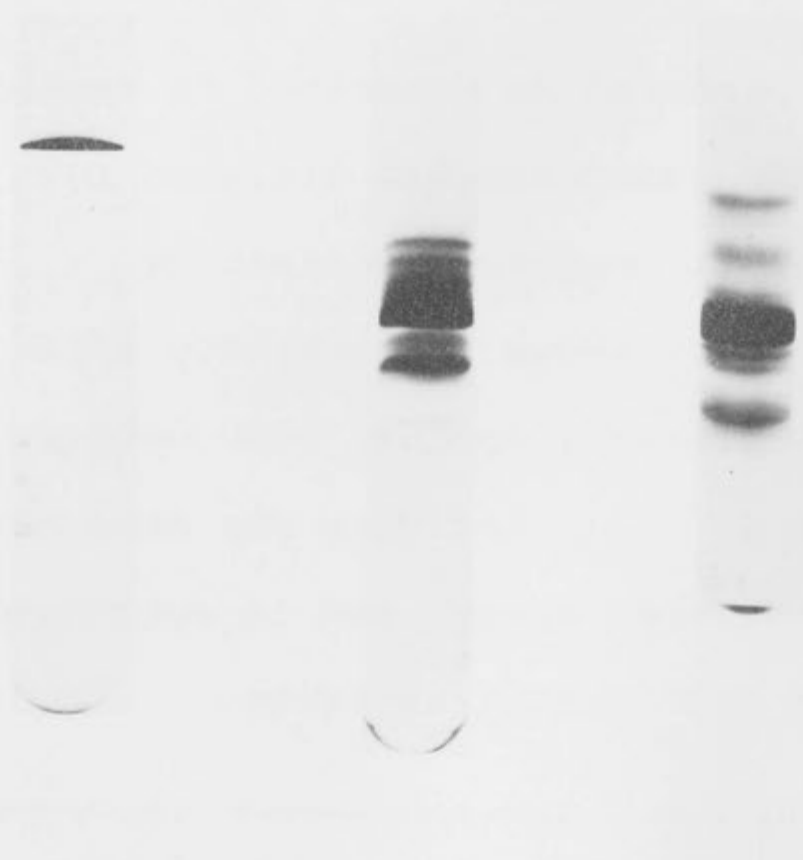
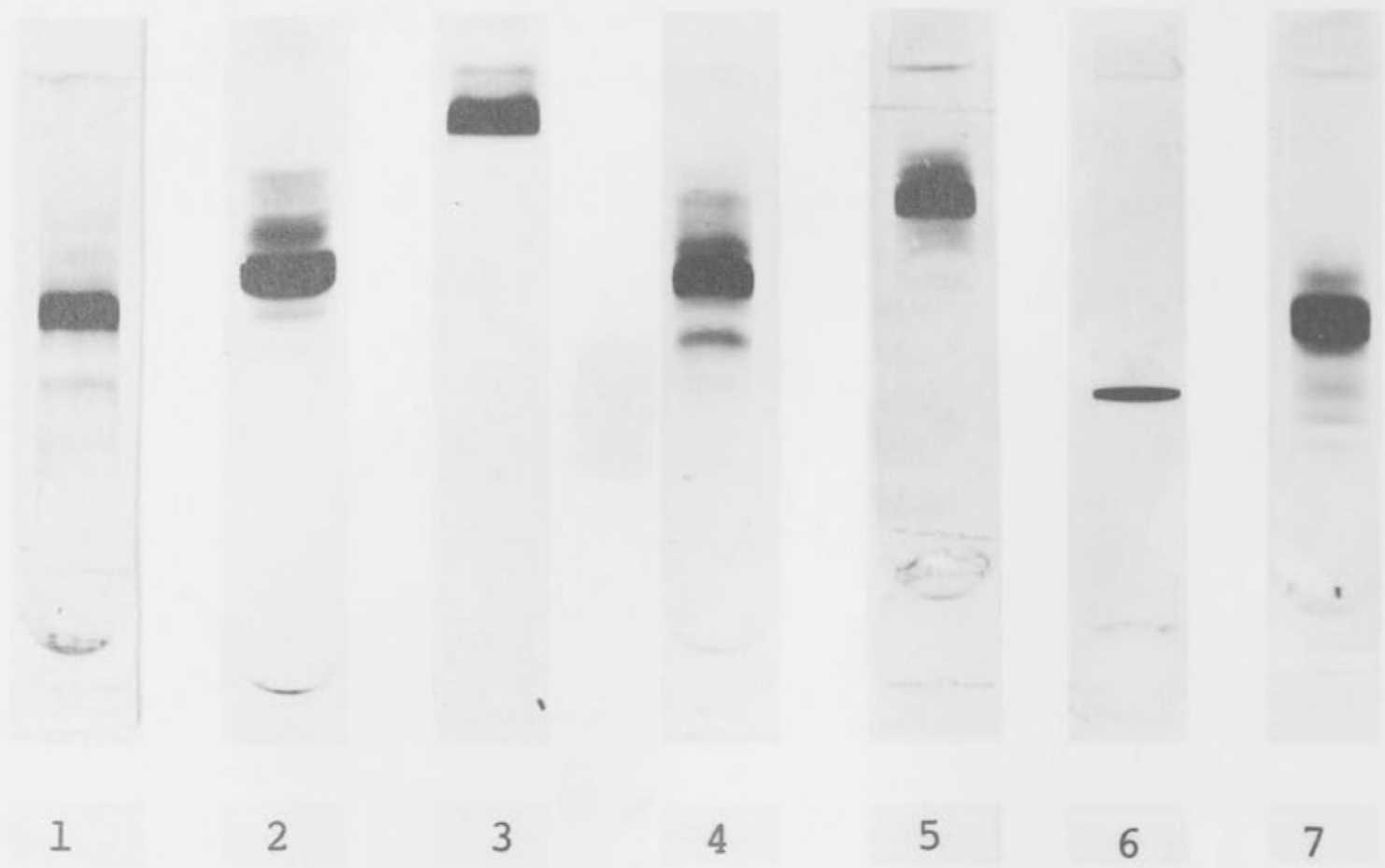


FIG. II.7. Electrophoresis of ribonucleotide reductase at 3° in Tris-glycine or TEA-TES buffer.
Gels 1 and 2, Tris-glycine; gel 3, TEA-TES. The buffers for gel 1 were prepared by the use of the anion concentrations of Thelander (1967) and adjusted at 0°C to the pH values shown in column 1, Table II.1 by the addition of solid Tris. Buffers for gels 2 and 3 were prepared as shown in Table II.1, columns 1 and 2, respectively.


1
2
3
4
5
6
7

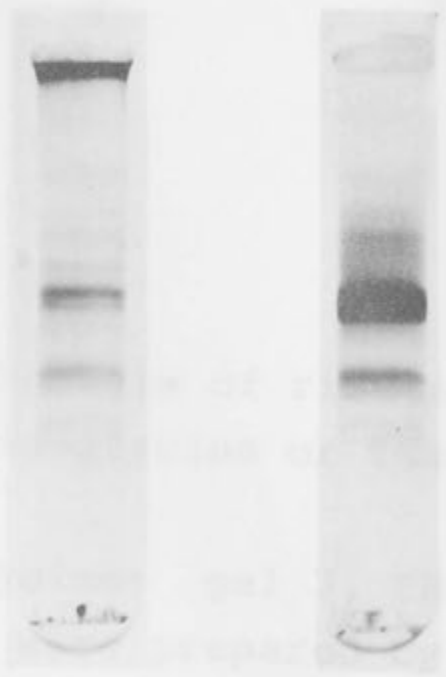
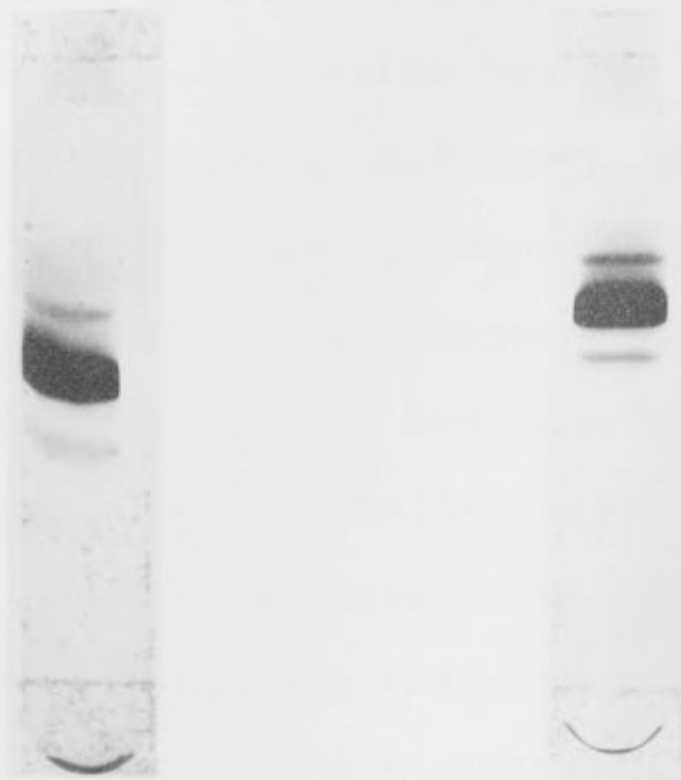

8
9

FIG. II.8. Resolution obtained on polyacrylamide gel electrophoresis of ribonucleotide reductase with various buffer systems. Samples comprised 100 μg of reductase purified to step 7 as described in Methods. Gel 1, TEA-TES; gel 2, pyridine-MES; gel 3, TEA-DMG; gel 4, TEA-TES-phosphate; gel 5, imidazole-MES; gel 6, imidazole-alanine; gel 7, imidazole-TES. The effect of overnight dialysis against imidazole-acetate (0.16 m, pH 8.0, 0°C) on the electrophoretic mobility of the reductase in TEA-TES buffer (gel 8) may be compared with that of the untreated enzyme (gel 9).

25°C there is very little loss of activity over this range.

Gel electrophoresis of ribonucleotide reductase with alternative buffers. When the pH of the Tris-glycine system was lowered in an attempt to increase the stability of the reductase during electrophoresis, all resolution was lost (Fig. II.7). As the enzyme is stable in the pH range buffered by TES, a system was developed with a resolving gel pH of 6.8 to 8.0, and the resolution obtained at 3°C can be compared with that of the Tris-glycine system in Fig. II.7. The overall resolution obtained in the two systems is comparable, but the relative amount of protein of low mobility is much less when electrophoresed at the lower pH. The yellow protein represented by the band of highest mobility is well resolved in both systems.

Electrophorograms of reductase preparations performed in the buffer systems are shown in Fig. II.8. Protein resolution with pyridine-MES pH 5.8-7.0 is similar to that seen with TEA-TES, but the separated discs are not so well defined. The toxicity of pyridine restricts the use of this system in unventilated areas. Buffers containing imidazole as the cation permitted good resolution, but could not be



1 2

FIG. II.9. The effect of adding DMG buffer with the protein sample on the electrophoresis of ribonucleotide reductase.
Enzyme was purified as described in Methods, and electrophoresed in the TEA-TES chloride system.
Gel 1 : Sample in 0.01 M potassium phosphate buffer pH 7.3.
Gel 2 : Sample in 0.01 M potassium phosphate buffer and 0.035 M DMG buffer pH 7.3.

used for the reductase which is unstable in them. Incubation of the reductase in imidazole-acetate for 16 hours prior to electrophoresis in TEA-TES so changes the properties of the inactive reductase that it no longer enters the resolving gel.

The use of buffers containing DMG as the slow anion gave fair resolution with discrete band formation, but the rate of migration was impractically slow. However, the presence of DMG in the sample can enhance the quality of the resolution obtained with TEA-TES buffer, and as shown in Fig. II.9, reduces distortion during electrophoresis.

A summary of the results obtained with various buffers is shown in Table II.3. The TEA-TES buffer offered the best conditions for electrophoresis of the reductase, and has been used for the further purification of this enzyme.

The effect of gel pore-size on the resolution obtained. The concentration of acrylamide and bis acrylamide used in the preparation of acrylamide gel determines the pore-size of the gel and the value of the latter which gives optimal resolution is related to the molecular weight of the proteins being separated. The optimal gel composition for the pro-

TABLE II.3. Buffer systems investigated for use in the electrophoresis of ribonucleotide reductase

Cation	Slow anion	Fast anion	pH upper buffer	pH stacking gel	pH resolving gel	pH lower buffer	Final pH resolving gel	Resolution	Percent initial activity†
Tris*	glycine	Cl^-	8.3	6.9	8.9	8.1	9-10	good	20
Tris**	glycine	Cl^-	8.8	7.4	9.5	8.7	9.5-10	good	50
Tris***	glycine	Cl^-	8.3	6.9	8.8	8.1		Nil	
Tris* (0.02 M)	glycine	Cl^-	8.4	8.4	8.4	8.4	8.4-10	good	-
Imidazole	glycyl glycine	Cl^-	6.8	6.3	7.3	7.3	7.3-8.5	Nil	
Imidazole	alanine	Cl^-	6.8	6.3	7.3	7.3	7.3-8.5	Nil	
Imidazole	TES	Cl^-	6.3	5.8	6.8	6.1	7.0-8.0	good	38
Imidazole	TES	Phosphate	6.3	5.8	6.8	6.1	7.0-8.0	good	70
Imidazole	TES	Acetate	6.3	5.8	6.8	6.1	7.0-8.0	good	8
Imidazole	MES	Cl	5.3	4.8	5.8	5.1	6-7.5	good	
Imidazole	MES	Phosphate	5.3	4.8	5.8	5.1	6-7.5	good	
Pyridine	MES	Cl	5.3	4.8	5.8	5.1	6-7.5	good	
TEA	MES	Cl	5.3	4.8	5.8	5.1	6-7.5	Nil	
TEA	DMG	Cl	6.3	5.8	6.8	5.8	7.0-8.0	poor	
TEA	TES	Cl	6.3	5.8	6.8	5.8	7.0-8.0	good	100

†Percent enzyme activity remaining after 7-16 hours exposure to the resolving gel buffer at 0°C.

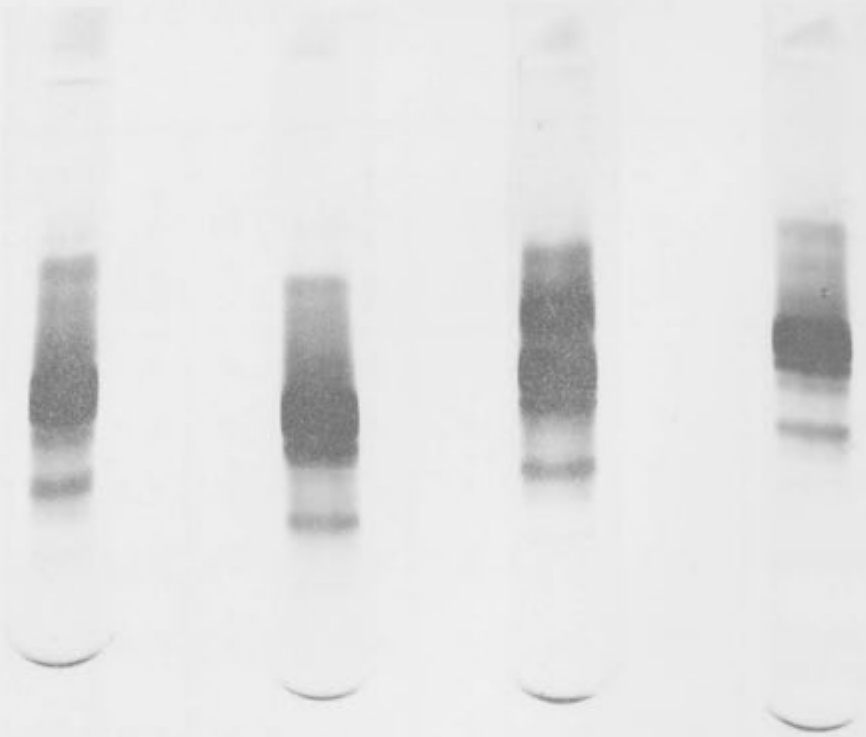
*Buffers were adjusted to the pH indicated at room temperature and electrophoresed at 25°C. Tris used was 0.02 M.

**Buffers were adjusted to the pH indicated at room temperature (25°C) but electrophoresed at 3°C.

***Buffers were adjusted to the pH indicated at 0°C and electrophoresed at 3°C.

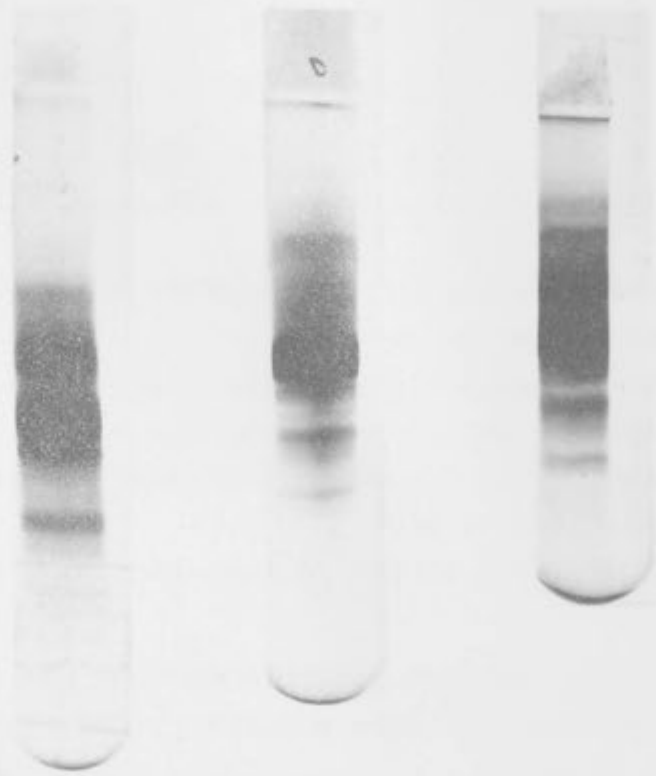
The molarity of each anionic buffer component was the same as given in Table II.1, except for the Tris-glycine system pH 8.4, where the Tris was 0.02 M.

A



1 2 3 4

B



1 2 3

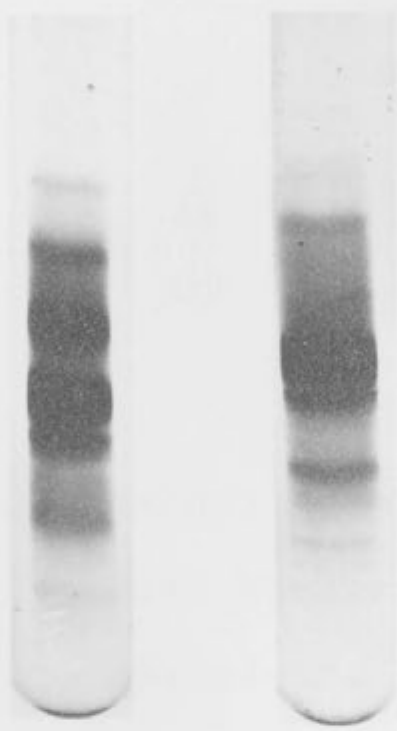
FIG. II.10. Resolution of a ribonucleotide reductase preparation on gels polymerised from solutions of various concentrations of acrylamide and bis acrylamide. Samples comprised 100 µg of reductase purified to step 7 as described in Methods.

A. Bis acrylamide concentration 0.1%

Acrylamide concentration : Gel 1, 7½%; gel 2, 8%; gel 3, 9%; gel 4, 10%.

B. Acrylamide concentration 9%.

Bis acrylamide concentration : Gel 1, 0.1%; gel 2, 0.2%; gel 3, 0.3%.



1 2

FIG. II.11. Resolution of a reductase preparation on gels polymerised with different catalysts.
Gel 1 : Polymerisation by ammonium persulphate.
Gel 2 : Polymerisation by illuminated riboflavin.
Sample in each case contained 100 µg protein, purified to step 7 as described in Methods.

teins present in reductase preparations was determined experimentally. As shown in Fig. II.10, a resolving gel prepared from 9% acrylamide and 0.1% bis acrylamide (W/V) offered the best resolution as judged by the separation of the predominant protein from the minor contaminant which migrates immediately ahead of it in the TEA-TES system.

The effect of catalysts used in gel polymerisation on ribonucleotide reductase. Since ammonium persulphate, widely used as a polymerisation catalyst, is a powerful oxidising agent, possible inactivation of enzyme by residual persulphate in the gel was investigated. The mixture of proteins disclosed by analytical electrophoresis in gels polymerised by ammonium persulphate-TEMED was compared with that seen after electrophoresis in gels polymerised by illuminated riboflavin-TEMED with the results shown in Fig. II.11. The greatly increased proportion of bands with low mobility in the persulphate-polymerised gel suggests that persulphate or its products converts proteins present in the reductase preparations to forms with lower mobility, the presence of these proteins being much less evident in gels polymerised with riboflavin.

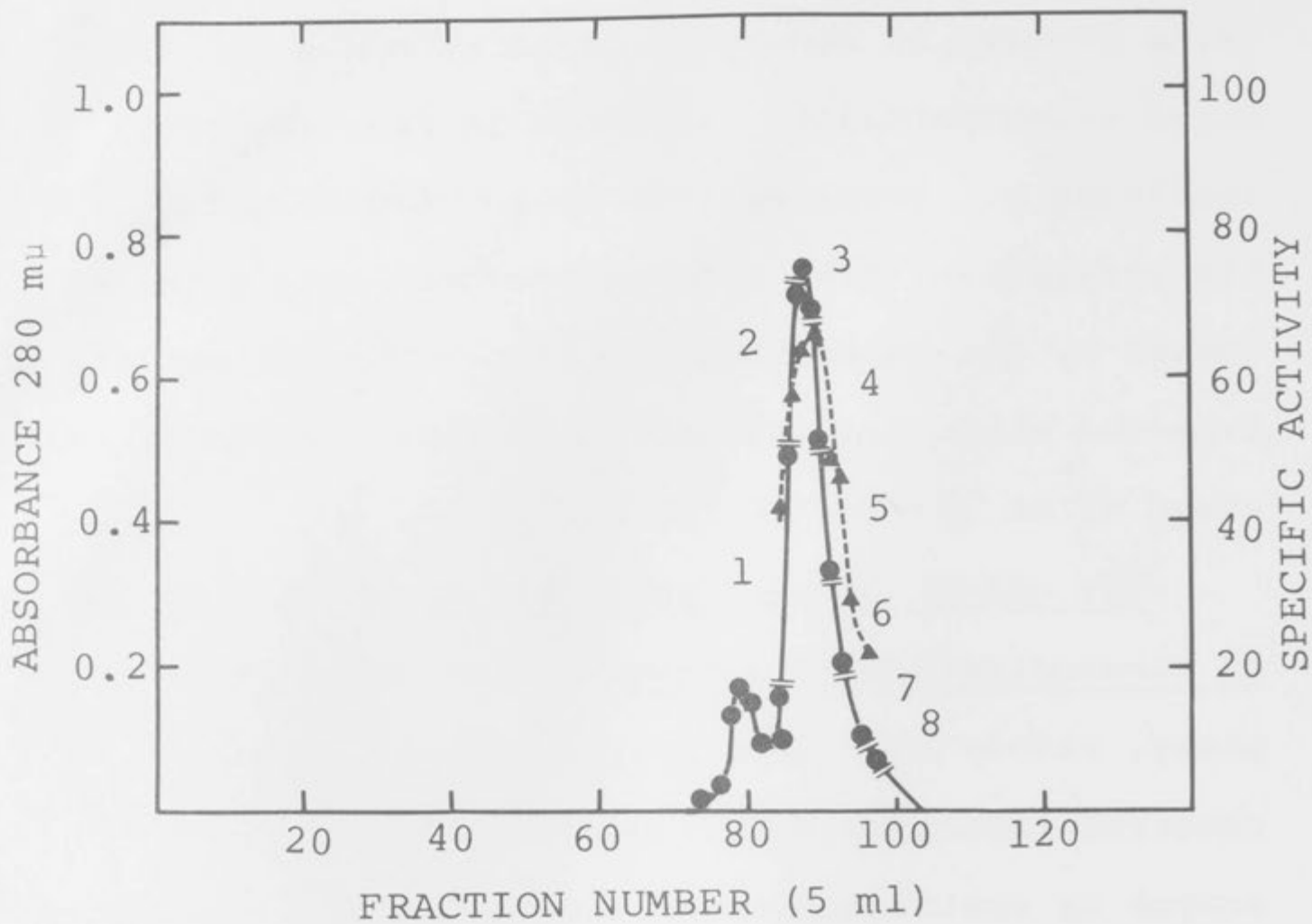


FIG. II.12a. Preparative electrophoresis of ribonucleotide reductase.
Standard TEA-TES buffer (Table II.1) was used. Sample contained 33 mg protein of specific activity, 45 μmoles ATP reduced/hr/mg.
●—●, Absorbance of eluted fractions at 280 mμ;
▲····▲, Specific activity of concentrated fractions.

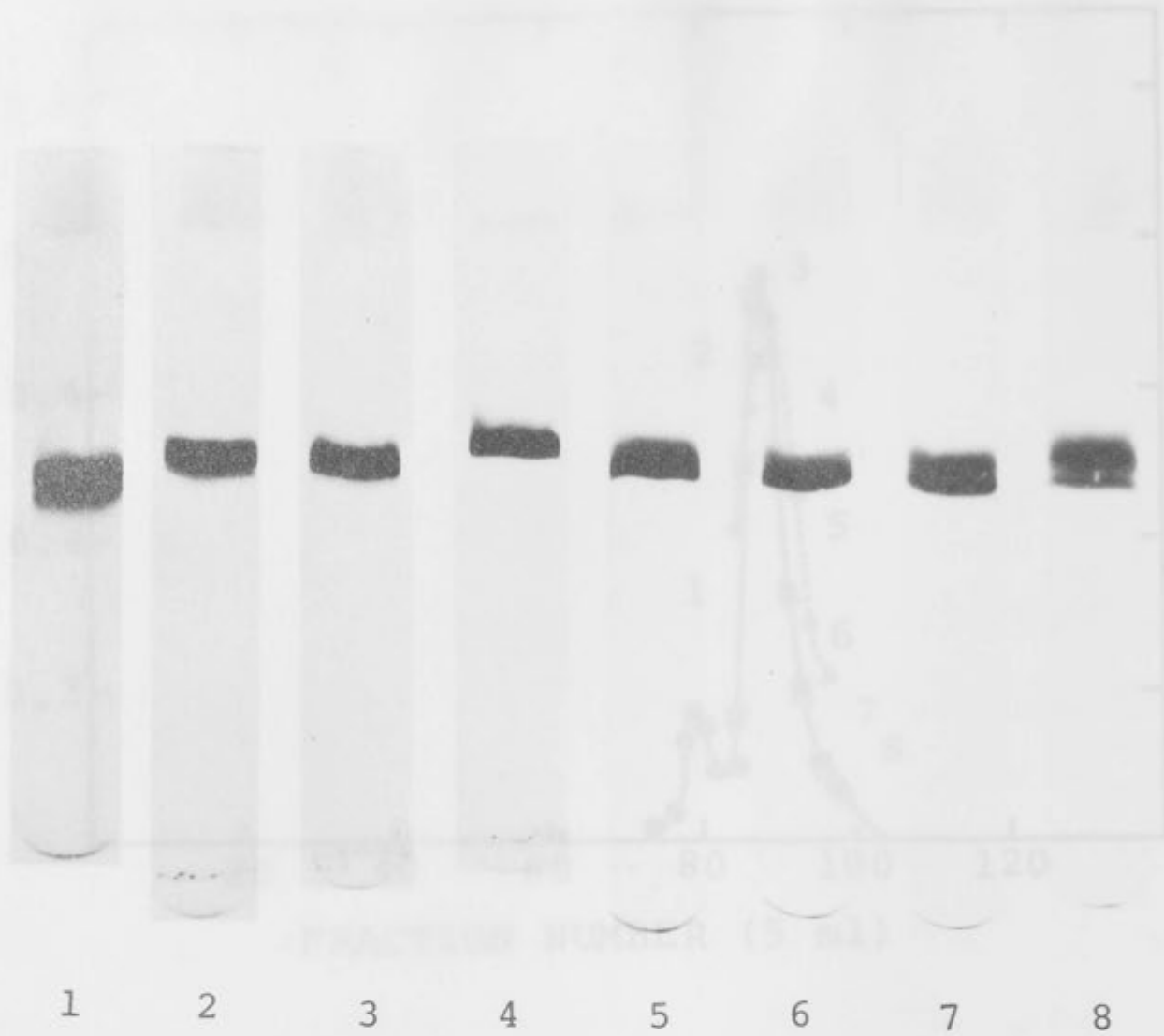


FIG. II.12b. Gel electrophoresis of proteins concentrated after preparative electrophoresis of ribonucleotide reductase.
Gels correspond to the numbered areas on Fig. II.12a. Standard TEA-TES buffer (Table II.1) was used. Samples contained 100 µg protein. Gel (a) represents the original mixture prior to preparative electrophoresis.

The stability of the reductase in the presence of riboflavin was investigated with the results shown in Table II.4. Although completely stable in the presence of riboflavin in the dark, the reductase is inactivated when illuminated even in the absence of added riboflavin, perhaps due to sensitization by an intensely yellow protein present in the preparations.

Ribonucleotide reductase is quite stable in the presence of TEMED at the concentrations used in the gel. The concentration of this agent was therefore maintained at the level used by Thelander (1967) (Table II.1).

Preparative electrophoresis of ribonucleotide reductase. Results of the preparative electrophoresis of the reductase purified as described in Methods is shown in Fig. II.12a. The small yellow protein peak of high mobility has not been completely resolved from the larger peak containing active protein. The variance in the specific activity among the active fractions (values shown in upper curve) indicates that the predominant protein fraction which includes the active enzyme, is not homogeneous. The composition of protein fractions from portions of the main peak are shown in Fig. II.12b. Only three fractions

TABLE II.4. The stability of ribonucleotide reductase in the presence of riboflavin

A reductase preparation (30 mg/ml) was diluted 50-fold with 0.1 M DMG pH 7.3. Riboflavin was added to the tubes shown to a final concentration of 13.3 μM. Samples were illuminated for 4 hours through the wall of a small beaker containing ice water. Diffuse illumination was provided by a 100 watt daylight fluorescent lamp at a distance of 1 meter; strong illumination by an 8 watt fluorescent lamp at 2 cms. Assay was performed by the colorimetric method.

Treatments		Percent initial activity
Riboflavin	Illumination	
Present	Dark	105
Present	Diffuse	79
Absent	Strong	65
Present	Strong	21

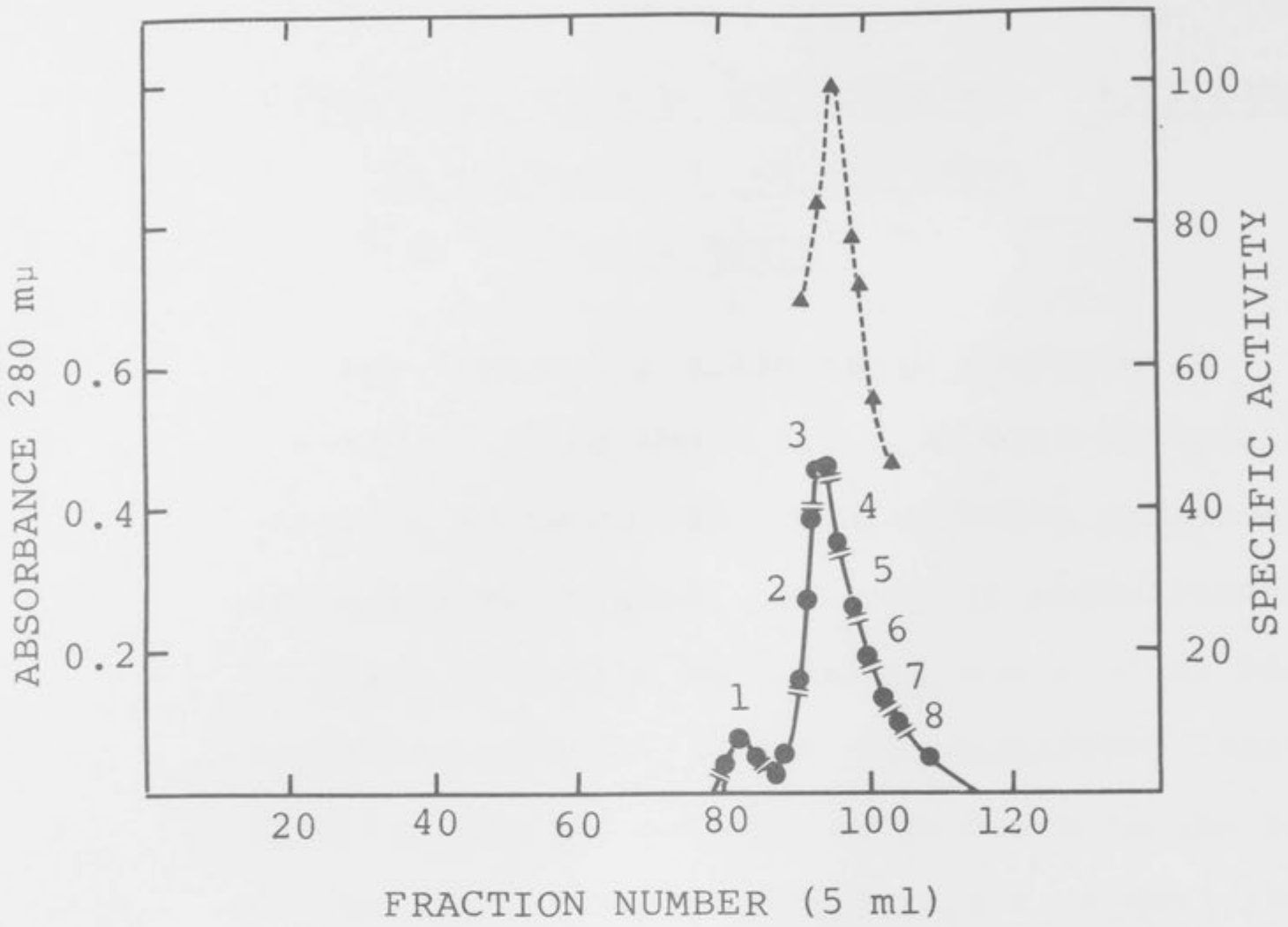


FIG. II.13a. Preparative re-electrophoresis of ribonucleotide reductase.
Standard TEA-TES buffer (Table II.1) was used. Sample contained 23 mg of protein of specific activity 70, which was obtained from combined fractions with contaminants of low mobility from the activity peaks of previous electrophoreses.
●—●, Absorbance of eluted fractions at 280 mμ;
▲····▲, Specific activity of concentrated fractions.

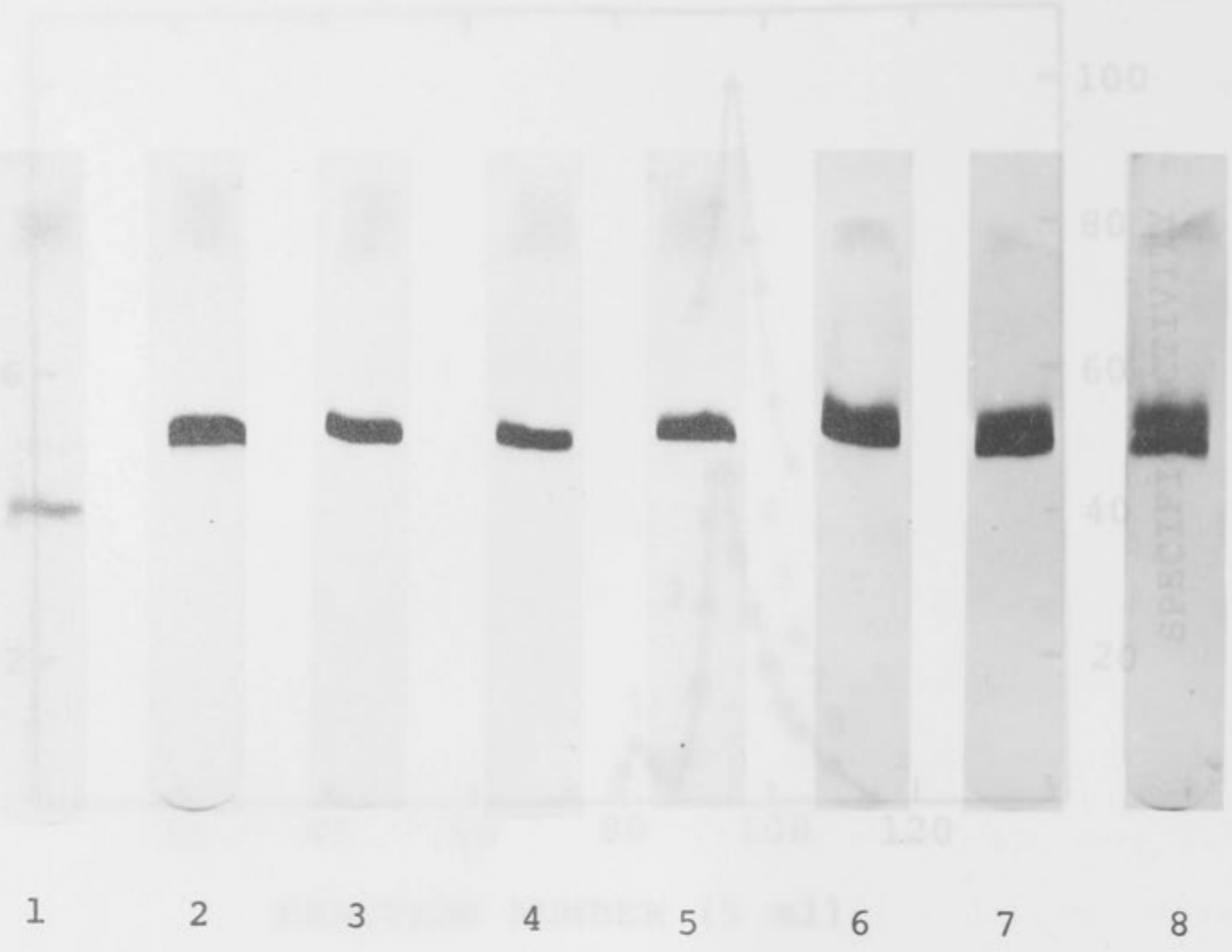
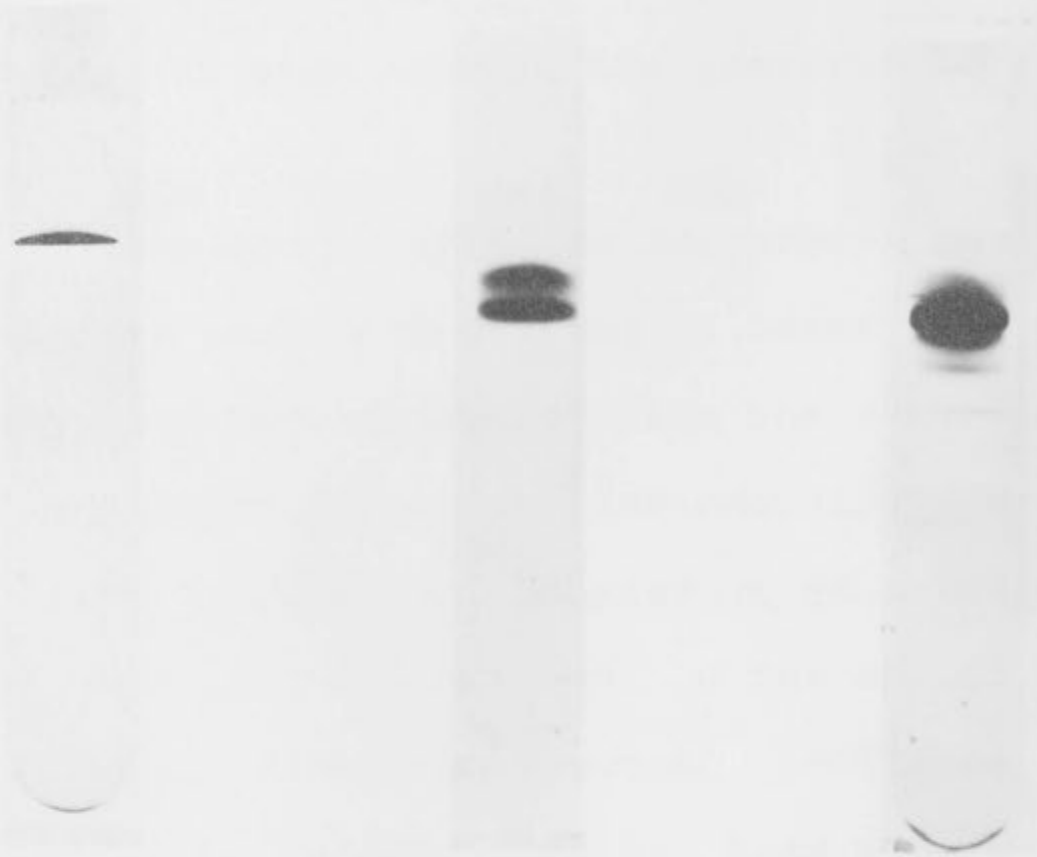


FIG. II.13b. Gel electrophoresis of protein concentrated after preparative electrophoresis of ribonucleotide reductase.
Gels correspond to the numbered areas on Fig. II.13a. Standard TEA-TES buffer (Table II.1) was used. Samples contained 100 µg protein.

of high specific activity contain protein which migrates as a single band, and the recovery of protein in these fractions was 33% of the applied sample. Total recovery of activity and protein were 80% and 70%, respectively.

The yield of electrophoretically-homogeneous protein can be increased by pooling fractions containing similar mixtures and again electrophoresing. The results of a re-electrophoresis of pooled fractions mainly contaminated by protein of low mobility are shown in Fig. II.13a and b. Protein and activity recovery were each 70%. Recovery of protein which migrates as a single band was 8%. Similar recoveries were obtained from re-electrophoresis of pooled fractions containing contaminants of high mobility.

Active reductase which migrates as a single band on gel electrophoresis can be prepared from enzyme with a specific activity of 50 to 110. The expectation that all such homogeneous preparations would exhibit the same high specific activity was not realised, the range of values extending from 67 to 140.



1 2 3

FIG. II.14. Gel electrophoresis of ribonucleotide reductase in Tris-glycine buffer.
Samples were purified by preparative electrophoresis with TEA-TES buffer. The homogeneous preparation was electrophoresed as follows :

Gel 1 : TEA-TES 3°C. Buffers adjusted at 0°C.
Gel 2 : Tris-glycine 25°C.
Gel 3 : Tris-glycine 3°C.

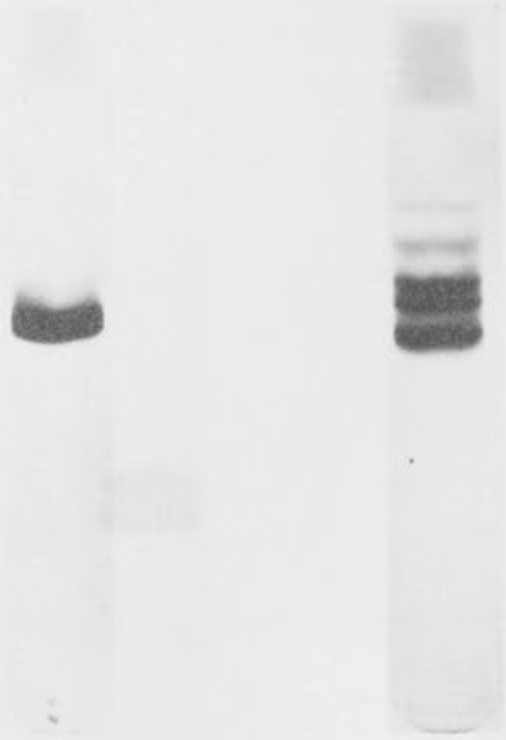


FIG. II.15. Modification of ribonucleotide reductase during dialysis against Tris-glycine buffer.

Homogeneous ribonucleotide reductase (6 mg) in 1 ml of 0.1 M DMG (pH 7.3) was dialysed for 16 hours at 25°C against 100 volumes of Tris-glycine buffer, pH 8.9, prepared as described in Methods. The protein solution was dialysed at 3°C for a further four hours against 500 volumes of 0.1 M DMG, pH 7.3. The protein (100 µg) was electrophoresed in TEA-TES-chloride, pH 7-8 (gel 2) and may be compared with untreated enzyme (gel 1).

Stability of homogeneous ribonucleotide reductase in Tris-glycine buffer. The effect of electrophoresis in Tris-glycine buffer is investigated more easily with pure material. Reductase purified as described by preparative electrophoresis in TEA-TES to a single migrating species gave rise to a more slowly migrating protein when electrophoresed in Tris-glycine at 25°C (Fig. II.14). This slow component is not seen after electrophoresis at 3°C, but some fine discs of high mobility are produced which could result from protein inactivated during electrophoresis.

The effect of Tris-glycine buffers was investigated further by dialysis of reductase which was electrophoretically homogeneous in the TEA-TES system against Tris-glycine buffer pH 8.9 at 25°C prior to electrophoresis in the TEA-TES system. The production of protein of low mobility (Fig. II.15) is far greater than can be demonstrated after the briefer exposure to Tris-glycine buffer that occurs during analytical electrophoresis.

Preparative electrophoresis in the Tris-glycine system was performed on electrophoretically purified ribonucleotide reductase which migrated as a single band in TEA-TES and the quantitative recoveries of

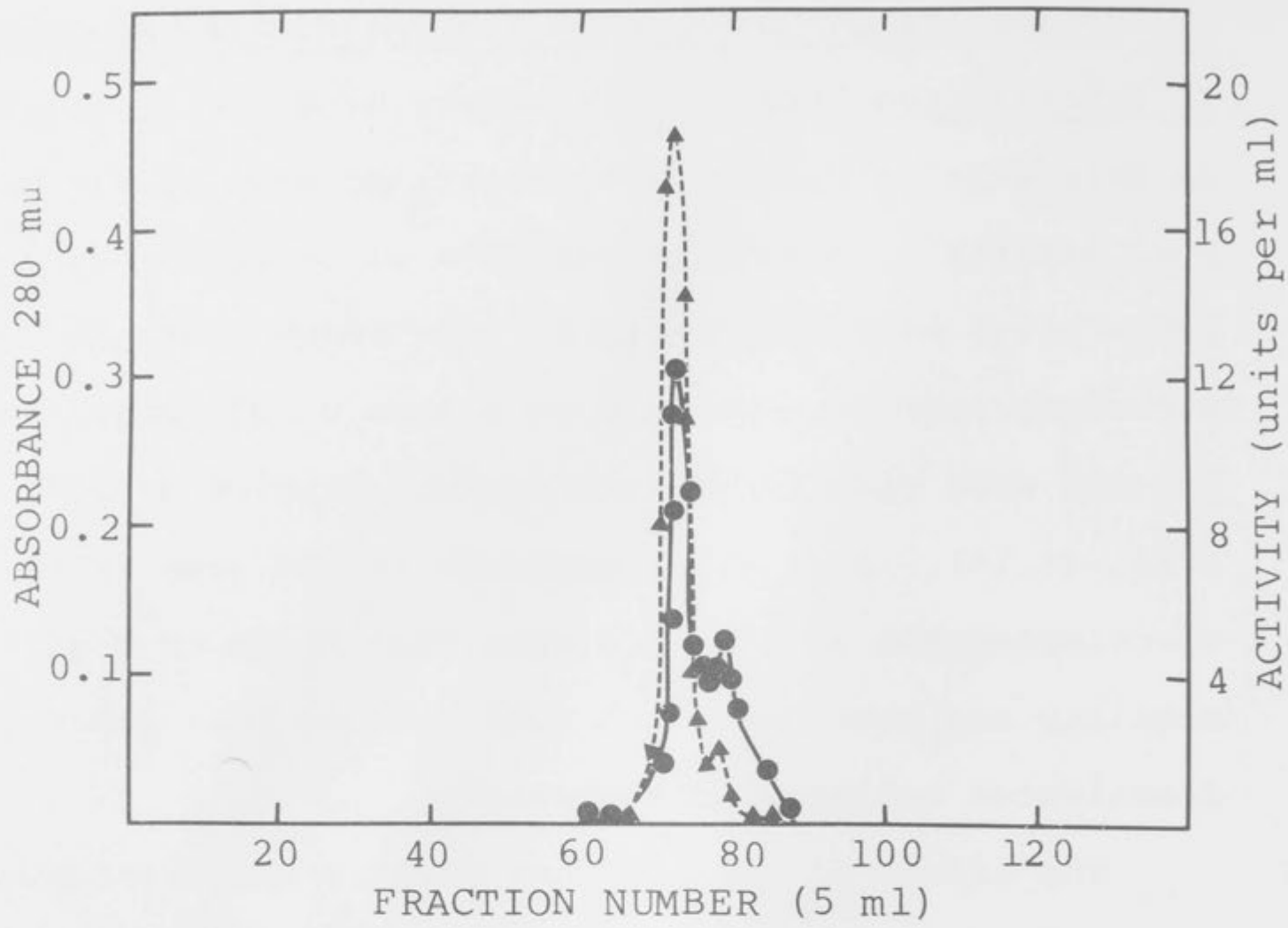


FIG. II.16. Preparative electrophoresis of ribonucleotide reductase, using the buffer system of Thelander (1967).
The electrophoretically homogeneous sample was prepared by preparative electrophoresis at pH 6.8, standard conditions (Table II.1). Sample size was 10.9 mg. Fractions were eluted with Tris-phosphate buffer, pH 8.1.
●—●, Absorbance 280 mμ; ▲—▲, Activity.

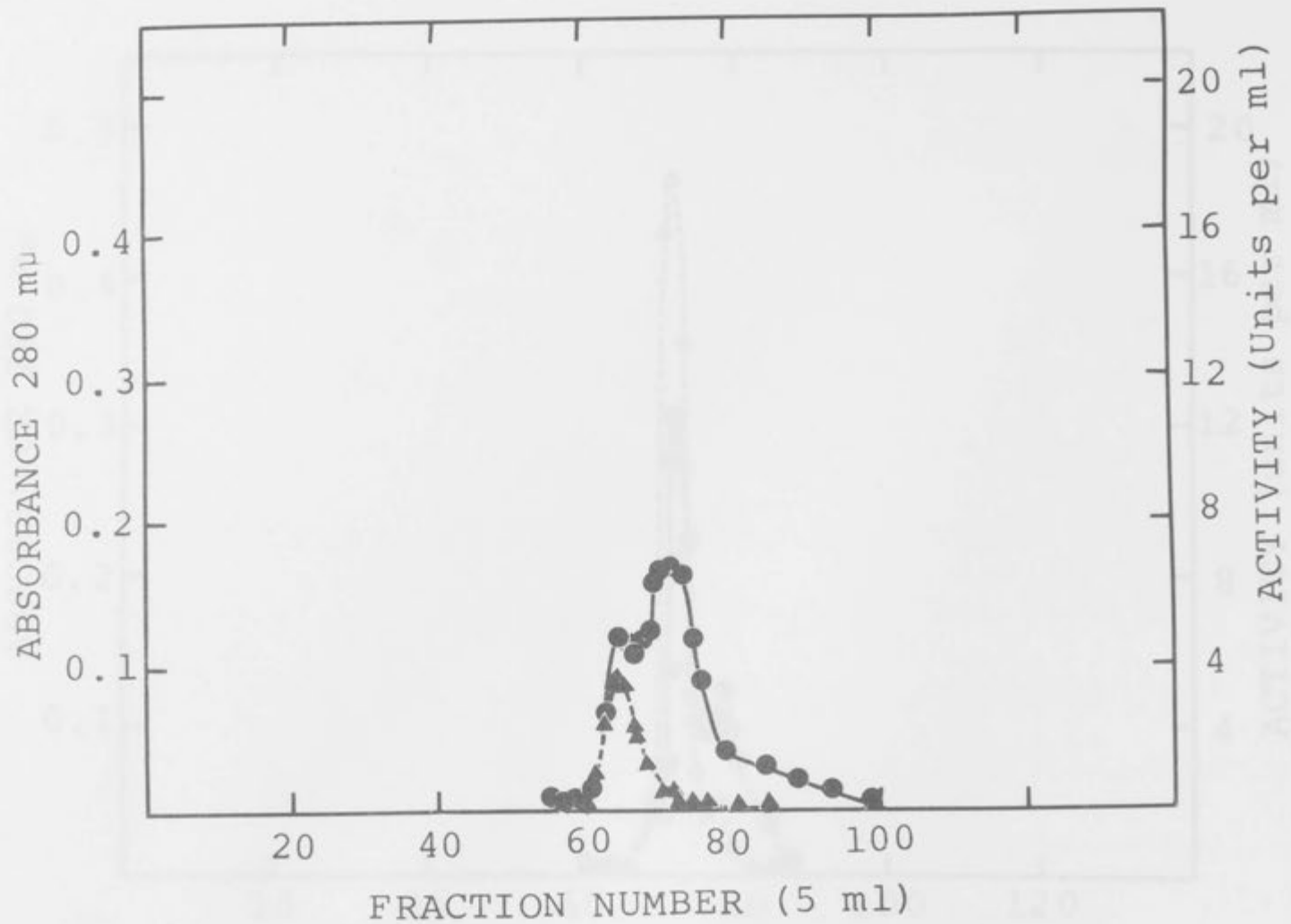


FIG. II.17. Preparative electrophoresis of ribonucleotide reductase.
Buffer conditions were identical as for Fig. II.16 (Thelander, 1967), except that the electrophoresis was performed at 25°C. Sample size was 12 mg of electrophoretically homogeneous protein. Fractions were eluted with Tris-phosphate buffer, pH 8.1. ●—●, Absorbance 280 mμ; ▲···▲, Activity.

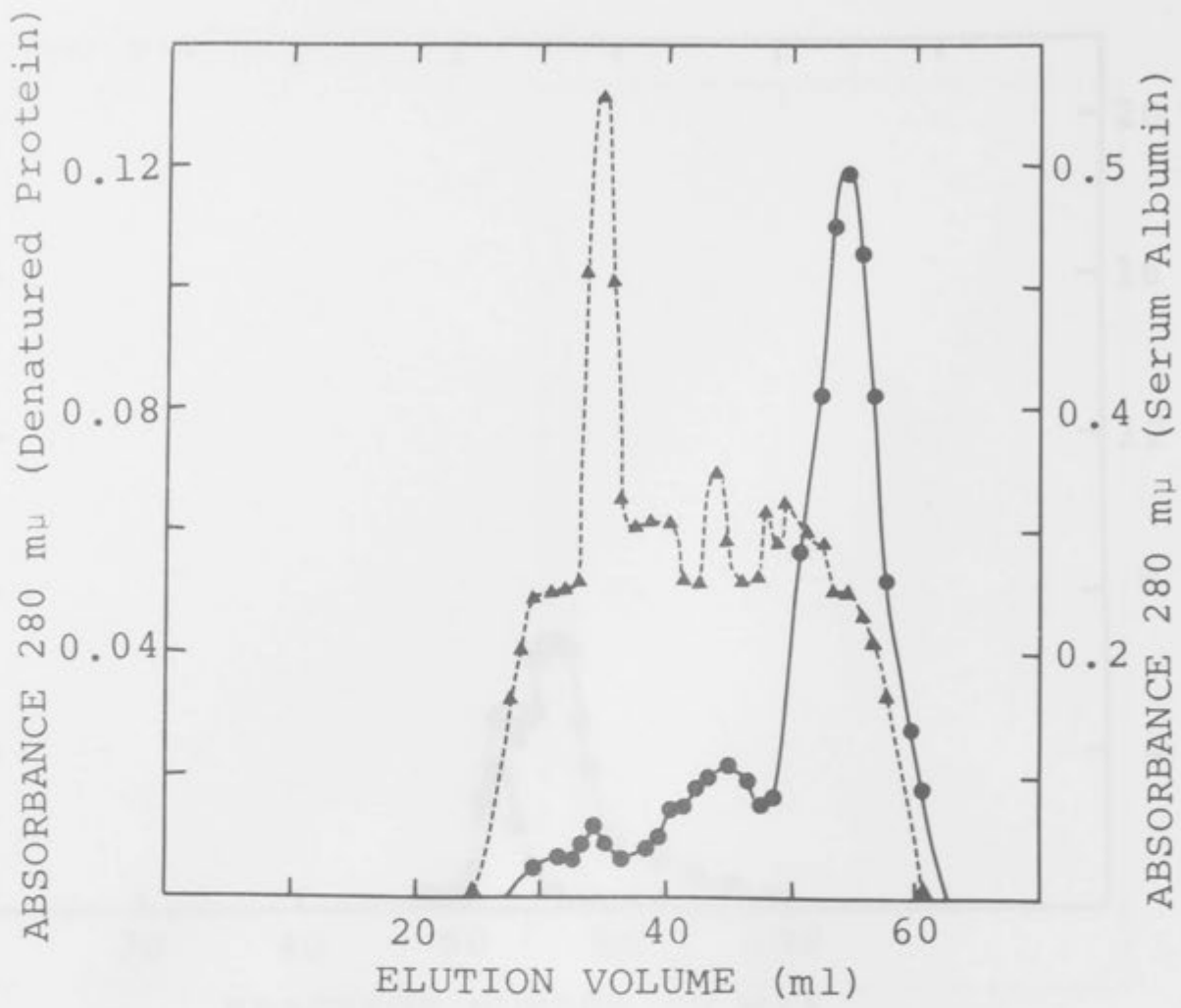


FIG. II.18. Gel filtration of inactivated ribonucleotide reductase.
Protein sample (2 mg) was concentrated by pressure dialysis from the second protein peak shown in Fig. II.16, and chromatographed on a 100 x 1 cm column of Sephadex G200, previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. This buffer was also used to elute the fractions. The elution profile obtained (▲···▲) is shown with that of a serum albumin monomer-dimer-trimer mixture (●—●).

protein and activity at 0°C were 70% and 53%, respectively. As shown in Fig. II.16, a small amount of protein of low activity was incompletely separated from the bulk of the active material. Although the specific activity of the peak tube was 67 compared with 61 of the applied sample, the poor recovery renders the method impractical for preparative use. Recovery of activity is even less (20%) when electrophoresis is performed at 25°C. As shown in Fig. II.17, activity is associated with the smaller protein of higher mobility. The specific activity in the most active fraction of 38 is considerably less than the applied protein (specific activity 61).

The slower mobility of the inactive protein suggests that this material has a decreased negative charge, an increased size, or both. The molecular weight of the inactive protein was investigated by gel filtration on Sephadex G200 by the method of Andrews (1964). Since the elution volume was found to be less than that of serum albumin dimer (Fig. II. 18), the inactive material appears to have a molecular weight in excess of 140,000 as compared to that of 70,000 for the inactive enzyme (Vitols et al., 1967b).

Protein concentrated from the inactive peak was not homogeneous on Sephadex G200 chromatography. This is consistent with the numerous proteins seen on gel electrophoresis following prolonged exposure to Tris-glycine buffer (Fig. II.15).

Discussion

Buffer systems for the electrophoresis of enzymes. In a recent review of gel electrophoresis techniques (Blomendahl, 1967), the pH values of all gels tabulated for use with anionic buffer systems were above pH 8.2. Several of these, including the Tris-glycine system, were developed initially for the electrophoresis of unfractionated serum proteins. The high isoelectric point of gamma globulins necessitates a high operating pH in the buffers used so as to ensure that all proteins migrate into the resolving gel. Tris buffers have been widely advocated for the electrophoresis of enzymes (Davis, 1964; Thelander, 1967; Jovin *et al.*, 1964; Shuster *et al.*, 1967; Gordon *et al.*, 1967; Nishihara *et al.*, 1967; Blomendahl, 1967), and some enzymes are stable when electrophoresed at pH 8.9-10 (Thelander, 1967), but the 25% recovery obtained during the preparative

electrophoresis of deoxycytidine deaminase (Nishihara et al., 1967) is probably more typical. Biological activity is seldom investigated in the proteins resolved from serum during analytical gel electrophoresis, and all of the proteins represented on the electrophoroqram may not be present as such in the applied sample.

Tris buffers used in a discontinuous system do not provide a constant pH, electrophoresis being accompanied by a rise of 0.5 pH unit at 25°C (Williams et al., 1964). When the temperature of electrophoresis is lowered to 0-5°C to protect enzymes, there is a further rise of 0.5 pH unit in the resolving gel due to changes in the pK_a of Tris and glycine with temperature (Perrin, 1965; Bates et al., 1961; Datta et al., 1958). The combined effect in conjunction with the starting pH of 8.9 accounts for the pH 10 determined in the resolving gels during the present investigation. Stability studies on ribonucleotide reductase exposed to Tris buffer at the pH of the resolving gel clearly indicate denaturation and electrophoresis of the reductase following such exposure demonstrates the retarded mobility of the inactivated form. Although altered protein of lowered mobility can be produced by other commonly used gel

constituents, the artifacts seen during electrophoresis of ribonucleotide reductase in Tris buffers are caused by the high operational pH, and the production of this modified, inactive protein renders the buffer unfit for use with this enzyme.

Electrophoretic mobility in polyacrylamide gels is influenced in part by the charge of the migrating species, and differences between charges on the various proteins will be greatest in the vicinity of their isoelectric point. However, because migration rates are impractically slow very near the isoelectric point, for optimal resolution a compromise working pH must be found that is sufficiently removed from the isoelectric point to ensure adequate mobility, but not so far as to minimise the charge differences which can enhance resolution. For enzymes such as ribonucleotide reductase with an isoelectric point below 5, charge differences between the enzyme and its contaminants are minimised by performing electrophoresis in the Tris buffer system which ultimately reaches pH 10. In the search for alternative buffer systems which can operate below pH 8.0, several have been shown to have high resolving power. One such system is imidazole-TES and although ribonucleotide

reductase is specifically denatured by imidazole above pH 7.0, this buffer system may well be useful for the resolution of proteins not subject to such inactivation. Pyridine-MES also provides excellent resolution between pH 5.5 and 7.0 but must be used with due regard for the toxicity of pyridine. TEA-TES buffer afforded the best resolution of the buffers tested for use with polyacrylamide gel electrophoresis of ribonucleotide reductase. The enzyme is stable in the buffering range of TES (pK_a at 20°C 7.5, Good et al., 1966), and although the pK_a of TEA is high (8.3 at 0°C, Bjerrum et al., 1948) compared with the experimentally determined working range of the resolving gel (pH 6.8-7.8), recovery of activity is satisfactory and the system does not produce artifacts.

The effect of other gel components on enzymes. Electrophoresis of ribonucleotide reductase in gels polymerised with ammonium persulphate as catalyst has been shown to produce modified protein of slower mobility. Similar findings have been reported from electrophoresis of clostridio peptidase (Mitchell, 1967) and yeast enolase (Brewer, 1967) and chick interferon is inactivated by persulphate (Fantes et al., 1967). Of the alternative methods available

for the polymerisation of acrylamide solutions (Oster, 1954; Oster et al., 1957) the addition of riboflavin provides the simplest conditions for visible light photopolymerisations and does not affect ribonucleotide reductase under dark conditions. Furthermore, the mixture of components for gel polymerisation can be poured and layered easily, provided it is protected from strong light which induces polymerisation. In contrast, when polymerisation is induced with ammonium persulphate, the reaction commences immediately the reagents are mixed, and was found to be so rapid in the presence of high concentrations of a tertiary amine such as TEA or TEMED, that distortion of the upper gel surface occurs. TEMED and EDTA did not affect the activity of the reductase and were maintained at the levels used by Thelander (1967), but their addition to the gels is probably superfluous.

A gel polymerised from 9% acrylamide and 0.1% bis acrylamide solution (W/V) was found experimentally to provide optimal resolution of the reductase from contaminating proteins. The reductase (M.W. 70,000, Vitols et al., 1967b) is similar to serum albumin in size (M.W. 68,000, Oncley et al., 1947) as well as

in mobility, and the above result may be compared with those determined for the latter protein. Albumin is separated optimally from components of faster and slower mobility in gels cast from 9% cyanogum solution (Margolis et al., 1967) or from 8-10% acrylamide and 0.1% bis acrylamide (W/V) (Morris, 1966). Ingram et al. (1967) have shown that a gel cast from 9% cyanogum solution decreases the mobility of serum albumin to 50% of that in free solution.

The preparative application of the TEA-TES system has provided the only method by which the reductase can be purified to electrophoretic homogeneity. Because the species to be separated have such similar mobilities, the protein load which was routinely used (30 mg) was lower than reported for the electrophoresis of preparations containing proteins of widely different mobility (Shuster et al., 1967; Gordon et al., 1967).

Homogeneity of ribonucleotide reductase. When electrophoresed in an innocuous buffer system, preparations of ribonucleotide reductase, purified by conventional methods, have been shown to be heterogeneous despite the preliminary observation of a single boundary during sedimentation velocity determinations in the ultracentrifuge. This discrepancy

is not surprising as the ultracentrifuge distinguishes molecules by size and shape, whereas polyacrylamide gel electrophoresis differentiates on the basis of charge in addition to size and shape.

Electrophoretically homogeneous material has been obtained by electrophoresis of material of widely different specific activity but contrary to expectation, all such "pure" material did not exhibit the same high specific activity. Instead, electrophoresis raised the specific activity of all preparations by 20-30%, irrespective of the specific activity of the sample electrophoresed. This indicates that these preparations are not homogeneous, but contain protein of at least two different specific activities. The uniform mobility of these preparations in a system of high resolving power demonstrates the similarity of the constituent proteins and suggests that the contaminant is an inactive or less active form of the enzyme.

Summary

Polyacrylamide-gel electrophoresis in Tris-glycine buffer at high pH was investigated with regard to the stability of ribonucleotide reductase. Activity is lost when this enzyme is exposed to this environment.

New buffer systems were sought which combine the high resolving power of the discontinuous system of Ornstein (1964) with an operating pH which offers greater stability for the electrophoresed proteins. Several systems with potential practical application have been developed and investigated with particular reference to ribonucleotide reductase where resolution of a single main species was required. Optimal conditions to effect this separation were determined.

The application of the most favourable of these systems to preparative electrophoresis has provided the only known means of increasing the specific activity of ribonucleotide reductase highly purified by conventional methods. Preparative polyacrylamide-gel electrophoresis of ribonucleotide reductase has provided milligram quantities of electrophoretically homogeneous reductase.

The re-electrophoresis of this homogeneous preparation in Tris-glycine buffer clearly indicates the formation of multiple inactive species associated with a loss of total activity. These findings indicate that, in general, the use of this buffer for electrophoresis as a criterion of purity must be exercised with caution.

CHAPTER III

THE PROPERTIES OF COBAMIDE-DEPENDENT RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE FROM *LACTOBACILLUS LEICHMANNII*

Introduction

Kinetic studies on ribonucleotide reductase of *Lactobacillus leichmannii* (Goulian and Beck, 1966; Beck, 1967; Vitols *et al.*, 1967a) indicate the binding of nucleotide activators at a site different from the substrate site. This allosteric effect has been presumed to operate by means of a conformational change in the enzyme or its subunits (Beck, 1967; Vitols *et al.*, 1967a; Blakley, 1967), although there is scant evidence from physical measurements to support this hypothesis.

The ribonucleotide reductase of *Escherichia coli* has been shown to consist of two non-identical proteins, each of which has been extensively purified (Holmgren *et al.*, 1965; Brown *et al.*, 1967). Both proteins are required for activity. Although these reductases isolated from the two sources bear few similarities apart from hydrogen donor specificity (Blakley and Vitols, 1968), the physical properties of the *L. leichmannii* reductase reported by Goulian

and Beck (1966) have been interpreted as evidence for a similar aggregated structure in this enzyme (Larsson and Reichard, 1967). Goulian and Beck (1966) reported a molecular weight of 110,000 determined by equilibrium sedimentation while a preliminary communication from this laboratory (Blakley et al., 1965) reported a molecular weight of 25,000 determined on an unfractionated extract by the method of Leach and O'Shea (1965). Although Larsson and Reichard (1967) have concluded that this discrepancy may indicate different polymer structure of the proteins isolated by the two techniques, subsequent analysis of a highly purified extract by sedimentation velocity indicated a molecular weight of about 70,000 (Vitols et al., 1967b) which makes the above hypothesis less tenable.

Gerhart and Schachman (1968) and Weber (1968a) have used sedimentation analysis in the presence of mercurials or 8 M urea containing 0.01 M mercaptoethanol to study the subunit structure of aspartate transcarbamylase. Gerhart and Schachman have also used sedimentation analysis for an elegant demonstration of conformational changes. In aspartate transcarbamylase the binding of substrates or modifiers causes up to a 3 percent change in the

sedimentation coefficient of the protein.

Difficulties in detecting similar effects with ribonucleotide reductase arise because of the much lower measured $\bar{s}$ values (2.8 S at 4° compared with 11.2 S for aspartate transcarbamylase at 25°) and the consequently small change expected in $\bar{s}$ due to ligand binding. This is accentuated by the fact the expected experimental variation in the measurement of $\bar{s}$ is independent of the magnitude of this parameter. A greater uncertainty therefore exists in interpreting the significance of differences between the $\bar{s}$ of treated and control reductase than in the case of aspartate transcarbamylase.

Various physical methods have been used to assess the purity of the electrophoretically homogeneous ribonucleotide reductase, to measure its molecular weight and investigate it for aggregation-disaggregation phenomena. Because of the well documented allosterism of the reductase physical evidence was sought, despite the above difficulties in detecting significant differences in the relatively small molecule, to substantiate the hypothesis that ligand-binding induces conformational changes. The reactivity of enzyme sulphydryl groups to various reagents has also been

investigated as an index of ligand-induced conformational changes.

Experimental Procedure

Materials

Electrophoretically homogeneous ribonucleotide reductase was prepared as described in Chapter II. p-Hydroxymercuribenzoate (PMB) and 5,5'-bis-dithionitrobenzoic acid (DTNB) were purchased from Sigma, St. Louis, and methylmercuribromide (MMB), methylmercuric iodide (MMI) and phenylmercuric acetate were purchased from K and K Laboratories, Plainview. Nucleotides were purchased from P-L Biochemicals, Milwaukee. Ribonuclease A, cytochrome C and ovalbumin were purchased from Sigma, St. Louis; soybean trypsin inhibitor from Worthington Biochemicals, Freehold; human serum albumin from Pentex Incorporated, Kankakee, and human γ-globulin was a gift from Commonwealth Serum Laboratories, Melbourne; $^{14}C_1$-iodoacetic acid was purchased from the New England Nuclear Corporation, Boston, and iodoacetamide from Fluka, Buchs.

Scintillation fluid was prepared according to Bray (1960).

Stock solutions of mercurials (10 mM) were prepared daily by dissolving the required amount of mercurial in 0.1 M KOH which contained 0.4 M $(NH_4)_2SO_4$. After the mercurial had dissolved, the solution was neutralised by the addition of KH_2PO_4. The concentration of PMB in the stock solutions was determined spectrophotometrically at 232 mμ ($\varepsilon = 1.69 \times 10^4$ at pH 7.0 (Boyer, 1954)). Concentration of the other mercurials was calculated on the assumption that they were 95% pure.

Methods

Gel filtration of ribonucleotide reductase. The methods of Andrews (1964) and Determann (1966) were used to estimate the molecular weight of ribonucleotide reductase. Bead form sephadex was swollen in water for minimum periods of 24 or 48 hours for G100 and G200 respectively, and the fines removed by decantation. The swollen sephadex was then equilibrated in the working buffer used in the individual experiments, 0.05 M potassium phosphate pH 7.3 containing 1 mM EDTA ($\tau/2 = 0.13$) or 0.1 M sodium dimethyl glutarate (DMG) pH 7.3 ($\tau/2 = 0.27$). Columns were prepared by adding the gel slurry to a reservoir above the buffer-filled column. The reservoir contents were

mechanically stirred. Buffer flow was commenced when the settled bed occupied 10-20 percent of the total column volume and was continued until a constant bed volume was established (minimum of 24 hours). Column sizes, sample volumes and protein concentrations used in zonal studies are recorded with individual experiments. Collection of the first fraction was commenced immediately the sample was applied. Protein was eluted into weighed tubes at flow rates between 10 to 23 ml, cm^{-2} $hour^{-1}$ for G100, and 2 ml, cm^{-2} $hour^{-1}$ for G200 and the volume of each fraction determined from its mass. Elution profiles were determined by measurement of absorbance at 280 mμ and by activity determinations on suitable volumes of effluent. In zonal studies, elution volumes were measured from the point of application of the sample to the maximum concentration of protein or enzyme activity. The following proteins were used as reference compounds for the molecular weights shown : horse heart cytochrome C, mol. wt. 12,400 (Margoliash, 1962); ribonuclease, mol. wt. 13,700 (Hirs et al., 1956); trypsin inhibitor, mol. wt. 21,500 (Wu and Scheraga, 1962); ovalbumin, mol. wt. 45,000 (Warner, 1954); human serum albumin monomer, mol. wt. 69,000 (Oncley

et al., 1947) and dimer, mol. wt. 138,000; human γ-globulin, mol. wt. 160,000 (Phelps and Putnam, 1960). A linear relationship was obtained between elution volume on Sephadex G100 and log molecular weight over the range 12,400-69,000.

Other experiments were performed to include a plateau region in the elution profile (Winzor et al., 1963). A column 30 cm x 0.6 cm was used with a sample size of 10 ml. Fractions of 0.4 ml (range 0.37-0.42) were collected. Elution profiles were determined as described above, the elution volume being taken as the volume which corresponded to an enzyme activity (or absorbance at 280 mμ) of 50 percent of that in the plateau region.

All gel filtration experiments were performed at 4°.

Ultracentrifugation

All ultracentrifuge investigations and the associated data treatments were performed by Dr. J.R. Dunstone, Department of Physical Biochemistry, John Curtin School of Medical Research.

A Beckman Model E analytical ultracentrifuge was used with either 12 mm or 30 mm double-sector cells (aluminium-filled Epon centrepieces); one sector was

filled with solution and the other with equilibrium diffusate. Solutions were buffered with dimethylglutarate (0.1 M 3,3'-dimethylglutaric acid adjusted to pH 7.3 with NaOH; $\tau/2$, 0.27). Temperatures were controlled with the RTIC unit. Individual experimental conditions are given in the text.

Sedimentation velocity. Experiments were performed at 50,740 rpm. The weight-average sedimentation coefficients, $\bar{s}$, were calculated from the rates of movement of the square roots of the second moments of the schlieren patterns (Goldberg, 1953). When comparisons between experiments at different temperatures were required, the $\bar{s}$ values were corrected to 20° in water giving the values $\bar{s}_{20,w}$. Concentrations were determined refractometrically using peak areas (obtained by trapezoidal integration) and assuming a specific refractive increment of 0.0018 dl g^{-1}. In the determination of the concentration dependence of the sedimentation coefficient mean plateau-region concentrations were used.

As a test of homogeneity an analysis of the schlieren patterns was carried out (Baldwin, 1954). A sedimentation coefficient distribution (a plot of g*(S) vs S) was obtained at different times during an

experiment. The terms g*(S), an apparent differential distribution function, and S, a reduced coordinate with the same dimensions as the sedimentation coefficient, have been clearly defined (Nichol and Creeth, 1963; Baldwin, 1954; Signer and Gross, 1934). The curves of g*(S) vs S were very nearly symmetrical. The range of S decreased progressively with time indicating the significant contribution of diffusion to the spreading of the boundary. These effects were eliminated by extrapolating to infinite time (Baldwin, 1959). The quantity $[S - \bar{s}]^2$, where $\bar{s}$ is the weighted mean of the distributions of S, was plotted against $1/te^{\bar{s}\omega^2 t}$ at fixed values of g*(S)/g*(S) max (ω is the angular velocity). As the weighted means of the distributions of S did not vary significantly from the corresponding weight-average sedimentation coefficients the single symbol $\bar{s}$ has been used throughout. While the method accounts for the diffusional spreading of the boundary it does not account for the concentration-dependence of the sedimentation coefficient. This should not markedly affect the analysis because of the relative closeness of the measured sedimentation coefficient, and that at infinite dilution (Baldwin, 1959).

A boundary analysis procedure (Creeth and Pain, 1967) based on a simplified form (Van Holde, 1960) of an expression for the height and area of a gradient curve as a function of time (Fujita, 1959) accounts for small linear concentration-dependence effects and provides a further test of homogeneity and a method for determining apparent diffusion coefficients. This method has been applied to the schlieren patterns from the sedimentation velocity experiments. The apparent diffusion coefficients, D*, so obtained have been corrected to 20° in water giving the values $D^*_{20,w}$.

Equilibrium sedimentation. Experiments were performed at 14,210 rpm; Kel-F polymer oil was used as an inert base fluid; volumes of polymer oil, equilibrium diffusate and solution were taken so that the diffusate column just overlapped the solution column (1.5 mm) at both ends; most experiments were carried out in the DMG buffer, pH 7.3; the An-J equilibrium rotor and schlieren optics were used. Separate experiments with solutions of different initial concentrations were performed. Equilibrium was usually reached in 15 hours (33 hours in 7 M urea). Measurements (20-30) were made of the refractive index gradient at equal intervals throughout the solution columns using a Gaertner

toolmakers' microscope, type M2001, As-P.

The molecular weight at any point in the cell can be calculated from the slope, at that point, of a graph of ln c *vs* $r^2/2$ or of ln $\frac{1}{r} \cdot \frac{dc}{dr}$ *vs* $r^2/2$ by multiplying by the constant factor $RT/(1-\bar{V}\rho)\omega^2$; the former method gives point weight-average molecular weights and the latter point z-average molecular weights. In practice these graphs were almost linear, so apparent weight-average ($\bar{M}_w$) and z-average ($\bar{M}_z$) molecular weights were obtained from the slopes of the best straight lines through the data, the slopes being calculated by the method of least squares. Point concentrations were computed (Schachman, 1957) using initial concentrations obtained by measurements with a differential refractometer (Cecil and Ogston, 1951).

Partial specific volume. The partial specific volume of the protein required for the calculation of molecular weight was deduced from the amino acid composition using the method outlined by Schachman (1957). A value of 0.73 was obtained.

Amino acid analysis. Chromatographic analysis was performed by Mr. L.B. James. Samples for amino acid analysis were hydrolysed with 6 N HCl in evacuated tubes (Crestfield *et al.*, 1963) at 110° for 22 hours.

Analyses were performed by the method of Spackman et al., (1958) on a Beckman Model 120B amino acid analyser (Spinco Division, Beckman Instruments Inc., Palo Alto, California). The height of the long column was 50 cm and that of the short column, 12 cm. Buffer flow rate was 40 ml per hour. Determinations of serine and threonine in 22 and 70 hour hydrolysates did not vary by more than 2-3 percent with the increased hydrolysis time and no correction has been made for any loss of these amino acids during hydrolysis. No correction was made for possible incomplete cleavage of any peptide bonds. Each value for the number of amino acid residues shown in the text is the mean of three determinations for basic amino acids and eleven determinations in the case of neutral and acidic amino acids. All figures were within the range of accuracy expected from this method (± 3 percent).

The number of residues per mole has been calculated for a molecular weight of 76,000 and the number of residues of tryptophan has been assumed to equal the average of tyrosine and phenylalanine. In the case of some analyses where small quantities of S-carboxymethylcysteine were determined, the resistor card in the Honeywell recorder of the analyser was replaced with one

for the range 3.610-4.6385 V, resulting in a 10-fold increase in sensitivity for these peaks (Bell et al., 1968). The conventional resistor was used for the determination of other amino acids in these investigations.

Performic oxidation of ribonucleotide reductase

The method of Hirs (1956) was used to performic acid oxidise the reductase. Performic acid was prepared by adding 0.5 ml of H_2O_2 (100 vols) to 9.5 ml of formic acid and the mixture was allowed to stand at room temperature for 2 hours before being chilled to 0°. Ribonucleotide reductase (2 mg) was dissolved in 0.05 ml of formic acid (98 percent) and reacted with a twelve molar excess of the performic acid (about 0.1 ml) at 0° for two hours. The protein solution was lyophilised and the residue dissolved in 1 ml of 98 percent formic acid. 5 ml of water was added to destroy any residual performic acid and peroxides present and then re-lyophilised. This material was then hydrolysed for amino acid analysis or subjected to tryptic digestion for peptide mapping.

Alkylation of exposed sulphydryl groups. The number of exposed sulphydryl groups in the reductase under various conditions was assayed by the amino acid

analysis of S-carboxymethylcysteine following exposure to iodoacetic acid or iodoacetamide for 30 min at 0°. Brownson and Blakley (1967, unpublished results) have shown that iodoacetamide inactivation of the reduced reductase is complete after 10 minutes exposure at 0°. This modified procedure of Crestfield et al. (1963) was used, both in the presence and absence of 7 M urea. In these determinations, corrections were made for the presence of methionine sulphoxide in the S-carboxymethylcysteine peak. This was done by subtracting from this peak the number of residues by which the methionine peak was deficient. With this technique there was excellent agreement between the results obtained by amino acid analysis and the radioactivity determinations.

Preparation of reduced ribonucleotide reductase. The reductase (0.5-10 mg in 0.5-1 ml) was exposed to 30 mM reduced lipoate at 0° for 30 minutes. The mixture was then dialysed against 1000 volumes of potassium phosphate buffer (0.1 M pH 7.3) for 24 hours with changes of the buffer every 6-8 hours to remove excess lipoate.

Determination of ^{14}C-S-carboxymethylcysteine. Alkylation of the reductase was performed as described above with [1-^{14}C]iodoacetic acid S.A. 3.3 x 10^6 cpm/

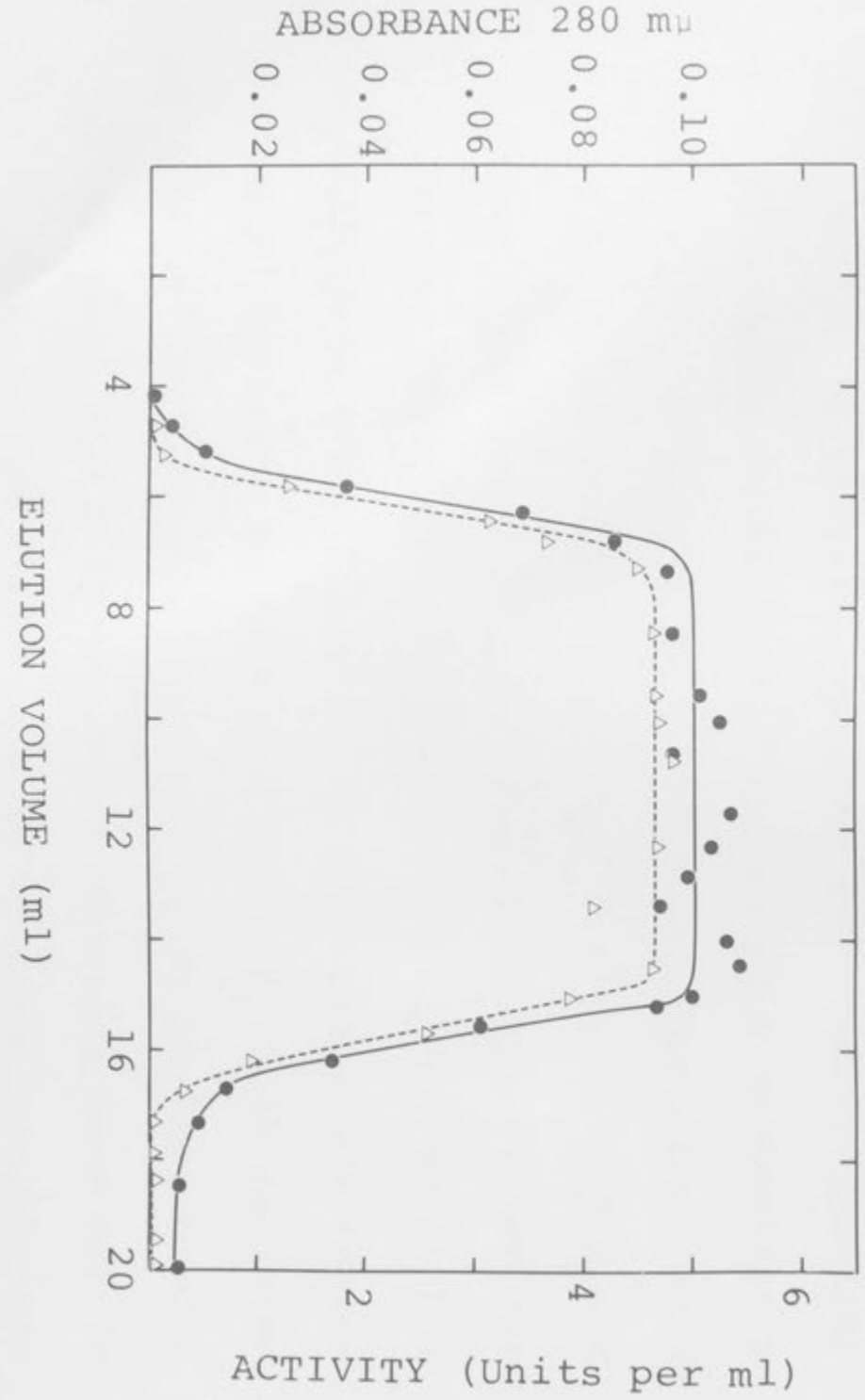
μmole. After dialysis and hydrolysis, the proportion of the radioactivity associated with the S-carboxymethyl-cysteine peak was measured on a portion of the sample by passage of the column effluent from the amino acid analysis through an anthracine-filled flow cell in a Nuclear Chicago 720 series liquid scintillation counter before colorimetric assay in the normal manner. The total radioactivity in the hydrolysate was measured in a Packard Tri-carb liquid scintillation spectrometer, and the counts in the hydrolysate and the original iodoacetic acid were measured in the same vials to control quenching. The total number of moles of protein present in the hydrolysate was calculated from its amino acid analysis.

Tryptic digestion. This was performed by Dr. D.C. Shaw, as were the peptide maps, by the method of Bell et al. (1968).

Detection of ninhydrin-staining peptides. A solution of the reagent is prepared by dissolving 0.5 g of ninhydrin in 500 ml of acetone to which either 1 ml of pyridine or 1 ml of glacial acetic acid were added. The electrophorogram was sprayed with this reagent and the paper hung in the dark for 24 hours to allow the development of ninhydrin-positive spots.

Detection of ninhydrin-negative peptides. The electrophorogram was placed in a jar in which chlorine gas was produced by reacting $KMnO_4$ with HCl, for an exposure time of 30 to 60 minutes. Excess chorine was then removed by overnight flushing in a stream of air. The paper was then sprayed with a 1 percent solution of soluble starch containing 1 percent Kl. Peptides stain blue-black.

Measurement of rates of reaction of DTNB with ribonucleotide reductase. The reaction of DTNB with the enzyme was measured spectrophotometrically by recording the rate of production of the chomophore at 412 mμ in a Cary 14 recording spectrophotometer (Ellman, 1959). The reaction mixture (1 ml) was contained in a glass cuvette of 10 mm light path (4 mm wide) and comprised 0.95 ml of DMG or potassium phosphate buffer pH 7.3, with the concentration of activators as shown in the individual experiments, and 10 mM DTNB. The solutions were mixed by six successive inversions and placed in the spectrophotometer at 0° or 35° and the balance adjusted to give a zero reading at 412 mμ against a reference cuvette of the same solution. The reaction was initiated by the addition of 0.05 ml of electrophoretically


ABSORBANCE 280 mμ
0.02
0.04
0.06
0.08
0.10
4
8
12
16
20
ELUTION VOLUME (ml)
2
4
6
ACTIVITY (Units per ml)

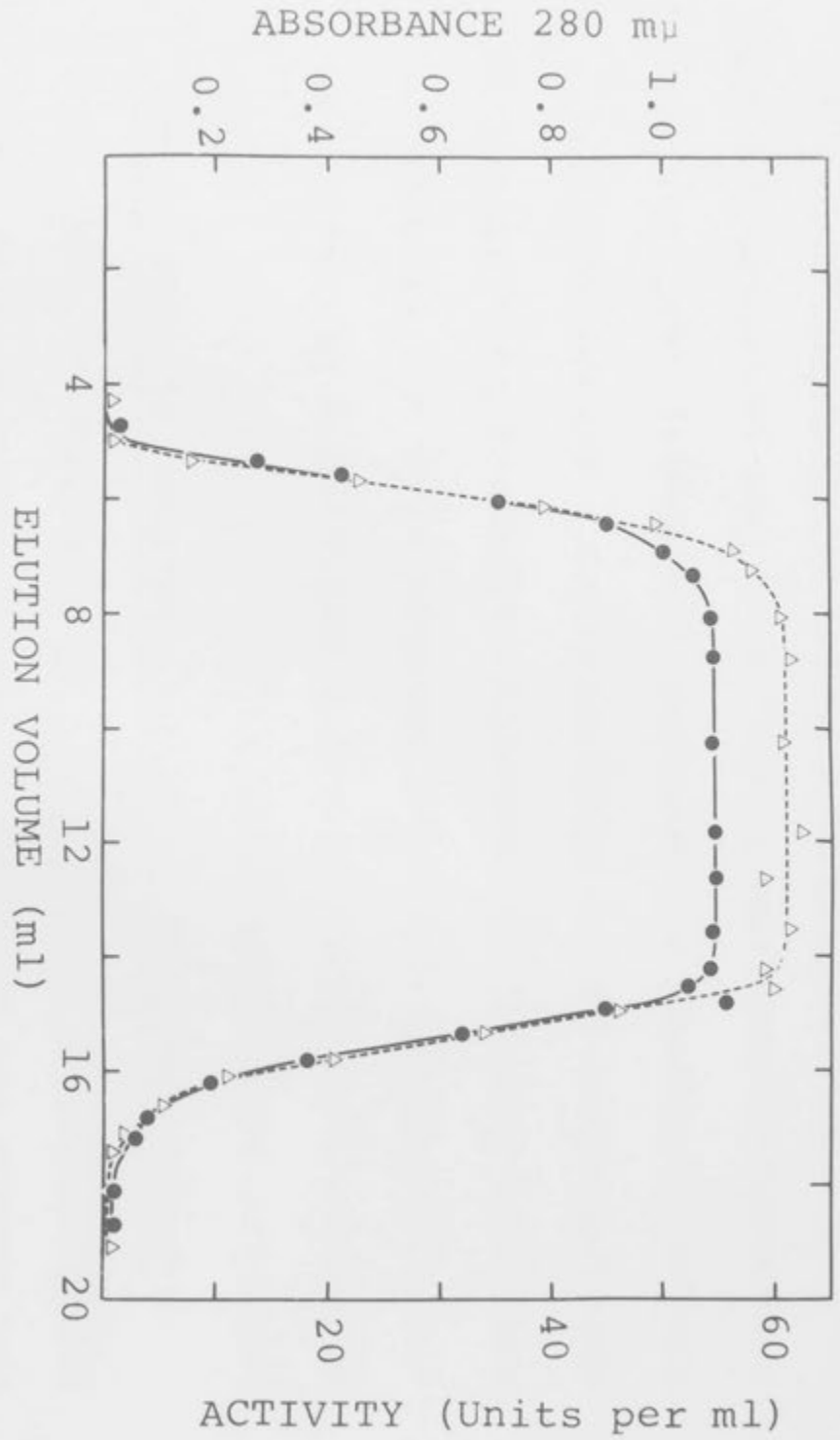

ABSORBANCE 280 mμ
0.2
0.4
0.6
0.8
1.0
4
8
12
16
20
ELUTION VOLUME (ml)
20
40
60
ACTIVITY (Units per ml)

FIG. III.1. Elution profiles of ribonucleotide reductase at concentrations of 1.2 mg/ml (A) and 0.09 mg/ml (B).

10 ml samples of the enzyme were chromatographed on a 0.6 x 30 cm column of Sephadex G100. Absorbance at 280 mμ, O—O, ribonucleotide reduction Δ——Δ as measured at 595 mμ by the use of the diphenylamine colorimetric assay with 0.02 ml and 0.2 ml of effluent in experiment A and B, respectively.

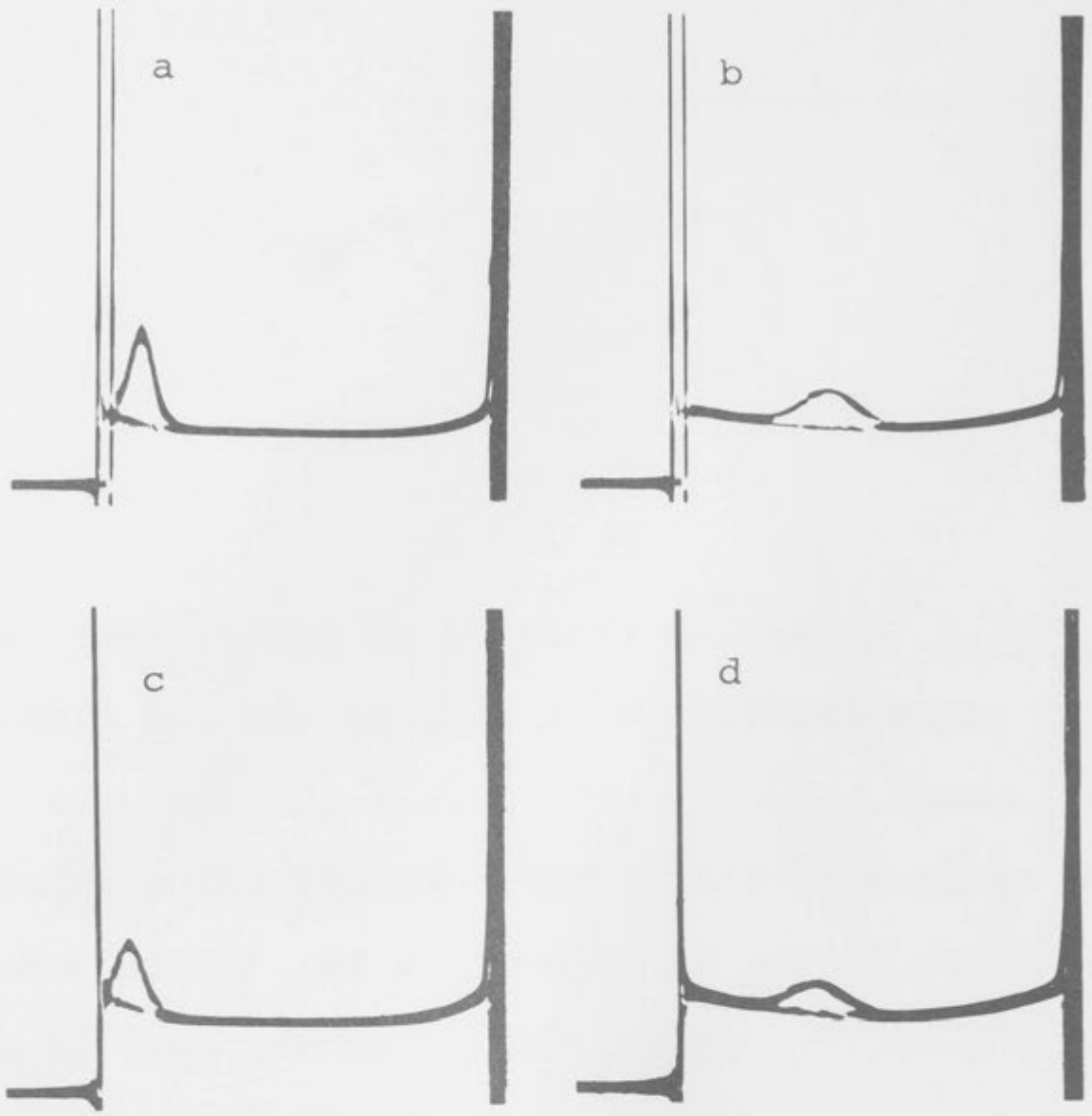


FIG. III.2. Schlieren patterns obtained during sedimentation velocity determinations on ribonucleotide reductase.
The speed was 50,740 rpm. Sedimentation from left to right.

a, b concentration 0.2 g dl^{-1}
Schlieren phase plate angle 70°
times after reaching speed 20 min, 108 min
temperature 4.1°

c, d concentration 0.13 g dl^{-1}
Schlieren phase plate angle 65°
times after reaching speed 19 min, 99 min
temperature 5.0°

homogeneous ribonucleotide reductase (protein concentration 20 mg/ml) in 0.1 M DMG, pH 7.3. In the case of the reduced enzyme, an equal volume of equilibrium diffusate was added to the reference cuvette. Again mixing was by six successive inversions and recording was commenced within 20 seconds of the addition of the reductase and was continued for 100 minutes. The number of sulphydryl groups was determined from ε_m = 13,600 (Ellman, 1959).

Results

Homogeneity of ribonucleotide reductase. The behaviour of electrophoretically homogeneous reductase was investigated by gel filtration on Sephadex G100. In zonal studies the elution patterns of protein and enzyme activity were both symmetrical and gave identical elution volumes. In plateau experiments, leading and trailing edges provided enantiographic patterns for both protein and activity as shown in Fig. III.1.

The homogeneity of the preparations was investigated further in the ultracentrifuge. As shown in Fig. III.2, sedimentation velocity experiments demonstrated single apparently symmetrical schlieren peaks, the area under which remained constant (after correction for radial dilution) for the duration of the experiment (3-4

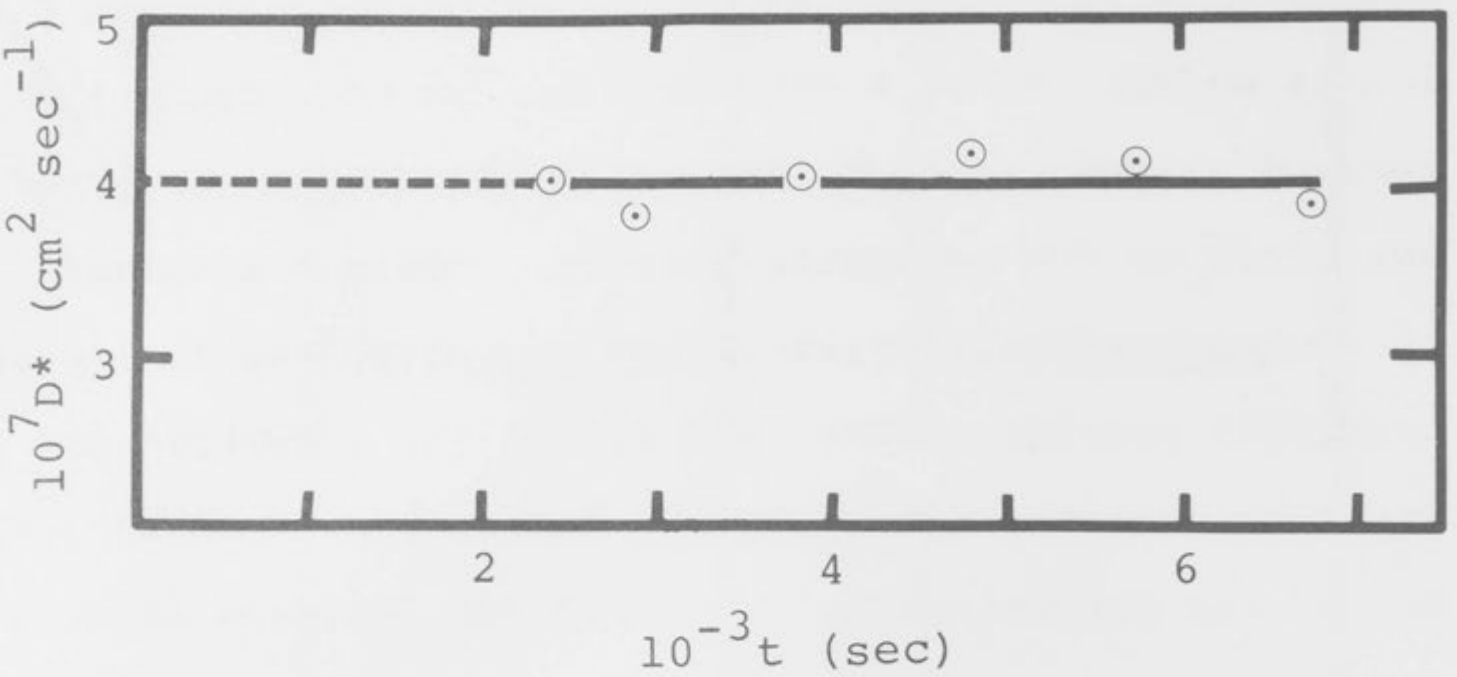


FIG. III.3. The constancy of the diffusion coefficient with time. Boundary analysis of Creeth and Pain (1967).
The apparent diffusion coefficient (D*) is plotted against time (t). Conditions were identical with those described for Fig. III.2a and b.

hours), a result indicating the absence of gross heterogeneity. An apparent content of 3 to 4 percent of slower sedimenting material was seen in some preparations, but as no corrections were made for the Johnston-Ogston effect (1946) the true proportion of these components is probably smaller.

The sedimentation coefficient of the reductase varied linearly with concentration over the range 0.03-0.3 g dl^{-1}, and from the least squares treatment of the experimental data the following expression for the concentration dependence of $\bar{s}_{20,w}$ was derived.

$$\bar{s}_{20,w} = 5.2\ (1-0.09\ C) \times 10^{-13} \qquad (1)$$

where C is the concentration of protein in g dl^{-1}. This relationship was used in the boundary analysis of Creeth and Pain (1967) where the apparent diffusion coefficient was calculated at various times and the results are shown in Fig. III.3. The criterion of homogeneity is the constancy of the apparent diffusion coefficient with time.

The apparent diffusion coefficient varied linearly with concentration in the range 0.03-0.3 g dl^{-1} and least squares treatment of the experimental data gives the following expression for the concentration depend-

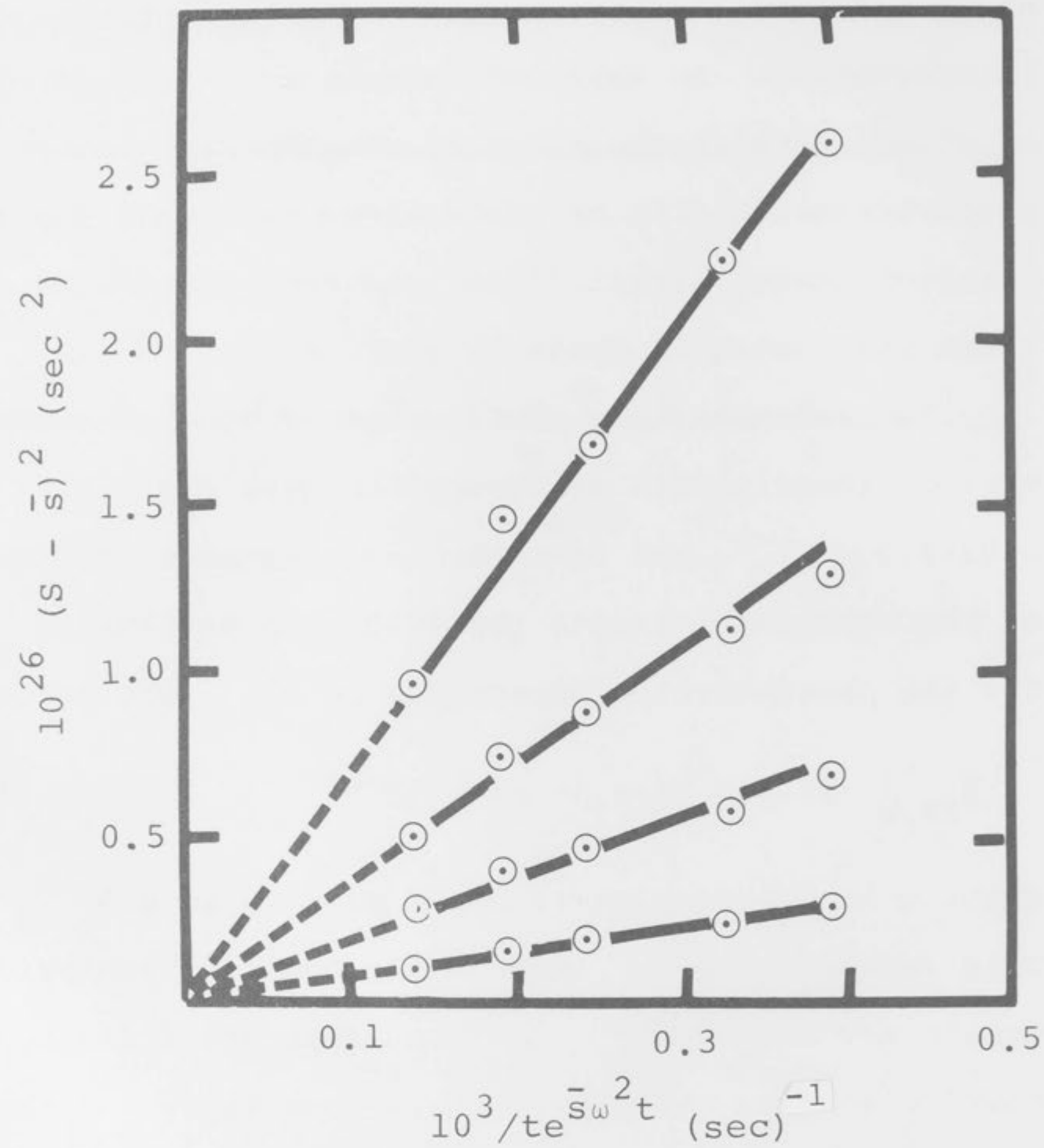


FIG. III.4. Boundary analysis of Baldwin (1954). The value $[S-\bar{s}]^2$ is plotted against $1/te^{\bar{s}\omega^2 t}$ for values of g*(S)/g*(S)Max of 0.2, 0.4, 0.6 and 0.8.

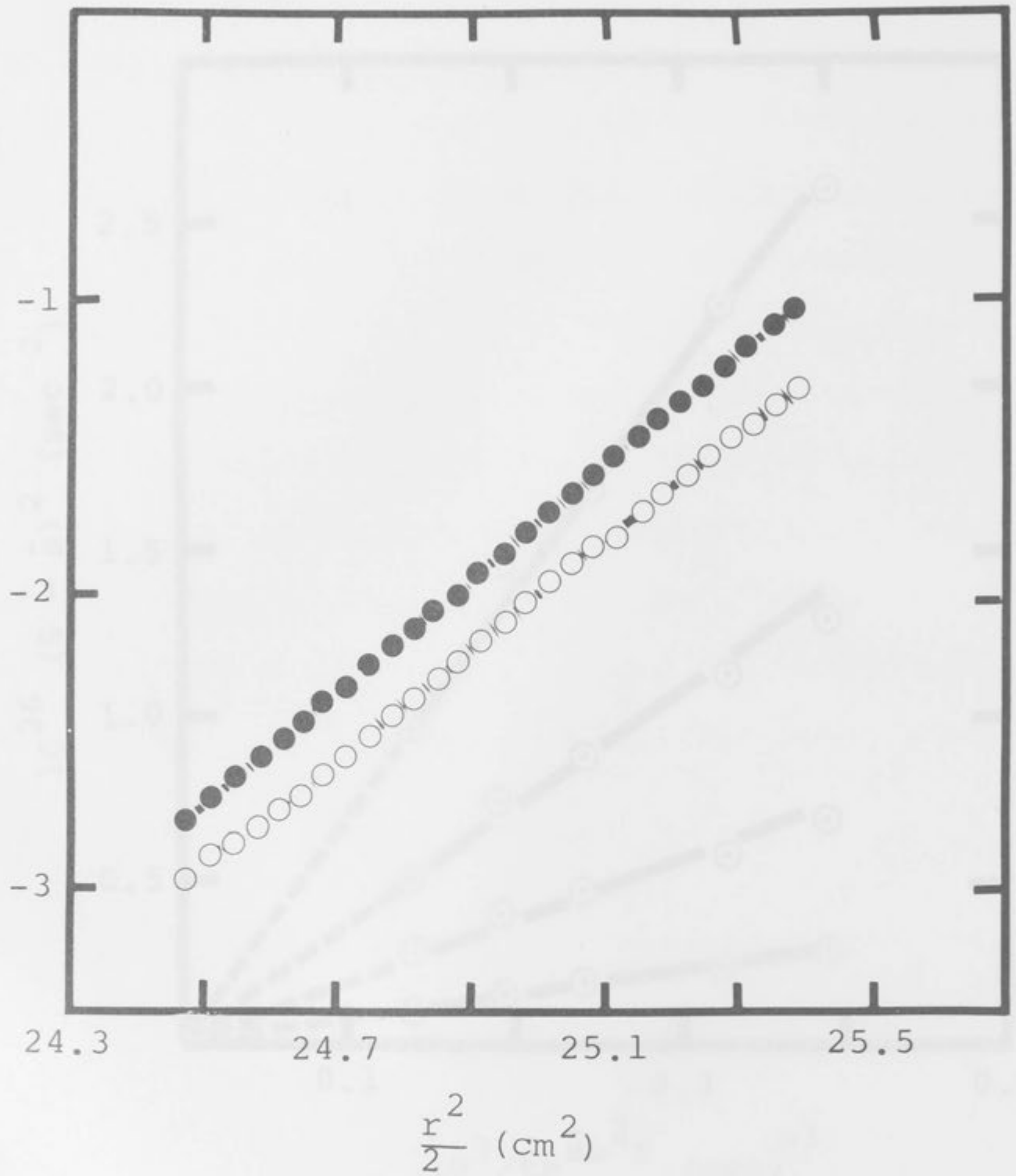


FIG. III.5. Equilibrium sedimentation analysis of ribonucleotide reductase.

●—● ln C. Initial concentration 0.2 g dl^{-1}.

○—○ ln $\frac{1}{r}.\frac{dc}{dr}$ Concentration range 0.06-0.5 g dl^{-1}.

ence of $D^*_{20,w}$.

$$D^*_{20,w} = 6.4\ (1 + 0.4\ C) \times 10^{-7} \qquad (2)$$

where C equals concentration in g dl^{-1}.

The results of the boundary analysis of Baldwin (1954) are shown in Fig. III.4, where $[S - \bar{s}]^2$ is plotted against $1/te^{\bar{s}\omega^2 t}$ for various values of g*(S)/g*(S) max and the extrapolations to infinite time are shown by the dashed lines. The values of $[S - \bar{s}]^2$ at infinite time are close to zero, indicating the absence of measurable heterogeneity in terms of sedimentation coefficient.

The plots of g*(S)/g*(S) max *vs* S were symmetrical which gives a further indication of the homogeneity.

Examination of the reductase by equilibrium sedimentation permitted the calculation of weight-average and z-average molecular weights from the slopes of the plots log c *vs* $r^2/2$ and $\log \frac{1}{r} \cdot \frac{dc}{dr}$ *vs* $r^2/2$, respectively (Fig. III.5). The z-average molecular weight, defined by the expression

$\bar{M}_z = \sum_i N_i M_i^3 / \sum_i N_i M_i^2$ and the weight average molecular weight, defined by

$\bar{M}_w = \sum_i N_i M_i^2 / \sum_i N_i M_i$

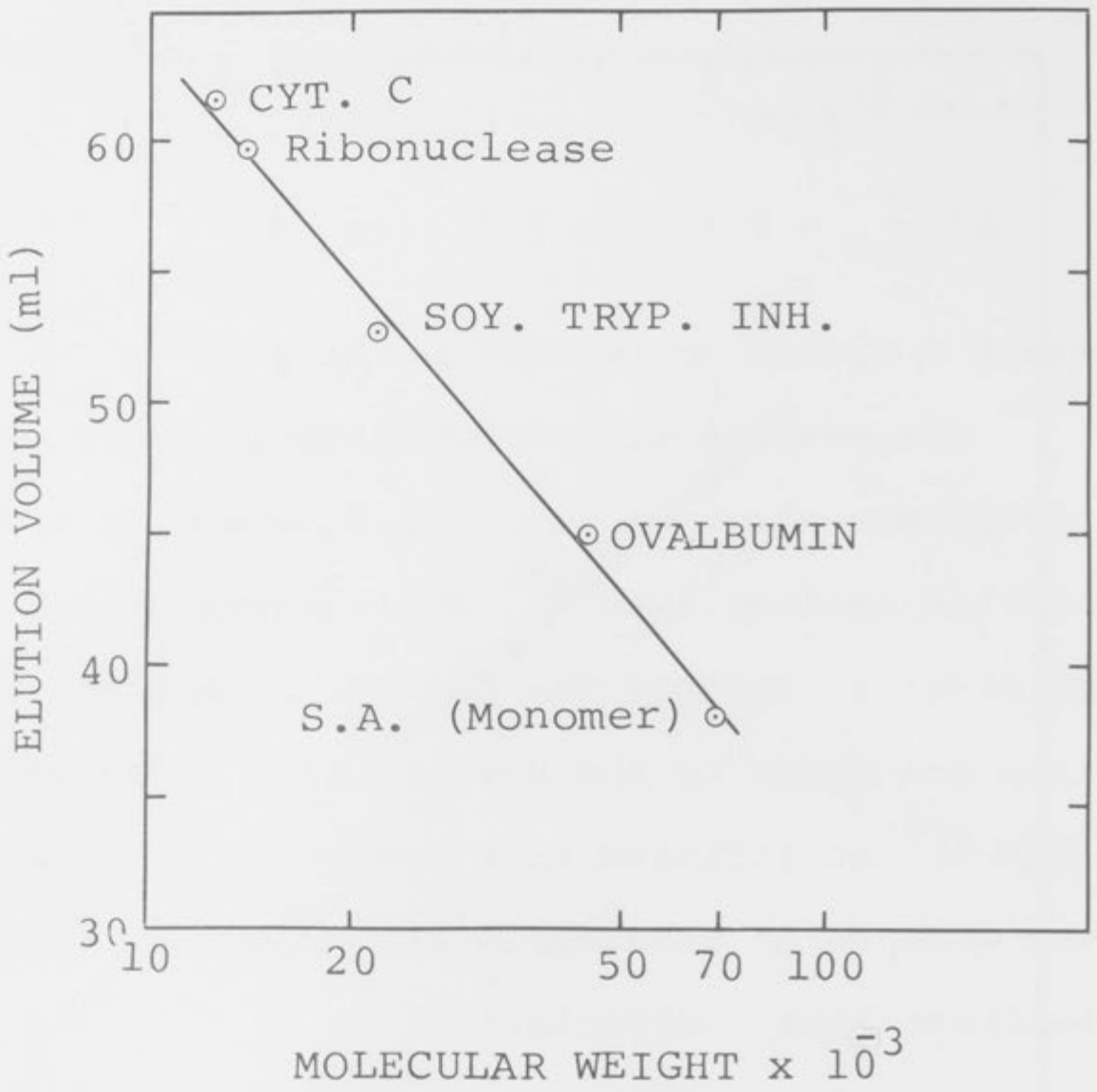


FIG. III.6. Relationship of elution volume of proteins from Sephadex G-100 and the log of the molecular weight.

The points indicate the experimentally determined elution volumes of the proteins shown, and the line was computed by the method of least squares.

where N_i is the number of molecules of kind i present in the mixture and M_i is their molecular weight, are equal if the material is homogeneous. If heterogeneous then invariably $\bar{M}_z > \bar{M}_w$ (Tanford, 1961). The linearity of the analytical plots and the similarity of the slopes (Fig. III.5) from which the $\bar{M}_w$ and $\bar{M}_z$ were calculated also indicate the homogeneity of the preparations.

Molecular weight of ribonucleotide reductase.

Comparison of the elution volume of the reductase (initial concentrations of 7, 0.7 and 0.07 mg/ml) from Sephadex G100 with those experimentally determined with proteins of known molecular weight (Andrews, 1964) suggests a molecular weight for the reductase of about 70,000 (Fig. III.6).

The relationships previously described were used to calculate the $\bar{s}_{20,w}$ (equation 1) and the $D^*_{20,w}$ (equation 2) at infinite dilution. The partial specific volume ($\bar{v}$) calculated as described in Methods, was 0.73. Substitution of these values into the Svedberg equation (Svedberg and Pedersen, 1940)

$$M = \frac{S^0 RT}{D^0 (1 - \bar{v}\rho_0)}$$

where M is the apparent molecular weight, R is the gas constant, T is the absolute temperature and ρ_0 is the density of the solvent, gives an apparent molecular weight of 73,000. Molecular weights calculated from equilibrium sedimentation at four different initial concentrations (0.03, 0.08, 0.13 and 0.20 g dl^{-1}) are shown in Table III.1. The weight-average and the z-average molecular weights are identical within the expected experimental variation of the method. The average value of molecular weight of the reductase from all estimations by gel filtration, sedimentation diffusion and equilibrium sedimentation is 76,000.

Amino acid composition and attempted determination of the chemical molecular weight of ribonucleotide reductase. A table of the number of residues determined for each amino acid for a molecular weight of 76,000 is given in Table III.2, column 1. The figures determined during this investigation may be compared with those of Goulian and Beck (1966) in column 2, which were estimated for an apparent molecular weight of 100,000. Column 3 of Table III.2 shows these figures proportionately reduced for molecular weight 76,000.

During the peptide mapping of the reductase by the method outlined by Bell et al. (1968), insoluble material

TABLE III.1. Molecular weights of ribonucleotide reductase by equilibrium sedimentation

Conditions	Initial concn. g dl^{-1}	Concn. range g dl^{-1}	Apparent mol. wt.	
			$\bar{M}_w$	$\bar{M}_z$
Dimethylglutarate	0.2	0.06-0.5	73,000	72,000
Dimethylglutarate	0.13	0.05-0.3	80,000	73,000
Dimethylglutarate	0.08	0.02-0.17	82,000	77,000
Dimethylglutarate	0.03	0.01-0.09	79,000	79,000
Dimethylglutarate + dGTP (1 mM)	0.07	-	-	78,000
Dimethylglutarate + urea (7 M)	0.16	-	-	76,000

TABLE III.2. Amino acid analysis of ribonucleotide reductase

Analyses were performed by the method described by Bell et al. (1968). Basic amino acids and half-cystine residues (the latter determined as cysteic acid after performic acid oxidation) are shown as average values from separate triplicate determinations. Values for acidic and neutral amino acids are the average of eleven estimations.

	Column 1	Column 2*	Column 3**
Lysine	37.9	98	74.5
Histidine	7.9	26	19.8
Arginine	29.8	15	11.4
Aspartic acid	77.0	105	79.6
Threonine	30.0	56	42.6
Serine	45.5	156	118.5
Glutamic acid	76.5	165	125.6
Proline	29.8	12	9.1
Glycine	52.8	157	119.2
Alanine	53.5	86	66.4
Half-cystine (as cysteic acid)	7.71	Not determined	-
Valine	40.2	25	19.0
Methionine	7.7	12	9.1
Isoleucine	36.8	32	24.3
Leucine	55.2	62	47.2
Tyrosine	23.8	None detected	-
Phenylalanine	27.8	21	15.9
Tryptophan	Not determined	Not determined	-
Mol. wt.	76,000	100,000	76,000

*Values published by Goulian and Beck (1966).

**Values of column 2 proportionately reduced to fit a molecular weight of 76,000.

was precipitated at pH 4.0 on the addition of acetic acid. This precipitate was soluble at pH 8.9 and electrophoresis at this pH in 2% $(NH_4)_2CO_3$ buffer followed by ascending chromatography in pyridine-isoamyl alcohol-water (35:35:30) revealed 38-40 ninhydrin-staining peptides. The basic amino acid content of the reductase (Table III.2, column 1) indicates a maximum of 65-70 cleavage points for complete tryptic digestion of a single polypeptide or two or more different constituent polypeptides for a molecular weight of 76,000. Although this initial result would seem compatible with a structure of two identical polypeptide chains about molecular weight 35,000, treatment of the peptide map with chlorine followed by staining with starch-potassium iodide indicated the presence of a large "core" peptide (s) which was estimated from the intensity and extent of the stain to constitute about 40% of the applied sample. The "core"-containing region of unstained maps was excised, macerated in 6 N HCl and hydrolysed after the removal of paper by filtration. Amino acid analysis of the hydrolysate indicated that insufficient material was eluted from the map by this technique to give a meaningful result. Gel filtration of a tryptic

digest of the reductase on Sephadex G100 did not reveal any material with an elution volume corresponding to the molecular weight of the native enzyme, but the elution volume of the first fractions containing material absorbing at 280 mμ, suggested a molecular weight for these substances of 20,000. A sample of the "core" was prepared by precipitation from the tryptic digest of the reductase with acetic acid at pH 4.0. This precipitate was then dissolved in ammonium bicarbonate (5 g NH_4HCO_3/l) and re-precipitated to minimise co-precipitation of soluble peptides. Amino acid content of the core, calculated for a molecular weight of 20,000 is shown in Table III.3. It is of interest that the core contains all of the histidine present in the original molecule, but no cysteine or cystine. The proportion of acidic amino acids has been considerably increased from 22% to 35%. The lysine and arginine residues remaining, suggest that up to ten cleavage points in the original molecule are resistant to tryptic digestion. Because of this poor reaction, coupled with the uncertainty of the molecular weight and homogeneity of the acid-insoluble fragment, no estimate of the minimal chemical molecular weight of ribonucleotide reductase can be made from this technique.

TABLE III.3. Amino acid analysis of the "core" peptide produced during tryptic digestion

Number of residues were calculated for molecular weight 20,000.

	No. of residues	
Lysine	3.67	(37.9)
Histidine	7.68	(7.9)
Arginine	4.84	(29.8)
Aspartic acid	20.3	(77.0)
Threonine	11.7	(30.0)
Serine	5.62	(45.5)
Glutamic acid	39.2	(76.5)
Proline	5.75	(29.8)
Glycine	16.2	(52.8)
Alanine	7.8	(53.5)
Half-cystine	Nil	(7.71)
Valine	5.15	(40.2)
Methionine	2.8	(7.7)
Isoleucine	15.9	(36.8)
Leucine	11.6	(55.2)
Tyrosine	Not det.	(23.8)
Phenylalanine	14.8	(27.8)
Tryptophan	Not det.	-

The number of residues in the original protein are shown in parenthesis.

The investigation of ribonucleotide reductase for aggregation or disaggregation under various conditions. Gel filtration of the reductase on Sephadex G100 at initial concentrations of 7, 0.7 and 0.07 mg/ml gave identical elution volumes on zonal analysis. As the protein was diluted approximately 10-fold during the experiment, this indicates that the active enzyme does not undergo any gross changes in size or shape when diluted to about 0.01 mg/ml. This finding was confirmed by "plateau" experiments at initial concentrations of 1.0 and 0.09 mg/ml (Fig. III.1), where each concentration gave identical elution volumes at both the leading and trailing profile. The leading and trailing edges of the profiles were enantiographic with respect to both activity and protein which indicates that the enzyme does not dissociate or associate over the range of concentrations investigated. The frontal analysis has the advantage that the maximum concentration is defined which in turn can directly influence the elution volume, whereas in the zonal study, the samples are subject to continuous dilution.

The weight-average sedimentation coefficient of the reductase determined by equilibrium sedimentation did not decrease on dilution and in fact, the

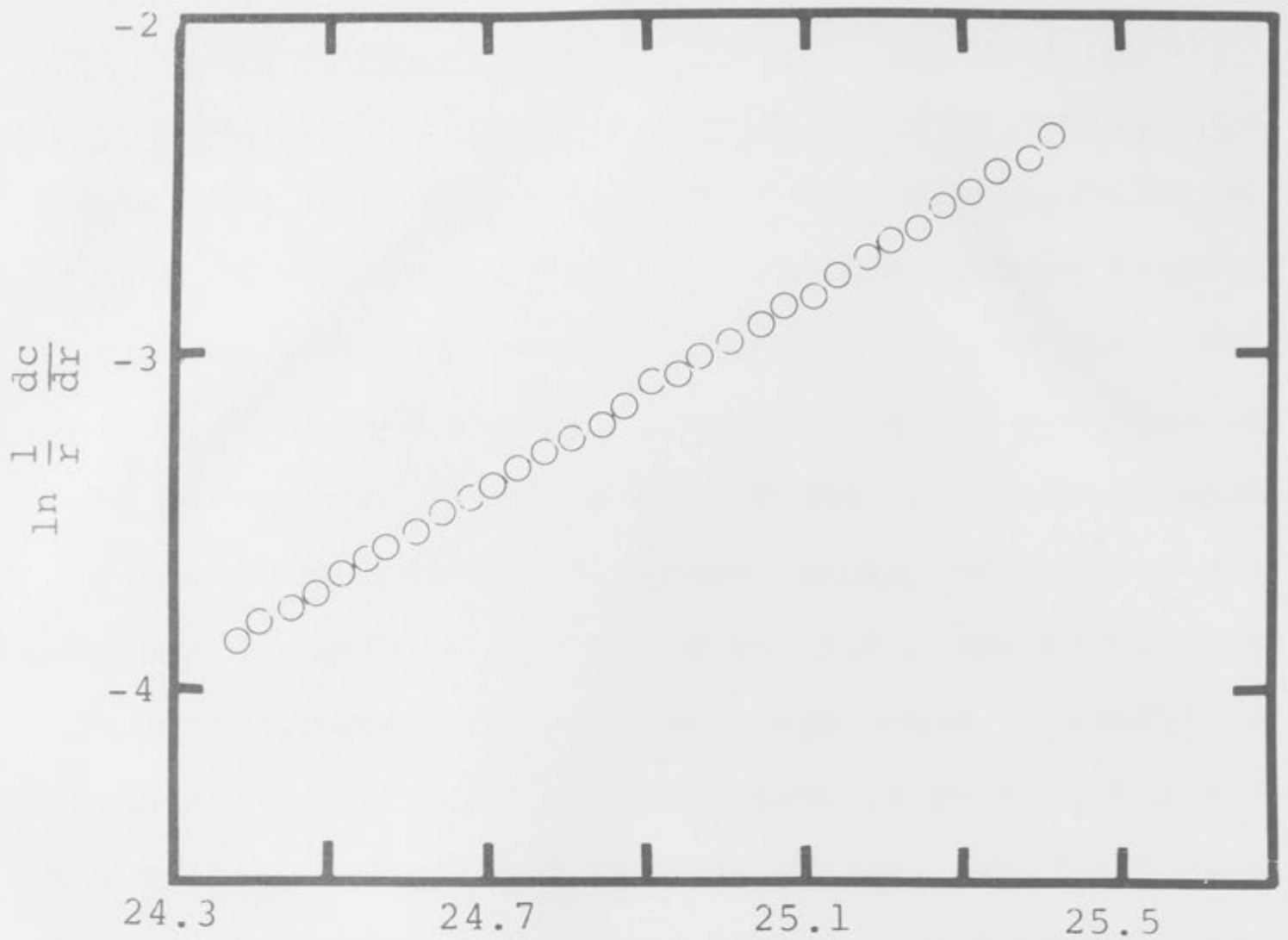


FIG. III.7. Equilibrium sedimentation analysis of ribonucleotide reductase (1.6 mg/ml) in 7 M urea and 10 mM mercaptoethanol.
The z-average molecular weight (76,000) was determined from the slope of the least squares line drawn through the experimental data.

concentration dependence was negative down to a concentration of 0.03 g dl^{-1} and there was a small negative (or no) concentration dependence of molecular weight down to 0.01 g dl^{-1}. This indicates that there is no aggregation or disaggregation over the range of concentrations investigated.

Exposure of the enzyme to 7 M urea containing 0.01 M mercaptoethanol produced no change in the apparent molecular weight of 76,000 as determined by equilibrium sedimentation and calculated from the slope of the plot $\ln \frac{1}{r} \cdot \frac{dc}{dr}$ vs $r^2/2$ (Fig. III.7 and Table III.1). The density of 7 M urea was obtained from the data of Kawahara and Tanford (1966) and the partial specific volume was assumed to be the same as that calculated for the enzyme in ordinary buffer (0.73). The true partial specific volume of the protein in 7 M urea is unknown, but it is likely that the real value is slightly higher than that used (see Schachman and Edelstein, 1966). If this is, in fact, the case, the use of a falsely low value would lead to a low apparent molecular weight. However, the observed molecular weight agrees well with those determined in buffer solution.

Sulphydryl groups of ribonucleotide reductase.

Vitols et al. (1967b) have shown that the enzyme, isolated as described in Chapter II Methods can be reduced by the action of a dithiol to a molecule which contains at least one sulphydryl group, readily accessible to iodoacetate, iodoacetamide and DTNB. Although this reduced form of the enzyme is present during activity determinations, they have clearly demonstrated that this thiol on the reduced protein does not function as the ultimate reductant in the production of deoxynucleotides. As it is possible that thiols and disulphides may be involved in the maintenance of quaternary structure of the enzyme, the reactivity of enzyme sulphydryl groups towards various reagents was investigated.

The total number of cysteine plus half cystine residues (calculated as cysteic acid after performic acid oxidation of the enzyme) was 8 per mole and the reactivity of the untreated enzyme with various reagents is shown in Table III.4. Iodoacetamide and iodoacetic acid react very poorly under conditions normally used for the S-carboxymethylation of proteins. However, on prolonged exposure of the enzyme to PMB or DTNB four SH groups react with these agents per mole

TABLE III.4. The reactivity of sulphydryl groups on unreduced ribonucleotide reductase with various reagents

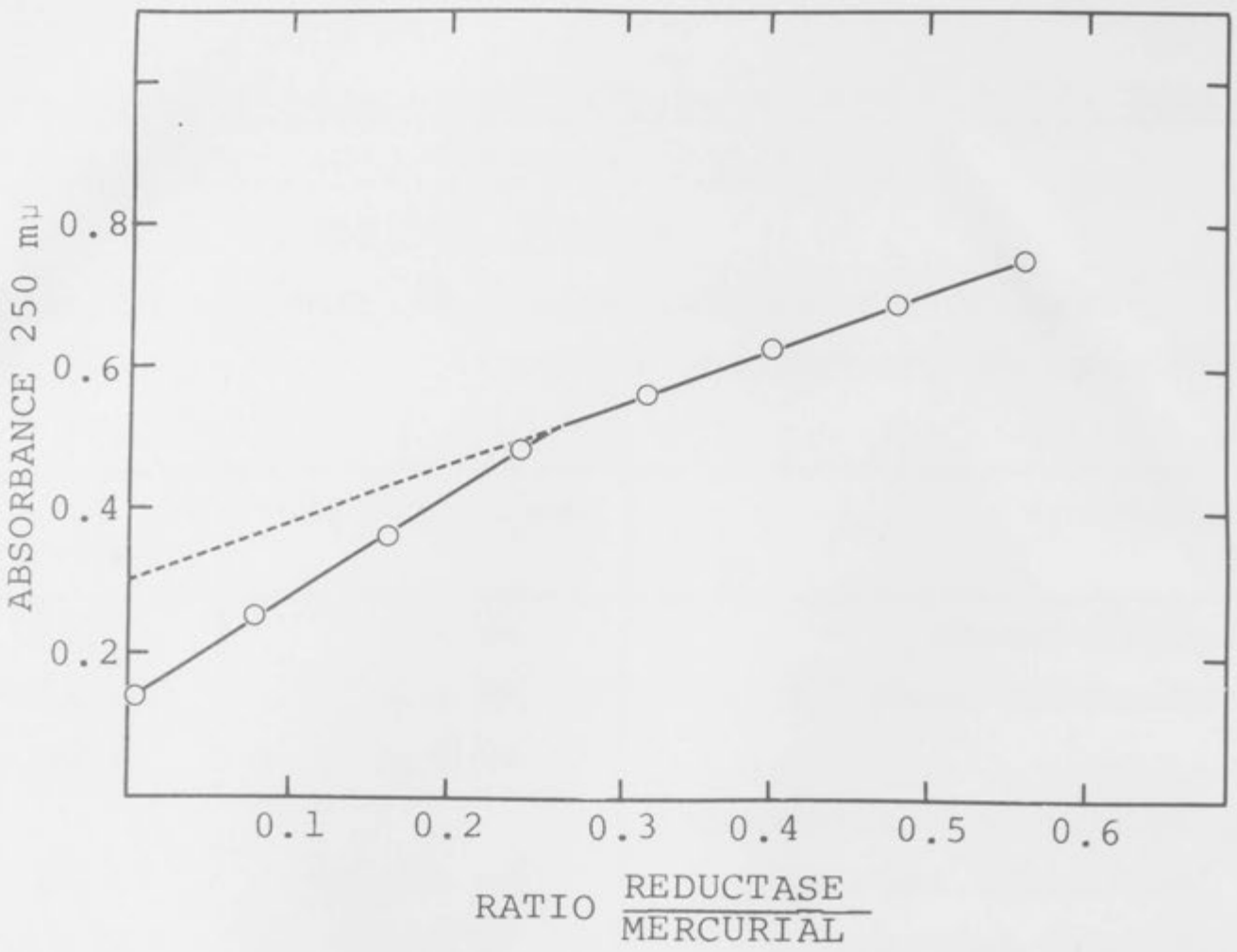
Results are expressed as the number of moles of -SH reacted per mole of enzyme.

Reagent	Reaction time	No. of moles/ SH/mole
Iodoacetamide	30 min	0.227
Iodoacetic acid ^{14}C	30 min	0.058*
	30 min	0.00
p-Hydroxymercuribenzoate		
mercurial excess	24 hours	3.57
enzyme excess	6 hours	4.2
DTNB	48 hours	3.8
Following exposure of enzyme to 7 M urea		
Iodoacetamide	30 min	Nil
p-Hydroxymercuribenzoate	3 min	4.7
DTNB	1.5 min	3.73

The reaction of the enzyme sulphydryl groups with iodoacetamide and iodoacetic acid was assayed by amino acid analysis of S-carboxymethylcysteine.

*Calculation of S-carboxymethylcysteine formed was determined radiometrically after amino acid analysis of enzyme treated with 1-^{14}C-iodoacetic acid.

Reactivity with PMB and DTNB were determined spectrophotometrically at 250 mμ and 412 mμ, respectively.


ABSORBANCE 250 mμ
0.8
0.6
0.4
0.2
0.1
0.2
0.3
0.4
0.5
0.6
RATIO REDUCTASE / MERCURIAL

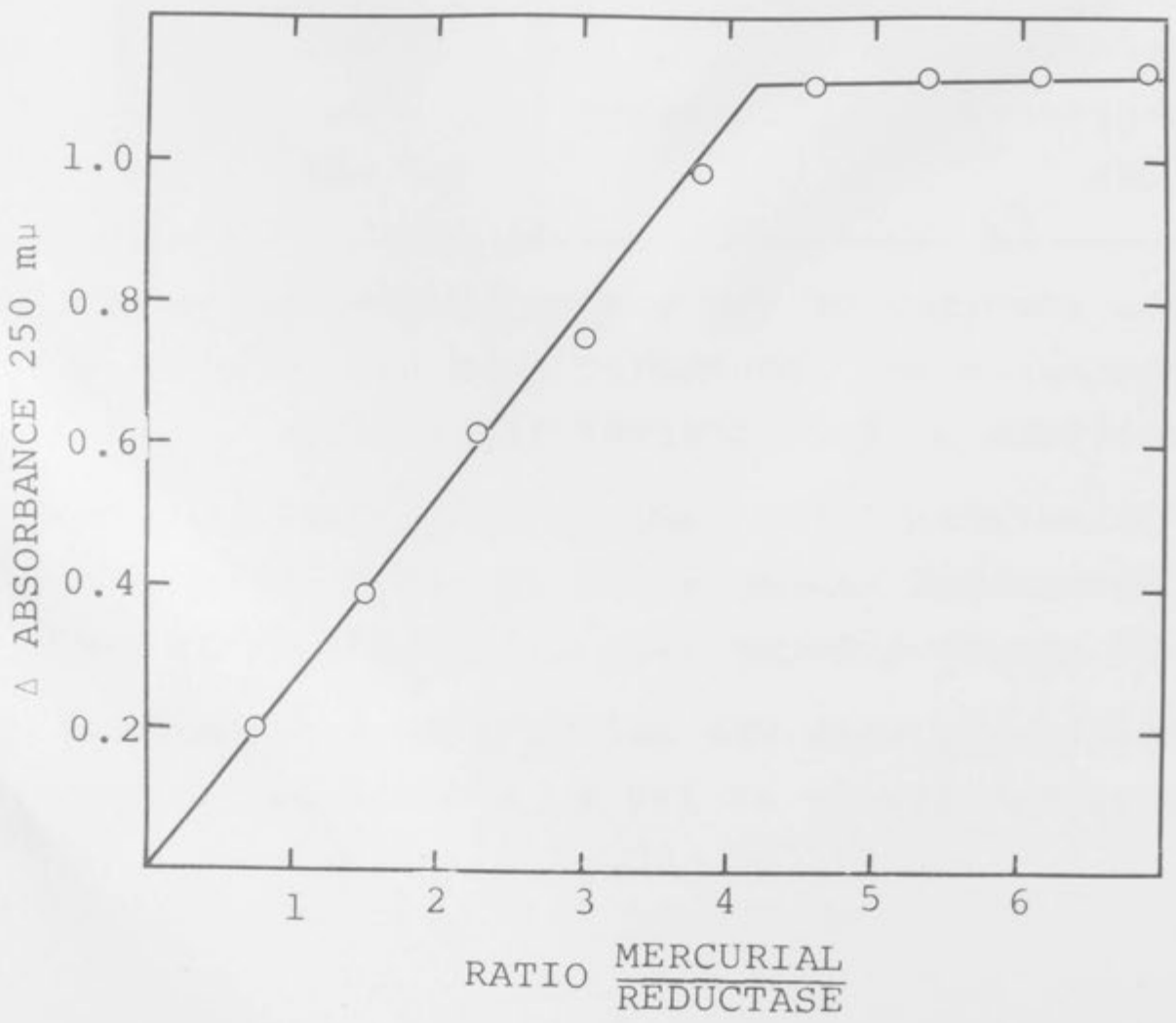

Δ ABSORBANCE 250 mμ
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
RATIO MERCURIAL / REDUCTASE

FIG. III.8a. Titration of sulphydryl groups of ribonucleotide reductase after the method of Boyer (1954). Cuvettes (of 1.5 ml capacity) contained in 1 ml 2.24×10^{-5} M PMB in 0.04 M potassium phosphate buffer (pH 7.0) and ribonucleotide reductase at concentrations up to 12.5×10^{-6} M (0.950 mg/ml). The absorption at 250 mμ was determined after 28 hours at 3°. The ordinate gives the absorbance at 250 mμ and the abscissa gives the molar ratio of reductase to PMB. The end point in the titration is indicated by the intersection of the two straight lines, which are the computed best fit for the data.

8b. Titration of sulphydryl groups of ribonucleotide reductase after the method of Benesch and Benesch (1962).
Cuvettes contained in 1 ml 0.04 M potassium phosphate buffer (pH 7.0) ribonucleotide reductase 13.4×10^{-6} M (1.0 mg/ml). The enzyme was omitted from the blank. PMB was added in small increments (10 mμmoles) to each cuvette and the difference in absorbance at 250 mμ measured in a Cary 14 recording spectrophotometer. The ordinate gives the absorbance change at 250 mμ on the addition of mercurial, and the abscissa gives the molar ratio of mercurial to enzyme. The end point of the titration is indicated by the intersection of the two straight lines.

of enzyme. The mercaptide reaction proceeds in the case of both mercurial excess (Fig. III.8a; Boyer, 1954) and enzyme excess (Fig. III.8b; Benesch and Benesch, 1962). When these experiments are performed in 7 M urea no reaction can be demonstrated with idoacetamide, but the time for complete reaction with PMB and DTNB is reduced to only a few minutes. Total number of groups reacted was unchanged with DTNB, but a slight increase in the apparent number of reactive SH groups was observed with PMB. The reactions with PMB and DTNB have been used to study the quaternary structure of the reductase.

The activity of mercurial-treated ribonucleotide reductase. Spectrophotometric investigation of mercaptide formation between PMB and the unreduced enzyme gave no indication of the nature of the reacted protein. Activity of the reductase was measured after exposure for one week to PMB (4-400 molal excess) at 0° by the use of the standard assay procedure (Methods, Chapter II). As the reaction mixture contains excess thiol (reduced lipoate, 30 mM), no attempt was made to remove excess mercurial prior to assay. Reductase exposed to a 4 to 40 molal excess of mercurial retained 92-94 percent of the activity of untreated controls, but with a 400-fold excess of mercurial, recovery was

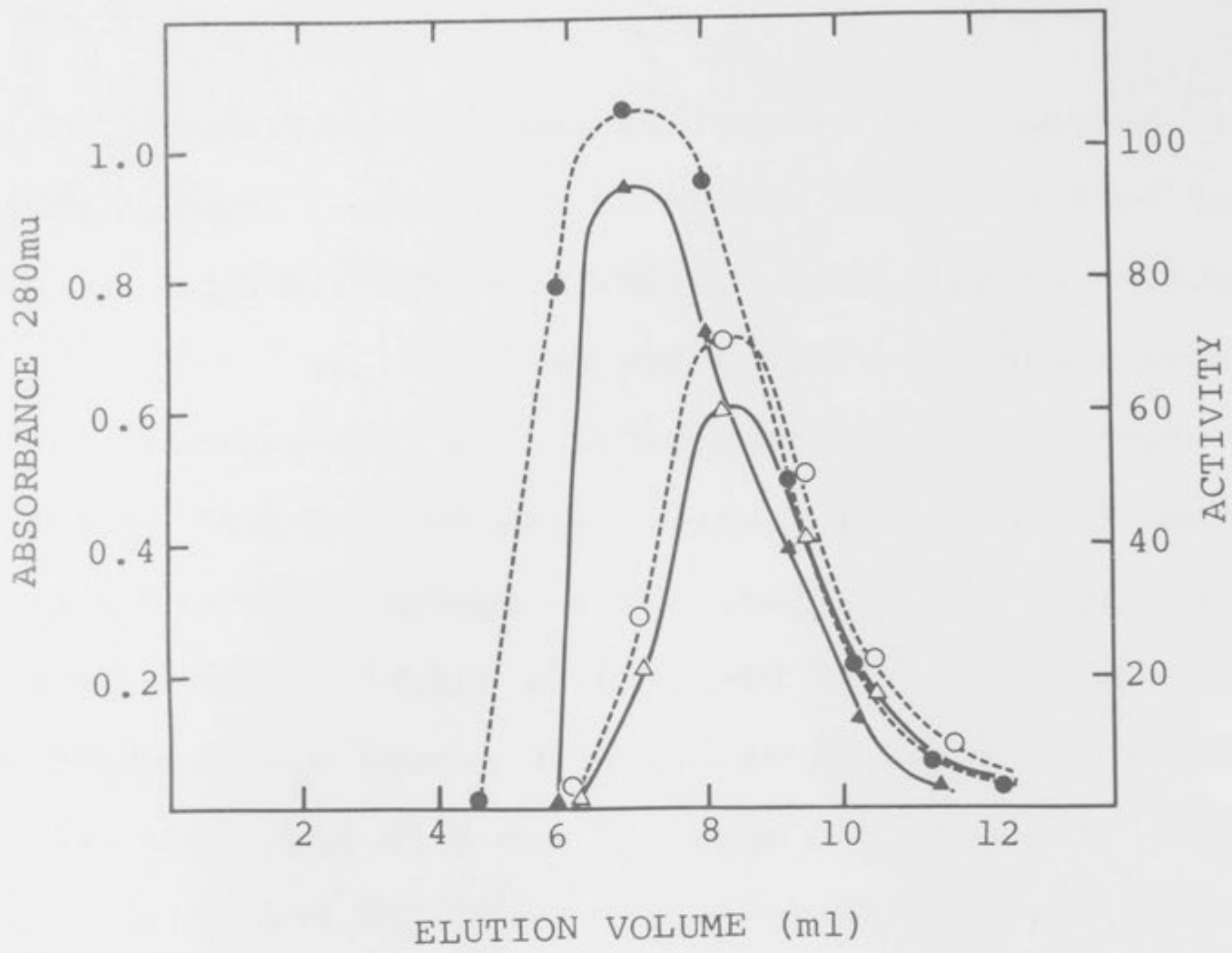


FIG. III.9. Gel filtration of mercurial-treated ribonucleotide reductase.
Electrophoretically homogeneous reductase (2.8 mg in 0.4 ml) was exposed for 16 hours to 1 mM PMB in 0.1 mM DMG, pH 7.3. The sample was applied to a 30 x 1 cm column of Sephadex G-200 prepared as described in Methods and equilibrated with 0.1 M DMG, pH 7.3, which was also used to elute the protein. The elution profiles of the mercurial-greated reductase, shown for absorbance at 280 mμ (●---●) and units of reductase activity per ml (▲—▲) may be compared with those for 2 mg of the untreated enzyme, O---O and Δ—Δ, respectively.

only 80 percent. It appears that after exposure to a high mercurial concentration, not all of the treated reductase is reconverted to an active form.

Gel filtration of mercurial-treated ribonucleotide reductase. The effect of PMB on the reductase could result in the production of enzyme with modified physical properties. This reagent has been used under other circumstances (Gerhart et al., 1968) to demonstrate subunit structure and a possible effect was the production of active protein of lower molecular weight. The elution volumes of mercurial-treated and native enzymes from a column of Sephadex G200 may be compared in Fig. III.9. The activity peak of the mercurial-treated sample is asymmetrical, and the elution volume is less than that of the native enzyme. The presence of inactive material with an elution volume less than the active enzyme suggests that the molecular weight of the latter is less than 200,000. The marked asymmetry of the mercurial-treated enzyme profile indicates the presence of at least two forms of potentially active enzyme. If the elution volume of the larger of these species is taken as 6.8 ml and that of the native enzyme (mol. wt. 76,000) as 8.3 ml, the substitution of these values into the equation

$$\text{Log } M = 6.698 - 0.987 \text{ (Ve/Vo)}$$

gives a molecular weight for the mercurial-treated enzyme of about 160,000. The above relationship (Determann, 1966) is the standard curve for Sephadex G200, where the ratio of the elution volumes (Ve/Vo) is plotted against log molecular weight.

The effect of PMB and DTNB on the sedimentation velocity of ribonucleotide reductase. As the gel filtration of the mercurial-treated enzyme had not yielded definite information with regard to its molecular weight, similarly treated enzyme was examined in the ultracentrifuge in the presence of 2.5 mM PMB (60 molal excess) and the results are shown in Table III.5. This treatment caused a change in the weight average sedimentation coefficient from 2.75 S to 2.84 S which is probably significant. Treatment of the reductase with 5 mM DTNB (120 molal excess) did not cause a significant change in the weight average sedimentation coefficient, but the areas under the schlieren peaks (corrected for radial dilution) of the samples treated with PMB of DTNB decreased during sedimentation. This progressive decrease in the ratio of the areas under the peaks of the treated samples to those of the corresponding reference material suggests the continuous rapid sedi-

TABLE III.5. Effect of various additions and treatments on the sedimentation velocity of ribonucleotide reductase

The initial concentration was 0.28 g dl^{-1} in all experiments. The temperature was controlled near 3° except where specified otherwise.

Addition or treatment	$\bar{s}$ (Svedberg units)		Area[a] ratio
	Treated	Untreated	
PMB (10 mM)	2.84 (2.82)[c]	2.75	0.68
DTNB (10 mM)	2.74 (2.73)[c]	2.75	0.83
pH 8.9, 16 hours, 25°	2.46	2.72	0.71
dGTP (5 mM)	2.65	2.68	0.99
dGTP (5 mM)[b]	5.52	5.63	-
ATP (10 mM)	2.74 (2.69)[c]	2.75	1.00

[a]For explanation see text.

[b]Run performed at 30°.

[c]Values in brackets are the measured values before correction for viscosity.

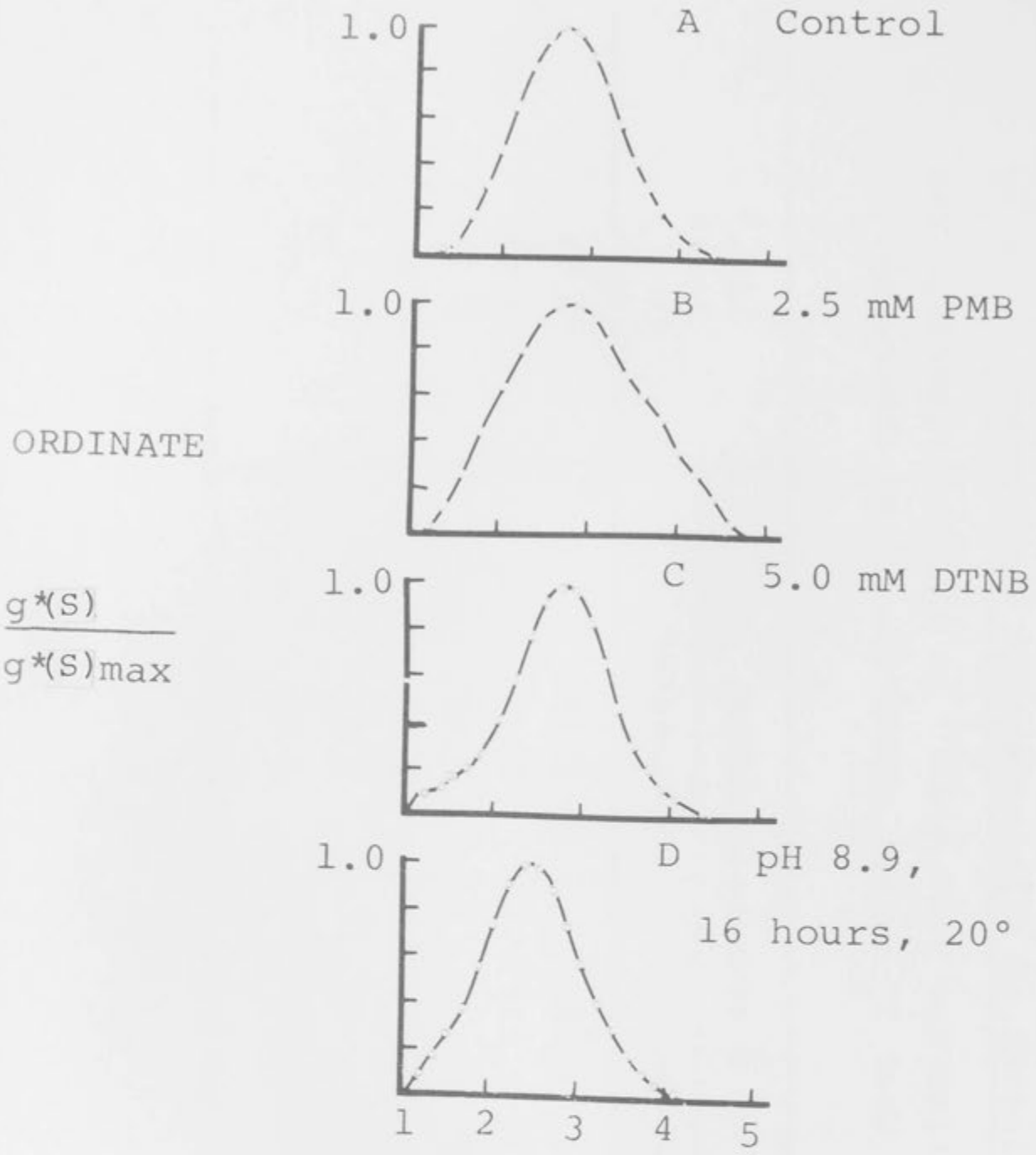


FIG. III.10. $g^*(S)/g^*(S)_{max}$ vs. S plots of ribonucleotide reductase in the presence of PMB and DTNB and after exposure to pH 8.9. Conditions were as stated in Table III.5.

mentation of high molecular weight material (Table III.5). This indicates the presence of other components, possibly high molecular weight aggregates, formed by these treatments.

Plots of g*(S)/g*(S) max vs S (Fig. III.10) clearly show the presence of components other than the original protein after the above treatments. After treatment with PMB, at least two new species with a larger weight average sedimentation coefficient have been formed, while the shape of the distribution about the lower values of S indicates the presence of modified material with a lower weight average sedimentation coefficient. The production of components with low $\bar{s}$ values is even more marked after treatment with DTNB, but here there is very little evidence for the production of high molecular weight material, except for the loss of area under the curve.

The effect of PMB and DTNB on the electrophoretic mobility of ribonucleotide reductase. Gel filtration and ultracentrifugal analysis of the mercurial-treated enzyme indicate that the predominant effect on the reductase is to produce an increase in the apparent molecular weight, with no evidence for the production of subunits. The samples prepared for the ultracentri-

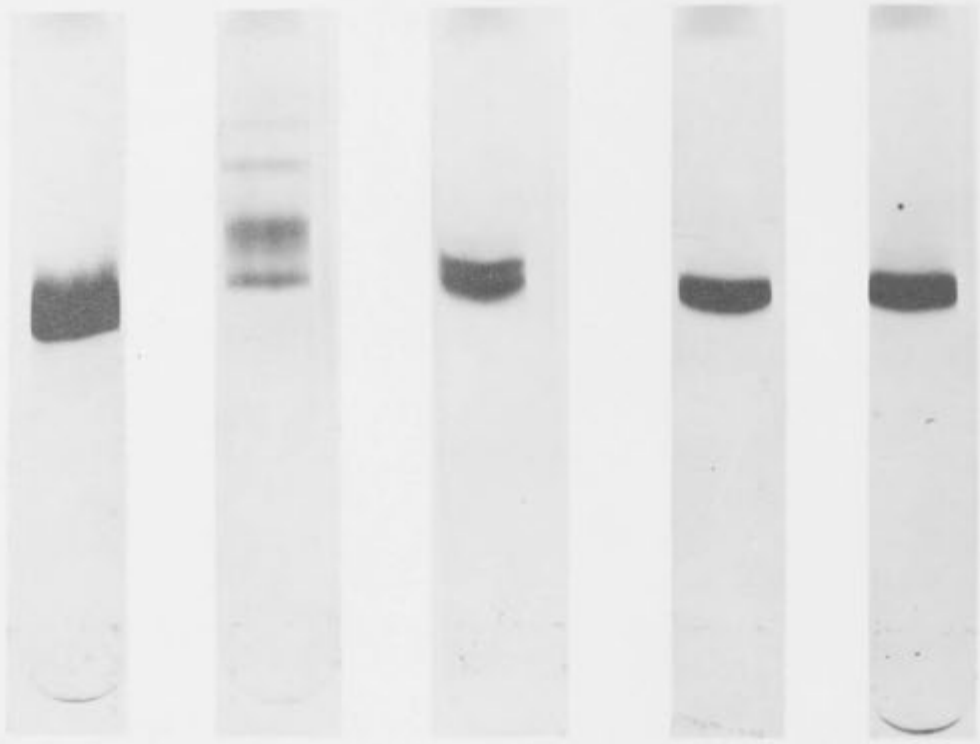
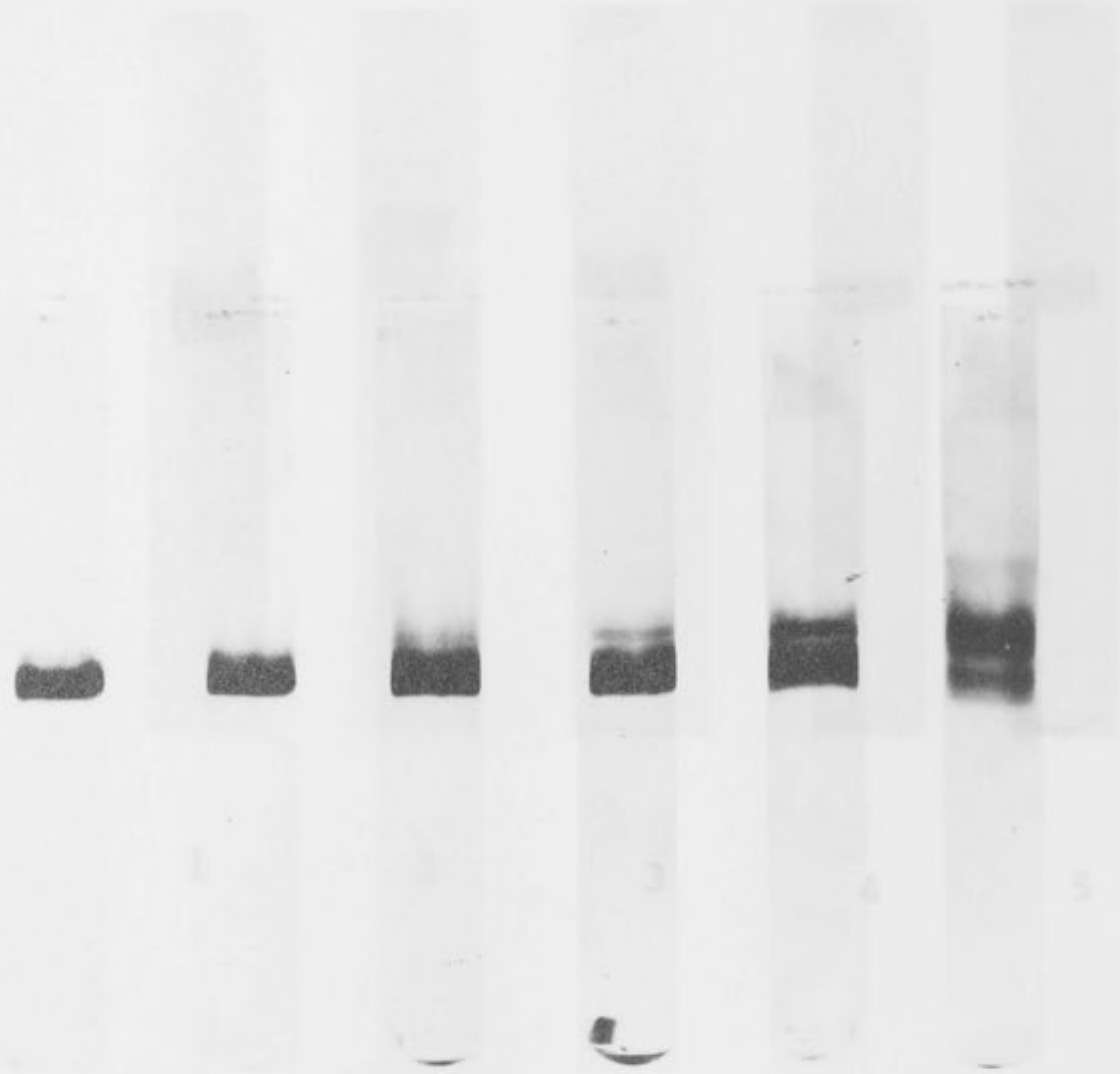


FIG. III.11. Electrophoretic analysis of the reductase used for the sedimentation analysis shown in Table III.5 and Fig. III.10. The reductase was exposed to the various treatments for 16 hours : Gel 1, Control; gel 2, 2.5 mM PMB (60 molal excess); gel 3, 5 mM DTNB (120 molal excess); gel 4, 5 mM dGTP; gel 5, 10 mM ATP.



0 0.5 1.0 2.0 8.0 40.0

MOLAL EXCESS $\frac{\text{PMB}}{\text{ENZYME}}$

FIG. III.12. The effect of PMB on the electophoretic mobility of ribonucleotide reductase. Electrophoretically homogeneous enzyme (80 µg) was exposed to PMB for 4 hours at 0° in the molal excess as shown above, and electrophoresed as previously described (Chapter II). Gel 1, untreated enzyme; gels 2-6, increasing concentration of PMB.

fuge experiments recorded in Table III.5 and Fig. III.10 were electrophoresed on polyacrylamide in the standard TEA-TES buffer system (Chapter II) without further treatment (Fig. III.11). The mercurial-treated enzyme contained six electrophoretically separable components. Treatment of the reductase with DTNB produced only two components. None of the components exhibited mobility greater than that of the native enzyme. Although simultaneous charge modifications may mask the demonstration of altered mobility in polyacrylamide due to change in size, the absence of altered protein of high mobility confirms the stability of the reductase to disaggregation by sulphydryl reagents.

Partial conversion of electrophoretically homogeneous ribonucleotide reductase can be effected at low concentrations of mercurial (1:1 ratio) and the transformation is almost complete at 40 molal excess of mercurial (Fig. III.12). Exposure of the enzyme to mercurials present in a molar excess greater than 20 produces some protein of even slower mobility as shown in gel 6 (Fig. III.12). The final concentration of mercurial used in this experiment was lower than that used for Fig. III.11, and the reaction time was much

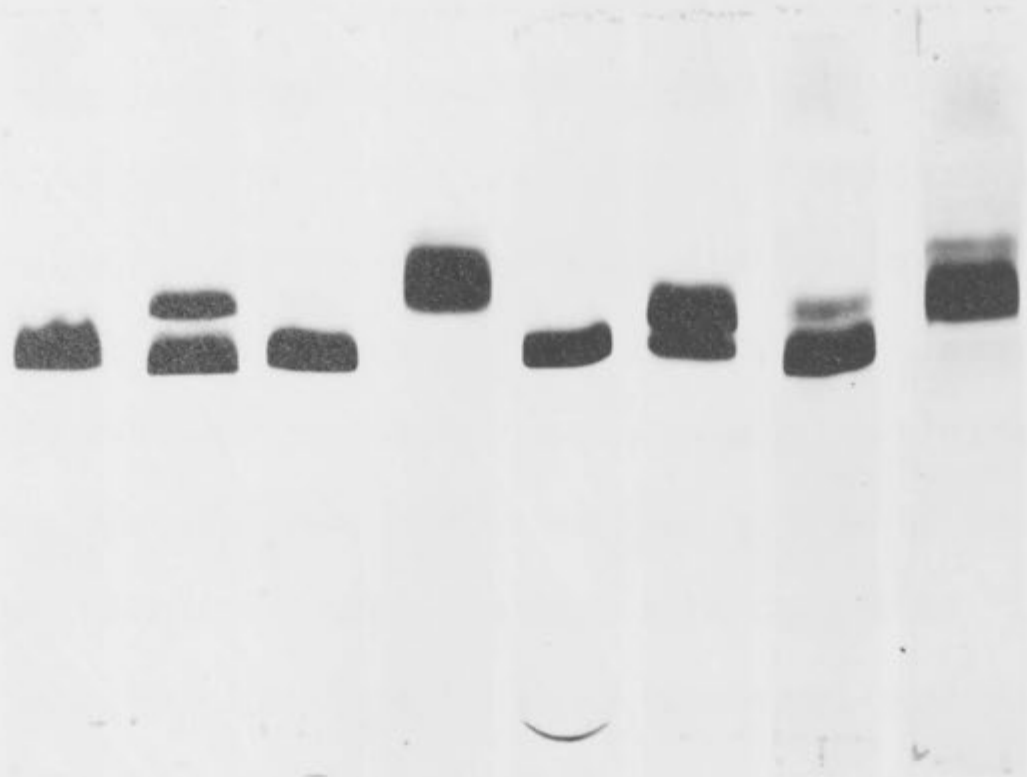


FIG. III.13. The effect of various mercurials on the mobility of ribonucleotide reductase during polyacrylamide gel electrophoresis. Electrophoretically homogeneous reductase (100 µg) was exposed to mercurials in the concentrations shown for 2½ hours at 0° prior to electrophoresis as described previously.
1, control; 2, PMB, 5 mM (40 molal excess); 3, MMB, 0.5 mM (5 molal excess); 4, MMB, 3.3 mM (30 molal excess); 5, MMI, 0.5 mM (5 molal excess); 6, MMI, 3.3 mM (30 molal excess); 7, PMA, 0.5 mM (5 molal excess); 8, PMA, 3.3 mM (30 molal excess).

less, which may account for the absence of the species of very low mobility seen in Fig. III.11. Prolonged exposure of the reductase (1 week) to various concentrations of PMB up to a 40 molal excess, did not appear to affect the relative proportions of the various bands used, and at lower mercurial concentrations did not cause the production of the very low mobility. The various forms of the mercurial-enzyme complex and their proportions were unchanged subsequent to the removal of excess mercurial by prolonged dialysis when examined by polyacrylamide gel electrophoresis.

As PMB is itself a charged molecule, the possibility of charge modification on the enzyme-mercaptide complex was considered. However, the similarity of the effects obtained by reaction of the enzyme with other mercurials (Fig. III.13) indicates that the resolution of the modified proteins is little affected by the charge on the mercurial.

Despite the major changes in size and electrophoretic mobility of the mercurial-treated enzyme, the modified protein is activated after treatment with substrate thiol. Electrophoresis of the mercurial-treated reductase after exposure to a thiol demonstrates the reconversion of the protein to electrophoretic homogeneity with a mobility

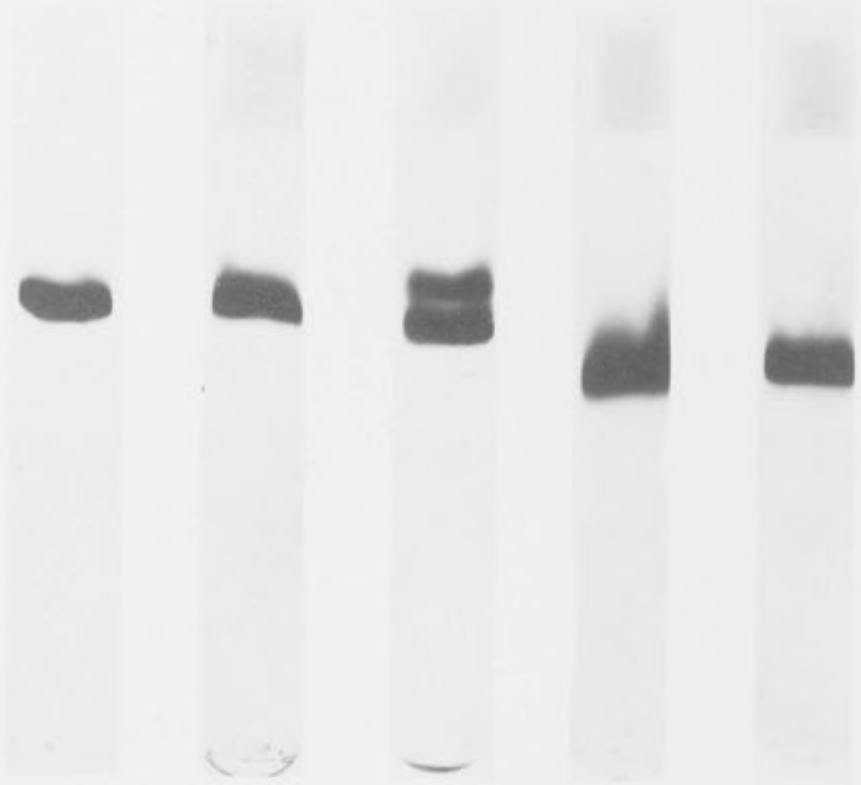


FIG. III.14. The effect of thiols on native and mercurial-treated ribonucleotide reductase. 100 µg samples of electrophoretically homogeneous enzyme were treated at 0° as follows, and electrophoresed as previously described : Gel 1, untreated enzyme; gel 2, the enzyme was exposed to 30 mM dithiothreitol for 4 hours prior to electrophoresis; gel 3, enzyme was exposed to 5 mM PMB for 4 hours prior to electrophoresis; gel 4, as for 3, but 30 mM dithiothreitol and 30 mM thioglycollic acid added to the sample prior to application to the gel. Gel 5 shows the effect of dithiothreitol (30 mM) and thioglycollic acid (30 mM) on the native enzyme.

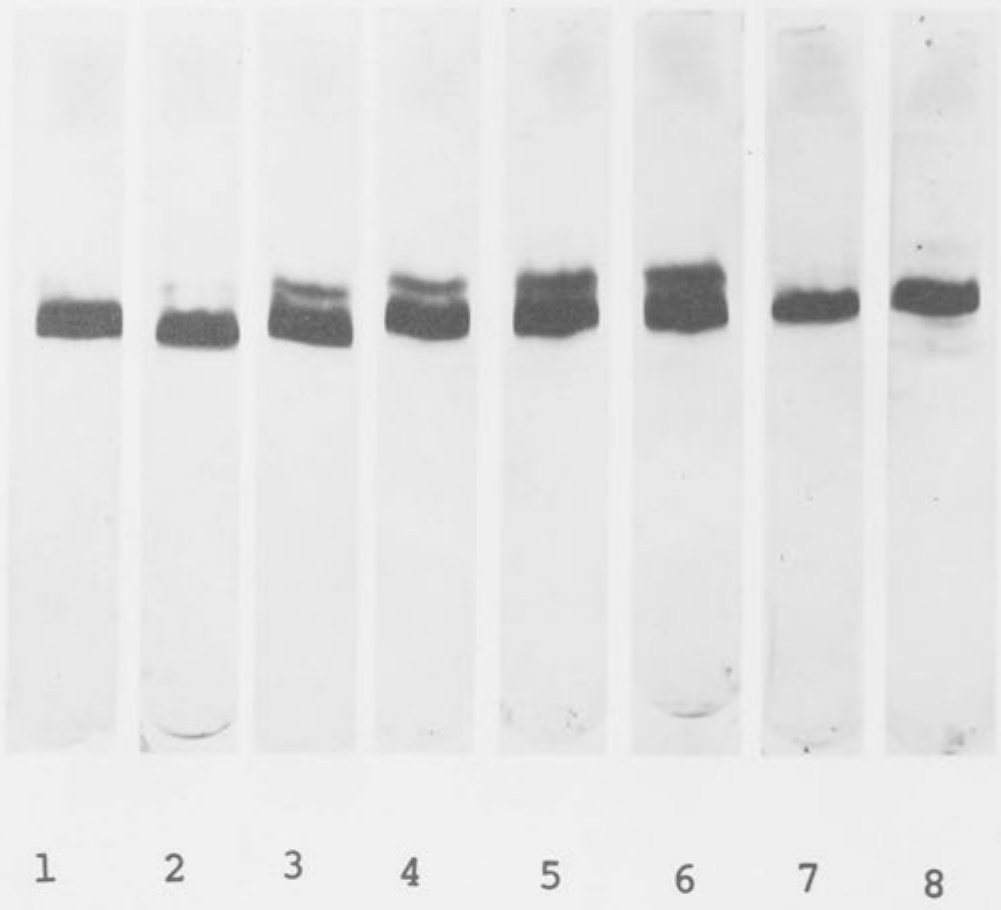


FIG. III.15. The mobility of ribonucleotide reductase after reaction with DTNB, and the effect of reduced lipoate on the mobility of treated and untreated enzyme.
Electrophoretically homogeneous reductase (100 µg) was exposed to DTNB in concentrations shown below for 4 hours prior to electrophoresis as previously described. 1, untreated enzyme; 2-7 reacted with DTNB. 2, 0.2 mM; 3, 0.5 mM; 4, 1.0 mM; 5, 2.0 mM; 6, 5.0 mM; 7, 5.0 mM, but reduced lipoate (30 mM) added prior to electrophoresis. 8 shows the effect of reduced lipoate on the untreated enzyme. The maximum concentration of DTNB used (5 mM) represents a molal excess of 40.

equal to that of the thiol-treated native enzyme (Fig. III.14).

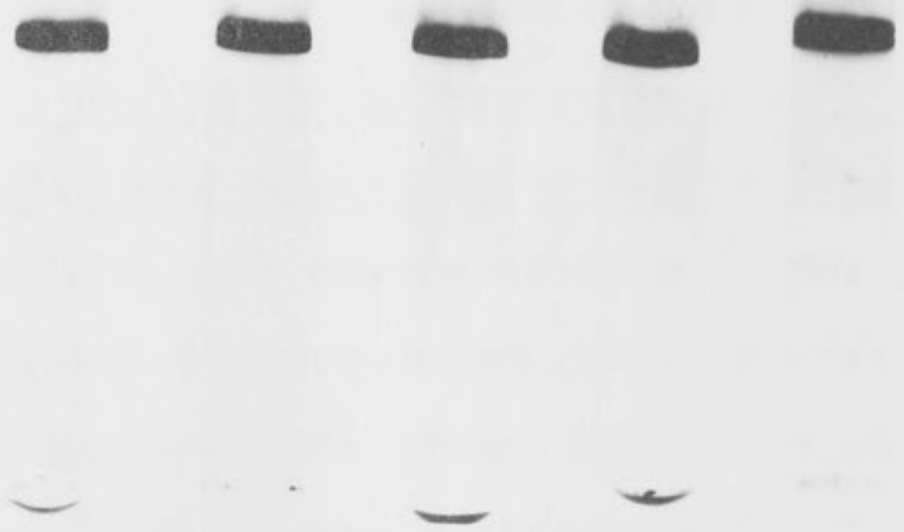
Although DTNB reacts more slowly with the reductase than does PMB, prolonged exposure of the reductase to a 20-fold excess of DTNB produces material with electrophoretic properties very similar to the material first produced after mercaptide formation. Again the effect can be completely reversed by thiols (Fig. III.15).

The formation of four mercaptide bonds on ribonucleotide reductase is not associated with any significant reduction of molecular weight of the protein but species of higher molecular weight are formed. The effect is almost completely reversible in the presence of thiols. A similar, but less marked effect can be demonstrated with DTNB.

The effect of Tris-glycine buffer, pH 8.9 on the sedimentation velocity of ribonucleotide reductase. This system was investigated in the ultracentrifuge for comparison with the gel filtration (Sephadex G200) and electrophoresis results described in Chapter II (Figs. II.15 and II.18). After this treatment, the $\bar{s}$ was decreased from 2.72 S to 2.46 S (Table III.5) despite the demonstration of material of very high molecular weight by gel filtration. The shape of the

g*(S)/g*(S) max vs S plot indicates the presence of species of low sedimentation coefficient. In addition, the progressive decrease in the area under the curve compared with the untreated enzyme (Table III.5) again suggests the continuous rapid sedimentation of high molecular weight aggregates.

The effect of substrate and activator on the physical properties of ribonucleotide reductase. Preliminary investigations showed that dGTP is an activator for ATP reduction by the electrophoretically homogeneous reductase, a finding which indicates that the reductase purified by polyacrylamide gel electrophoresis has retained its modifier site. However, the addition of 5 mM dGTP to the reductase produced only very small changes in the sedimentation coefficient as compared with untreated enzyme sedimented simultaneously in a single ultracentrifuge experiment (Table III.1). As the error in determination of sedimentation coefficient is, to a first approximation, independent of the sedimentation coefficient and is probably in the order of 0.03-0.05 S (Creeth et al., 1967; Gerhart et al., 1968) the observed differences are probably accountable in terms of experimental error. Plots of g*(S)/g*(S) max vs S for the treated reductase were identical with the distribution



1 2 3 4 5

FIG. III.16. Electrophoretic mobility of ribonucleotide reductase in the presence of increasing concentrations of GTP.
100 µg samples of electrophoretically homogeneous reductase were electrophoresed as described in Methods. Distance of migration was measured from the junction of the stacking and resolving gels to the centre of the protein band.
Gels 1 & 5, untreated enzyme; gel 2, .05 mM GTP; gel 3, 0.2 mM GTP; gel 4, 5 mM GTP.
A similar effect was observed with dGTP.

of the untreated enzyme. The molecular weight ($\bar{M}_z$) of 78,000 determined by equilibrium sedimentation of the enzyme in the presence of 1 mM dGTP does not differ significantly from that of the untreated enzyme (Table III.1). Similar negative results were obtained during the investigation of effects of 10 mM ATP on the sedimentation of the reductase. The electrophoretic mobility and resolution of protein treated with 5 mM dGTP or 10 mM ATP were unchanged (Fig. III.10).

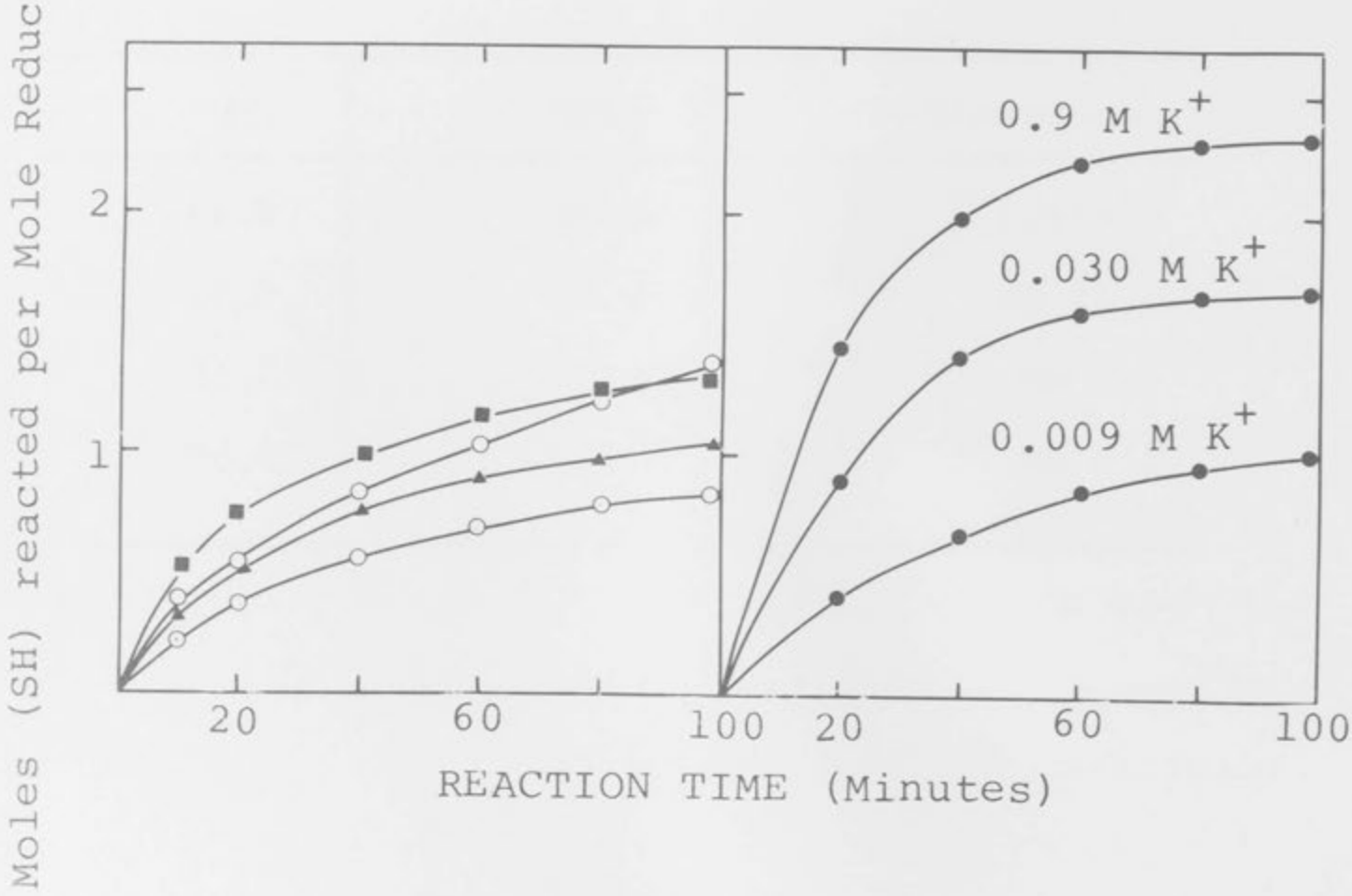
The electrophoretic mobility of the reductase in the presence of GTP was investigated on polyacrylamide gels. From the behaviour of the ultracentrifuge, it was expected that no pronounced changes would be observed. The absolute mobility of the reductase was only very slightly increased by such treatment, and the single protein is not resolved into multiple bands (Fig. III.16). In the assessment of electrophoretic mobility by this technique, it is sometimes difficult to exclude the possibility of two effects cancelling one another, such as a reduced molecular size occurring simultaneously with a diminution in the charge of the migrating species or in the effective strength of the electric field operating on the particle. The charged nucleotide in the upper buffer and eventually in the gel, probably

makes a significant contribution to the conductivity of the gels, particularly at the higher concentrations used, with a consequent effect on the mobility of other migrating species. In an attempt to overcome this difficulty, protein mobilities were related to that of the marker dye (bromphenol blue) which diminished with increasing concentrations of nucleotide and the results are shown in Table III.6. The eighteen percent increase observed in the relative mobility of the protein in the presence of GTP (or dGTP) is difficult to interpret because of the number of factors which may influence this determination.

As the substrate and activator nucleotides could not be shown to have a measurable effect on the physical properties of the reductase, an attempt was made to investigate the effect of activators on the rate of reaction of the thiol groups on the molecule with various sulphydryl reagents. The addition of dGTP to the reaction mixture rendered accurate absorbance measurements at 250 mμ difficult because of the high blank value. Alkali-metal ions are also activators of the reductase (Blakley, 1966a) and were used in concentrations up to 2 M during the reaction with PMB. However, reaction rates were so fast that comparison was

TABLE III.6. The relative electrophoretic mobility of ribonucleotide reductase on polyacrylamide gel in the presence of dGTP and GTP compared with that of bromphenol blue

Nucleotide	dGTP	GTP
Control	0.50	0.49
0.05 mM	0.52	0.51
0.2 mM	0.55	0.55
1.0 mM	0.59	0.59



0.558 M Na^+ ○

0.03 M Na^+ + dGTP ▲

0.009 M Na^+ ⊙

FIG. III.17. The effect of alkali-metal ion concentration, 0.005 M dGTP and 7 M urea on the reactivity of sulphydryl groups to DTNB on the unreduced ribonucleotide reductase.

Cuvettes (1.5 ml capacity) contained in 1 ml 13.2 μM ribonucleotide reductase (1 mg), 10 mM DTNB. The following buffers were used :

Experiment A. Sodium dimethylglutarate, pH 7.3 at the concentrations shown on the individual curves. 0.005 M dGTP, ▲—▲ in 0.005 M Na DMG, pH 7.3. Experiments were performed at 0° except for ■—■ which was at 35°.

Experiment B. Potassium phosphate, pH 7.5 at the potassium concentrations shown on the curves. Experiments were performed at 35°.

Cuvettes were compared in a Cary 14 spectrophotometer with blanks, from which the enzyme had been omitted. Number of sulphydryl groups reacted was calculated from the extinction coefficient of 13.6×10^3 (Ellman, 1959).

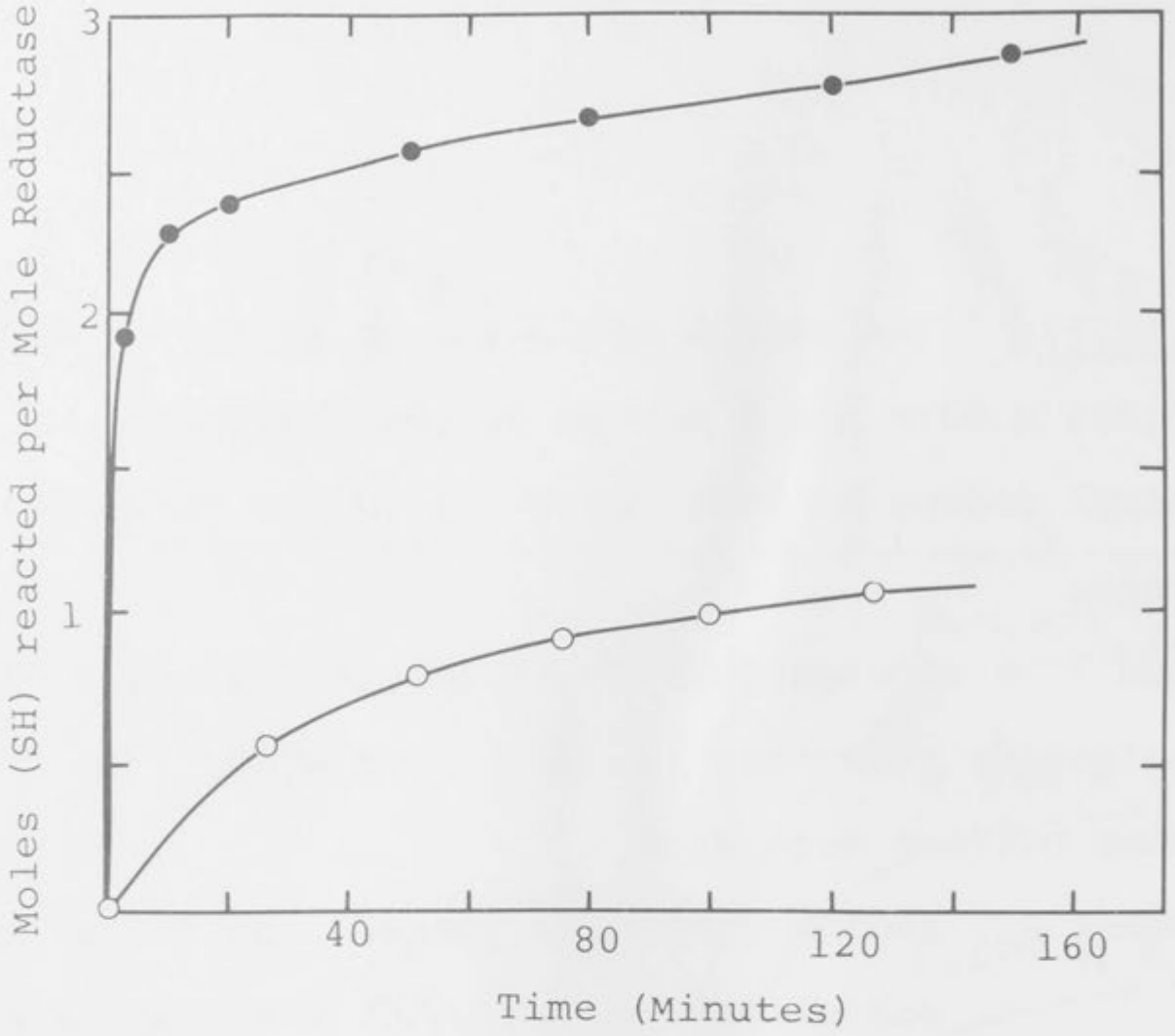


FIG. III.18. Rate of reaction of reduced ribonucleotide reductase.
The reduced enzyme was prepared by dialysis following exposure to 30 mM reduced lipoate. Cuvettes (1.5 ml capacity) contained in 1 ml 13.2 μM ribonucleotide reductase (1 mg) 0.1 M DMG (pH 7.3) and DTNB, 1 mM. Absorbance at 412 mμ was measured in a Cary 14 recording spectrophotometer against a blank in which the enzyme (0.05 ml) was replaced with equilibrium diffusate from the dialysis. The reaction rate of unreduced enzyme (lower curve) is included for comparison.

difficult, as much of the reaction was complete before recording could be commenced. The addition of sodium or potassium did not alter the total number of thiol groups reacting. Because of this rapid reaction with PMB, the effect of sodium, potassium and dGTP on the enzyme was assessed by the reaction rate of the reductase with DTNB, as shown in Fig. III.17. It can be seen that the reaction rate increases with increasing concentrations of alkali-metal ions. The effect of dGTP is difficult to interpret as the nucleotide (0.005 M) was added as the tetrasodium salt.

Sulphydryl reactions with reduced ribonucleotide reductase. Reduced enzyme was prepared by exposure to 30 mM reduced lipoate for 30 minutes, after which it was dialysed for 36 hours against 3 changes of 1000 volumes of buffer. The reactivity of sulphydryl groups on the reduced enzyme are shown in Table III.7. Iodoacetamide appears to be slightly more reactive than iodoacetic acid. The reaction of the reduced enzyme with DTNB is shown in Fig. III.18, and it may be compared with the rate of reaction of the unreduced reductase. After the rapid reaction with 2 moles of thiol per 76,000, the DTNB reaction with the reduced enzyme proceeds at the same rate as for the untreated reductase. This finding

TABLE III.7. Reactivity of reduced ribonucleotide reductase with various sulphydryl reagents

Reagent	Time of reaction	No. of groups reacted
Iodoacetamide	30 min	1.52 (0.227)
Iodoacetic acid	30 min	0.62 (Nil)
$^{14}C_1$	30 min	0.61 (0.58)*
DTNB	48 hours	6.17 (3.8)

Values shown in parenthesis represent the results with unreduced enzyme.

Assay procedures were the same as for Table III.3.

*Calculation of S-carboxymethylcysteine formed was determined radiometrically after amino acid analysis of reductase treated with $^{14}C_1$ iodoacetic acid.

is in agreement with the unpublished results of Morley and Blakley, that reduction of the enzyme does not alter the binding of the ligands.

Discussion

Homogeneity of ribonucleotide reductase. For all practical purposes, the electrophoretically homogeneous enzyme is pure and contains molecules indistinguishable with regard to size, shape and charge. Gel filtration by the method of Andrews (1964) and Winzor et al. (1963) confirm that there is no gross contamination nor any disaggregation of the molecule on dilution down to 10 µg/ml. Similar results were obtained in the ultracentrifuge by both sedimentation velocity and equilibrium experiments. The detection of 3 to 4 percent of contaminating protein of lower sedimentation velocity is probably an overestimate as no correction was made for the Johnston-Ogston effect. Boundary analyses by the methods of Creeth and Pain (1967) and Baldwin (1954) confirm a high degree of purity. These results, together with the similarity of the weight average and z-average molecular weights, and the linear plots from which they were calculated, not only substantiate the electrophoretic homogeneity of the preparations but confirm that there is no major change in molecular

weight of the proteins under the conditions of the analyses.

The "essentially pure" preparation of Goulian and Beck (1966) gives rise to multiple bands when electrophoresed on polyacrylamide gel by the method of Ornstein (1964), and the non-linearity of the Yphantis (1964) plot also suggests heterogeneity of the sample. Although Goulian and Beck have concluded that these results may represent an aggregation or cleavage of the pure protein, the findings could be due to impurities present in the sample or to inactivation-denaturation effects, for, as they have shown, exposure of the enzyme to the conditions used during electrophoresis causes irreversible inactivation. The temperature and duration of the electrophoresis were not reported by Goulian and Beck, but as has been described in Chapter II, polyacrylamide gel electrophoresis of ribonucleotide reductase in Tris-glycine buffer, pH 9.5 (Ornstein, 1964) does give rise to a separable inactive form, the production of which is minimal when the reductase is electrophoresed at 0°. Depending on the actual temperature of the experiments, the result could have arisen from initially homogeneous material. However, the reported conditions used by Goulian and Beck for ultracentrifugal analysis

should not have caused inactivation of the enzyme, and the Yphantis plot therefore offers the most faithful representation of the purity of the preparation, and the non-linearity may indicate genuine heterogeneity. In view of the above interpretation and the results of the present work, there is some reason to doubt the validity of their claim to purity and if, in fact, their preparation did contain extraneous material, the low specific activity reported would be explained.

Molecular weight of ribonucleotide reductase. The average molecular weight of the electrophoretically homogeneous reductase determined by all methods is 76,000 (range 70,000-82,000). Although the values obtained by sedimentation-diffusion and gel filtration are generally lower than those from equilibrium sedimentation, all estimates agreed within the limits of expected experimental variation. However, all of the results reported here differ markedly from the molecular weight of 110,000 reported by Goulian and Beck from the Yphantis analysis and supported by the sedimentation coefficient they report (when the latter is corrected for temperature and concentration according to the relationships described in the present work). Although it has been suggested

previously that the discrepancy between earlier reports on the molecular weight of the reductase from this laboratory and that of Goulian and Beck reflected the subunit structure of the reductase (Larsson and Reichard, 1967), the discrepancy between the present results and those of Goulian and Beck are such that they cannot be ascribed to aggregation-disaggregation of one form to produce the other.

Attempts to determine the minimal chemical molecular weight by peptide mapping after tryptic digestion were unsuccessful due to incomplete hydrolysis of the molecule, resulting in a large, acid-insoluble core. Gel filtration analysis of this core peptide on Sephadex G100 indicates a molecular weight of about 20,000. Amino acid analysis of the core shows the absence of cysteine or cystine, which precludes the use of ethylene-imine (Raftery and Cole, 1966) to increase the number of tryptic cleavage points on the molecule.

No amino acid was present in the original molecule as a single residue per 76,000 molecular weight, which prevents the determination of a minimal chemical molecular weight from the amino acid analysis. Methionine, histidine and half-cystine, the residues present as the smallest percentage, are all present as

eight residues per 76,000 molecular weight. Likewise, the "core" does not have any single residues per 20,000 molecular weight.

Amino acid analysis of ribonucleotide reductase. The high proportion of acidic residues present in the molecule explains the low isoelectric point of the reductase (below pH 4.0) and the behaviour on DEAE ion-exchangers. These analyses differ from those of Goulian and Beck, even when compared as estimates for the same molecular weight. The discrepancy cannot be explained by contamination of the preparations unless the concentration of impurity is extremely high, but even gross heterogeneity would not explain the absence of tyrosine as reported by Goulian and Beck.

The stability of the reductase to aggregation-disaggregation. The reductase was particularly stable under all conditions investigated. The negative dependence of weight average sedimentation coefficient on concentration down to 0.03 g dl^{-1} and the negative (or no) concentration dependence of the molecular weight to 0.01 g dl^{-1} indicate the absence of dissociation down to these concentrations. The range of concentrations studied did include the physiological concentration of the enzyme in the cell. From the total activity

extractable from a given weight of cell paste and an assumed specific activity of 200 for the pure reductase, intracellular reductase at the point of harvest would be one to three mg per ml. In addition to the stability on dilution, the molecular weight remained constant (76,000) in 7 M urea containing 10 mM mercaptoethanol and there was no apparent change in the weight average sedimentation coefficient in the presence of PMB.

Ultracentrifugal analysis of the reductase in the presence of substrate or modifier nucleotides did not reveal any increased tendency for dissociation or aggregation under these conditions.

Ligand induced conformational changes. Conformational changes, if they occurred under the conditions used, were not sufficiently great to be observed as a significant change in sedimentation coefficient. Constancy of the molecular weight in the presence of modifiers excluded aggregation-disaggregation. Even if the ligand-induced conformational changes in ribonucleotide reductase had been of the same order as those demonstrated by Gerhart and Schachman (1968) on aspartate transcarbamylase the larger experimental variation relative to the absolute $\bar{s}$ value would preclude their detection on the smaller reductase molecule. The change

in relative mobility on polyacrylamide gel electrophoresis in the presence of ligand is suggestive of physical modification, but the necessity to include variable amounts of a charged material into the electrolytic system renders interpretation difficult and minimises the significance of this result.

Gerhart and Schachman (1968) have demonstrated an effect of ligands on the rate of reaction of the sulphydryl groups of aspartate transcarbamylase and have interpreted the results as a reflection of conformational changes within the molecule. As in the case of the aspartate transcarbamylase regulatory subunit, the rate of reaction of electrophoretically homogeneous ribonucleotide reductase with PMB is too fast to be accurately measured by conventional spectrophotometric techniques. The slower reaction of DTNB with the sulphydryl groups can be easily determined and has been used to monitor conformational change of the reductase in the presence of activators. The reaction rate is increased in the presence of 5 mM dGTP, but similar effects can be produced by the addition of alkali-metal ions, which are themselves activators of the reductase (Blakley, 1966a). As the dGTP used was added as the tetrasodium salt, the separate effect of the metal ions and the nucleotide

cannot be differentiated, but the combined effect of dGTP and sodium in increasing the rate of reaction of the reductase with DTNB is consistent with an induced conformational change.

The oxidation state of the eight half-cystine residues of ribonucleotide reductase. On the basis of the reaction of the reductase with PMB and DTNB, it appears that four cysteine groups are present per mole of reductase. The failure of the other four groups to react with mercurial even in the presence of 7 M urea suggests that they exist in the oxidised state, probably as two cystine residues. Although Schachman (1963) considers this type of protein unlikely, he does not categorically exclude the presence of both oxidation states of cysteine in the same protein molecule. In this particular instance, the relative inaccessibility of the sulphydryl groups and the negatively charged protein may minimise random thiol-disulphide exchange which appears to be the chief factor in excluding this mixed oxidation state in catalytically functional molecules.

The production of two extra groups which will react with DTNB after reduction of the enzyme with reduced lipoate, indicates that at least one disulphide

bridge is cleaved during the reduction, and provides further evidence for the presence of both oxidation states of cysteine in the molecule.

Different rates of reaction of enzyme sulphydryl groups with different reagents. The number of sulphydryl groups reacting with the reagents used, varied between 0-4 per 76,000 molecular weight. As the rate of reaction of all the reagents used is very rapid with low molecular weight thiols, this difference in reaction rate appears to reflect varying degrees of accessibility of the thiol groups to the reagents used. The most rapid reaction rate was provided by PMB, a molecule with both a positive and a negative charge, separated by the relatively bulky benzoic acid ring. It is possible that the positive charge of the mercurial could be "directed" by the negative charges on the protein, while the negatively charged group on the mercurial is sufficiently distant so as not to affect the reaction. DTNB, as well as lacking the positive charge, is a more bulky molecule which probably explains its longer reaction time. The poor reaction of iodoacetamide and iodoacetic acid with the unreduced reductase is unexplained. The difference observed between the reaction of iodoacetamide and iodoacetic acid with the reduced enzyme seems to

indicate that the charged group on the iodoacetic acid adversely affects the rate of reaction in some way.

The inaccessibility of the thiol groups on the reductase may be related to possible involvement in aggregation of subunits as in aspartate transcarbamylase, participation in specific thiol-disulphide interchanges necessary for active configuration or burial by the tertiary structure of the enzyme.

The effect of PMB and DTNB on ribonucleotide reductase. Unlike aspartate transcarbamylase, ribonucleotide reductase does not dissociate into subunits in the presence of excess PMB. In 2-6 hours, mercaptide formation is complete as measured spectrophotometrically, and a four molal excess of mercurial produces 5-10 percent of material of lowered mobility when electrophoresed on polyacrylamide gel. As the excess of mercurial is raised, the proportion of the material of lowered mobility is increased at the expense of the protein having the same mobility as the untreated enzyme. The conversion of the enzyme to material with different electrophoretic properties is almost complete at 50-100 molal excess of PMB. No protein with a more rapid mobility was detected at any mercurial concentration. Gel filtration of the mercurial-reductase complex

on Sephadex G200 indicates that the only effect of the mercurial is to produce species of increased molecular weight, which confirms the interpretation of the bands of lowered mobility seen on polyacrylamide gels. The reduction of the area under the schlieren peak during sedimentation velocity determinations confirms the presence of aggregated forms of mercurial-enzyme complex. Because this increase in molecular size is so readily demonstrated by these three techniques, it is unlikely that it merely reflects a conformational rearrangement of the tertiary structure of the reductase. The effects are much more readily attributed to aggregation. This conclusion is also indicated by the estimated molecular weight of the aggregate (160,000) from the gel filtration studies.

If mercaptide formation is alone responsible for aggregation, exposure to conditions permitting maximum mercaptide formation should cause all of the molecules to aggregate. Whereas only a four molal excess of mercurial is sufficient for all the mercaptide formation detected, a 100 molal excess is required to produce total conversion of the mercurial-enzyme complex to the aggregated form. This requirement for excess mercurial suggests that aggregation is probably a result of some

other type of mercurial-protein interaction, such as that of a second mercurial with a sulphur atom already participating in a mercaptide linkage, with a sulphur atom of a disulphide bridge or with some other group. There are several explanations which could account for the presence of both aggregated and non-aggregated forms of the reductase-mercurial complex. Although some of these may be mutually exclusive, several could play a part in the observed behaviour.

1. Rate of formation of the mercurial-enzyme complex may be unfavourable.
2. The equilibrium constant of this reaction may favour unbound enzyme.
3. Aggregation of the mercurial-enzyme complex may be slow.
4. At equilibrium, the equilibrium may favour the non-aggregated form.
5. At low mercurial reductase ratio there may be insufficient mercurial added for the stoichiometric requirements of aggregate formation.

The finding that exposure of the reductase to the mercurial for one week does not appear to alter the proportion of aggregated species from that present after only 30 minutes exposure, excludes the

possibility of slow mercurial complex formation or slow aggregation. The stability of the reductase-mercurial aggregate when excess mercurial is removed by dialysis excludes the possibility that the equilibrium is unfavourable for complex formation. The results from polyacrylamide gel electrophoresis indicate that the equilibrium is not unfavourable for aggregation (explanation 4) as there is no apparent disaggregation as the non-aggregated form is removed from the aggregated electrophoretically. Although the preliminary experiments with the mercurial-enzyme complex on Sephadex G200 do not clearly indicate two separate species, the shape of the peak is consistent with the presence of two forms, as seen on polyacrylamide gel electrophoresis. However, the gel filtration results do not exclude the possibility of a very slow equilibrium between the several forms. If, as seems possible, there are stringent stoichiometric requirements, it is assumed that a very large number of mercurial molecules must be present to convert all of the enzymes to the species which aggregates.

The effect of mercurials (Perkins and Bertino, 1964, 1965; Kaufmann, 1964) and DTNB (Reyes and Heunnekens, 1967) in the enhancement of activity of

certain dihydrofolate reductases has been interpreted as resulting from an induced conformational change in the enzyme-mercurial or enzyme-DTNB complex. In the case of the ribonucleotide reductase-mercurial complex, the activity of the various forms cannot be measured directly because the high concentration of thiol present in the assay mixture causes immediate disruption of the mercurial-reductase complex to give active enzyme indistinguishable from untreated enzyme. If all of the protein is assumed to be in the mercurial complex form, whether aggregated or not, there is little physical evidence for a conformational change in the non-aggregated form, which appears to have the same electrophoretic mobility as the native enzyme. The slight increase in the $\bar{s}$ of the mercurial-treated enzyme is probably caused by the presence of the high molecular weight species.

Although not demonstratable by the physical methods used, small changes in conformation which presumably occur as a result of substitution of thiol groups may expose regions of the molecule, hitherto buried, which can participate in intermolecular reactions and promote aggregation. Even larger aggregates are produced when the reductase is exposed to more than a 40 molal excess of mercurial, despite the fact that at this concentrat-

ion not all of the mercurial-reductase complex has completed the first aggregation step. Treatment of the complexes with excess thiol prior to electrophoresis, can convert all of the aggregates back to protein which has the same electrophoretic mobility as the untreated enzyme. Although excess thiol can activate most of the mercurial complex, assay of the fractions eluted from Sephadex G200 during gel filtration of the mercurial-reductase complexes indicates that the largest aggregates cannot be reactivated, but that of molecular weight 160,000 is readily reactivated by the assay procedure. This suggests that removal of mercurial from the larger aggregates is not necessarily accompanied by reversion to the native, active configuration. A similar conclusion is indicated from the stability studies where a 400 molal excess caused a 20 percent loss of activity which could not be reversed with excess thiol. Some of the intramolecular bonds formed as a result of the mercurial reaction may therefore be irreversible, even after the removal of the mercurial.

The effect of DTNB on the physical properties of the reductase is less marked than that of mercurials. After prolonged exposure, four enzyme thiol groups react as measured spectrophotometrically. This DTNB-reductase

gives rise to two protein species when electrophoresed on polyacrylamide gel, the first corresponding in mobility to that of the native enzyme and the other to a species of higher molecular weight. It is possible that the large excess of DTNB required to cause aggregation simply reflects the difficulty in obtaining complete reaction of the four thiol groups. The occurrence of some other slow reaction that is necessary for aggregation and that only occurs when a large excess of reagent is present is also possible, however.

The ultracentrifugal analysis of the DTNB-reductase gave a main peak with an identical $\bar{s}$ to the untreated control. A small amount of material of lowered $\bar{s}$ was observed in the g*(S)/g*(S) max vs S plots. The appearance of this material could result from the formation of species of lower molecular weight or from substantial modification in the shape of the molecule, although the latter is less likely to produce a change in $\bar{s}$ of such magnitude. A more definite result is the demonstration of aggregates by the reduction in area under the peak. Again, as with the mercurial complex, reversal of these reactions with thiol indicates that substitution of the reductase with DTNB plays a large part in effecting the observed changes.

Although the g*(S)/g*(S) max *vs* S plots give a qualitative estimate of a small proportion of protein with low $\bar{s}$, the possible explanation that this resulted from disaggregation of the 76,000 molecular weight unit, was not substantiated by the more sensitive technique of polyacrylamide gel electrophoresis. Although it may be argued that in this technique the effect of charge on the migrating species can mask molecular size effects, change in charge in this instance, especially in regard to DTNB would be to increase the negative charge on the protein fragments, which would accentuate the increased mobility of such small fragments. The complete absence of material with a mobility greater than that of the untreated enzyme therefore clearly indicates the absence of any subunit produced by any of the treatments used.

Effect of pH 8.9 on ribonucleotide reductase. As described in Chapter II, exposure of the reductase to pH 8.9 causes the production of an irreversibly inactivated species of lower electrophoretic mobility on polyacrylamide gel electrophoresis. When examined in the ultracentrifuge, the weight average sedimentation coefficient is slightly reduced, indicating a slight decrease in the molecular weight or a conformational

change in this more slowly sedimenting material. Again reduction in the area under the peak suggests in addition the presence of high molecular weight forms. Formation of aggregates at the high pH may be attributed to thiol-disulphide interchange, or to ionisation of those groups on the molecule with the highest dissociation constants with a consequent increase in the repulsive forces on the enzyme leading to irreversible inactivation. Apart from the detection of material of low sedimentation coefficient, the ultracentrifugal analysis confirms the effects of pH 8.9 observed on polyacrylamide gel electrophoresis.

Consideration of ribonucleotide reductase as an allosteric enzyme in the light of current models. The small size of ribonucleotide reductase, whilst not excluding the presence of subunits, makes this type of structure less likely as the physical basis of the allosteric properties of the reductase. Because of the large number of substrates, modifiers and cofactors known to participate in the complete reaction, explanation of the data by the symmetry theory of Monod et al. (1965) would demand an extremely complicated model (cf. Koshland and Neet, 1968). The sequential theory of Koshland et al. (1966) where each modifier is assumed

to induce a slightly different conformational change, provides a simpler model to which the data might be fitted, but again this model requires the presence of subunits which it has not so far been possible to demonstrate. Likewise, the inability of the modifiers to produce demonstrable aggregation or disaggregation excludes the possibility of polymer equilibrium (Winzor et al., 1963) as an explanation for the allosteric properties of the enzyme.

Recently an alternative to allosterism and co-operativity was proposed for the interpretation of kinetic data (Sweeny and Fisher, 1968). This treatment only considers enzymes which give rise to sigmoidal plots of initial velocity against substrate concentration and assumes such reactions take place by several types of well characterised reaction mechanisms simultaneously. However, the action of ribonucleotide reductase is not described by such plots. Also, as the hypothesis does not consider the effect of allosteric modifiers, this mechanism is irrelevant to the discussion on this enzyme.

The kinetics of the ribonucleotide reductase of L. leichmannii indicate the combination of more than one molecule of ribonucleotide, with substrate activation after the binding of multiple sites. These

phenomena may be explained in terms of classical kinetics (c.f. Frieden, 1964; Worcel et al., 1965; Morrison, 1965; Sanwal and Cook, 1966) on the basis of a simple model comprising one catalytic site and one allosteric site to which substrate or modifier may bind. The fit of the data to this model gives no indication of the possible subunit structure of the reductase. However, the model described above provides the best fit to the available data with the least number of assumptions.

Summary

The purity of electrophoretically homogeneous ribonucleotide reductase was investigated by gel filtration and ultracentrifugation techniques, and was found to be more than 95% pure. Analytical ultracentrifugation and polyacrylamide-gel electrophoresis were used to investigate the physical properties of the reductase in the presence of substrate and allosteric activators. Measurement of the weight average sedimentation coefficient provided identical values in the presence and absence of these ligands. This indicates that there is no aggregation or disaggregation in the presence of activators and substrates under the conditions used.

The rate and extent of reaction of sulphydryl groups on the reductase was investigated. Increased rates of reaction were observed in the presence of dGTP and alkali-metal ions, indicating that these activators of the reaction facilitate a conformational change in the reductase. The possibility of subunit structure was investigated by examination of the molecular weight at low protein concentrations and in the presence of 7 M urea or excess PMB. In no case was material of lower molecular weight observed in significant amounts. However, PMB causes aggregation of the molecule to several species of higher molecular weight. The smallest aggregates may be reactivated in the presence of thiols while those of very high molecular weight remain inactive.

The chemical molecular weight of ribonucleotide reductase could not be determined by tryptic digestion and peptide mapping, due to incomplete hydrolysis by trypsin resulting in a large insoluble "core" peptide.

On the basis of these results, ribonucleotide reductase appears to be an allosteric protein where the mechanism does not depend on the participation of subunit structure.

CHAPTER IV

PURIFICATION AND PROPERTIES OF THIOREDOXIN AND THIOREDOXIN REDUCTASE OF LACTOBACILLUS LEICHMANNII

Introduction

The investigation of Reichard and his colleagues on reduction of ribonucleotides by cell-free extracts of Escherichia coli B led to the isolation and characterisation of the hydrogen donor system for this reaction. The reductant for ribonucleotides was found to be a small, heat-stable protein which, when oxidised, contains a single disulphide bridge and was designated thioredoxin (Laurent et al., 1964). This protein is reduced by NADPH in the presence of a specific flavoprotein, thioredoxin reductase (Moore et al., 1964) by the reaction shown in Fig. I.2. Thioredoxin is the specific hydrogen acceptor for the thioredoxin reductase, but together these proteins will catalyse the NADPH-dependent reduction of glutathione, lipoic acid, oxytocin and insulin (Moore et al., 1964).

Several other superficially similar reducing systems have been described. Black et al. (1960) have isolated three proteins from yeast which couple the reduction of methionine sulphoxide to NADPH. Two of these three proteins can also reduce several disul-

phides in the presence of NADPH, including hydroxyethyl disulphide, L- or D-cystine, homocystine, DL-lipoic acid, oxidised glutathione, oxytocin and the three disulphide bonds of insulin. Another three proteins constituting a similar system for the reduction of sulphate have also been isolated from yeast (Bandurshi et al., 1960). Two of these proteins are heat labile and the third is relatively heat stable. The reaction is accelerated by the addition of FAD. Asahi et al. (1961) describe two enzymes, A and B, which catalyse the reaction. The first step involves the reduction of a small, heat stable disulphide protein (Fraction C) by NADPH in the presence of enzyme A, this reaction being stimulated by FAD. Fraction C is the specific hydrogen acceptor for enzyme A, but the two proteins, A and C, in the presence of NADPH, reduce 5,5'-dithiobisnitrobenzoic acid (DTNB), lipoamide or, in the presence of enzyme B, 3'-phosphoadenosine-5'-phosphosulphate to sulphite.

Four proteins have been implicated in the transfer of electrons from glycine to NAD in Peptococcus glycinophilus (Baginski and Huennekens, 1966), one of which is a flavoprotein (Klein and Sagers, 1967). The reaction, involved in the oxidative decarboxyl-

ation of glycine, differs from those described above in pyridine nucleotide specificity. The flavoprotein can be reduced by NADH or by glycine, but reduction by the latter can only occur in the presence of the three other proteins of the system.

Thioredoxin reductase of E. coli has been obtained in pure form and has a molecular weight of 66,000 (Thelander, 1967, 1968). There is a resemblance between the properties of this enzyme and those of two other flavoproteins, glutathione reductase and lipoyl dehydrogenase (c.f. Colman and Black, 1965; Massey and Williams, 1965; Massey et al., 1962), all of which contain 2 moles of FAD per mole of protein and give similar visible-light-absorption spectra. All catalyse the reversible oxido-reduction of a disulphide bridge by pyridine nucleotides. Each is specific for a particular disulphide substrate, but thioredoxin reductase differs from the other two enzymes in that the latter have low molecular weight substrates. All three of these proteins, together with thioredoxin have been purified simultaneously from E. coli (Williams et al., 1967). In their comparison of these enzymes, Zanetti and Williams (1967) and Thelander (1968) conclude that a reducible intra-chain disulphide bridge provides an integral part of

the reaction mechanism, and although these enzymes catalyse apparently similar chemical reactions, the reaction mechanism of thioredoxin reductase may differ from that of glutathione reductase and lipoyl dehydrogenase.

The isolation and purification of the physiological hydrogen donor in the ribonucleoside diphosphate reductase system led to speculation that a similar system might be present in extracts of Lactobacillus leichmannii, especially as the reduced thioredoxin of E. coli can function as the hydrogen donor for ribonucleoside triphosphate reductase (Vitols and Blakley, 1965). Several preliminary reports (Blakley et al., 1965; Vitols and Blakley, 1965; Beck et al., 1966) described experiments designed to demonstrate the presence of such a system, but the results of all these experiments were negative. It was noted, however (Beck et al., 1966), that cell-free extracts of L. leichmannii contain an NADPH oxidase which was assumed to contribute to the difficulty of demonstrating the presence of an NADPH-dependent reducing system. Vitols and Blakley (1965) unsuccessfully attempted to demonstrate the reduction of nucleotides by crude extracts of L. leichmannii after the addition of

E. coli thioredoxin reductase and NADPH. As has been discussed previously, there are few similarities between the ribonucleotide reductases isolated from the two sources. It was of interest, therefore, to determine whether these dissimilarities extend to the function and structure of the hydrogen donor system. Cell-free extracts of L. leichmannii were therefore investigated for the presence of an NADPH-dependent reducing system. In this chapter, the isolation of the protein components of this system in substantially pure form is described.

Experimental Procedure

Materials

NADPH, NADP, NADH and nucleotides were purchased from P-L Biochemicals, Milwaukee. Streptomycin sulphate was purchased from Glaxo-Allenburys, Sydney, Australia. Catalase was purchased from Nutritional Biochemicals, Ohio. Glucose-6-phosphate dehydrogenase (G6PD) and glucose-6-phosphate (G6P) were purchased from Sigma, St. Louis.

Thioredoxin and thioredoxin reductase of E. coli, purified by the methods of Laurent et al. (1964) and Moore et al. (1964) were supplied by Dr. E. Vitols. Ribonucleotide reductase was prepared by the method described in Chapter II, and was supplied by Dr. R.L. Blakley.

Methods

Enzyme assay. Both enzyme (thioredoxin reductase) and substrate (thioredoxin) were assayed by the same procedure. Thioredoxin reductase activity was measured in the presence of excess thioredoxin, the latter being assayed in the presence of a fixed concentration of thioredoxin reductase isolated by Sephadex G-100 gel filtration equivalent to 10 µg of pure reductase. In the assay of thioredoxin in extracts prior to the removal of thioredoxin reductase, a slight positive error may arise due to the presence of a small amount of reductase in the sample. The NADPH-dependent reduction of DTNB was measured at 412 on a Cary model 14 or 15 recording spectrophotometer. The experimental cuvette contained 200 to 1500 µmoles of potassium phosphate buffer, pH 7.5, 10 µmoles of EDTA, 80 mµmoles of DTNB, 100 mµmoles of NADPH and thioredoxin and thioredoxin reductase in the amounts required for assay. Final volume was 1 ml. A unit of activity is defined as the amount of protein which will reduce one mµmole of DTNB per minute. Specific activity is defined as the number of units per milligram. In the calculation of these units, a molar extinction coefficient of 13.6×10^3 was used for reduced DTNB (Ellman, 1959).

The molarity of thioredoxin preparations was determined by the method of Laurent et al. (1964).

Protein determination. Protein concentrations were determined by the method of Gornall et al. (1949). Protein concentrations of fractions eluted from chromatography columns were estimated from the absorbance at 280 mμ.

Bacterial cultures. Cells of L. leichmannii, a mutant of ACTC 7830 strain, were grown on the defined medium described by Blakley (1965). This medium was autoclaved at 15 pounds pressure for 30 minutes. Cells were grown in 60 litre batches in still culture at 37° from a 0.0014 to 0.07% inoculum in active growth phase. When cell growth had produced an absorbance of 1.0 at 660 mμ, as measured on a Beckman DK2 spectrophotometer, the medium was vigorously agitated. Under these conditions active growth proceeds in a non-logarithmic fashion to an absorbance at 660 mμ of 2.5-3.0. After chilling the medium, cells were harvested in a Sharples ultracentrifuge at 3°. Wet cell paste (150-200 g) was resuspended in ten volumes of cold 0.05 M Tris-HCl buffer, pH 8.0, and the cells were collected by centrifugation at 3° for 10 minutes at 20,000 x g.

After resuspension in 6 volumes of cold 0.05 M Tris-HCl buffer, pH 8.0, the cells were disrupted in a Ribi (Sorvall) cell fractionator at 20,000 pounds per square inch. Particulate material was removed by centrifugation at 30,000 x g for 1 hour at 3°.

Preparative chromatography. Sephadex G-100 and G-50 were prepared and columns poured as described in Chapter III. These columns were supported from below by two layers of terylene gauze; flow rates of 2.0 and 2.5 ml per hour per cm^2 were obtained for G-100 and G-50, respectively.

DEAE-Sephadex A-50 was prepared in the phosphate form after cycling with acid and alkali, as described by the manufacturers, Pharmacia, Uppsala. Initial flow rates of 10 ml per hour were obtained with increasing flow as the eluting buffer became more concentrated.

Polyacryamide-gel electrophoresis. Preparative and analytical electrophoresis was performed as described in Chapter II.

Concentration of protein solutions. This was effected with an Amicon Diaflow apparatus incorporating membranes which will specifically retard molecular weight species in excess of 10,000 and 50,000 molecular weight for the concentration of

thioredoxin and thioredoxin reductase, respectively. Compressed helium was used in the ultrafiltration to minimise frothing on the release of pressure.

Oxygen consumption. A small oxygen electrode (Titron Instrument Co., Sandringham, Australia) was used to measure oxygen consumption by the method of Snoswell (1966). Total volume of the reaction mixture was 2.3 ml.

Amino acid analyses. The procedures described in Chapter III were used.

Spectrophotofluorimetry. Fluorimetric emission of thioredoxin after excitation at wavelength 280 mμ was measured on an Aminco-Bowman Spectrophotofluorimeter fitted with a Moseley X-Y recorder. Samples in 1.5 ml were examined in 1 cm x 1 cm light path quartz cuvettes at 10°.

Results

Joint purification of thioredoxin and thioredoxin reductase

Streptomycin sulphate precipitation of inactive protein. The cell-free extract was adjusted to pH 5.6 by the dropwise addition of 1 N acetic acid. Streptomycin sulphate solution (16 ml of a freshly prepared 5% solution per 100 ml of extract) was added to the stirred extract. The precipitate was removed by

centrifugation at 20,000 x g at 3° for 10 minutes. The decanted supernatant solution was adjusted to pH 7.3 by the dropwise addition of 1 N ammonium hydroxide. The purification of crude extract and the recovery of activity after streptomycin sulphate precipitation of nucleic acid and protein is shown in Table IV.1. The conditions used for preparation of E. coli thioredoxin and thioredoxin reductase are unsuitable for use with L. leichmannii extracts. With the amount of streptomycin sulphate used, no precipitation occurs at pH 8.0, but as the pH of the mixture is lowered, precipitation first occurs at pH 6.8. Recovery is good (>95%) at pH 5.6, but below pH 5.6 both recovery and increment in specific activity are diminished. Precipitation of protein by lowering the pH to 4.0 did not provide a method for separating thioredoxin and thioredoxin reductase as is the case with these proteins isolated from E. coli. Under all conditions used between pH 5.6 and pH 4.0 each protein was present in both soluble and insoluble fractions.

Ammonium sulphate precipitation of thioredoxin and thioredoxin reductase. The neutralised supernatant from the streptomycin sulphate fractionation was made 10 mM with respect to EDTA. Solid ammonium sulphate was then added mechanically at a continuous

TABLE IV.1. Removal of protein from the crude extract by streptomycin sulphate

	pH of precipitation	Specific activity	% Recovery
Control	-	12.7	100
Streptomycin sulphate	8.0	12.7	100
Streptomycin sulphate	5.6	18.4	95
Streptomycin sulphate	5.0	13.2	63

Streptomycin sulphate was added at the rate of 16 ml of 5% solution per 100 ml of extract at the pH indicated.

rate such that it was dissolved as rapidly as it was added. Stirring was continued for 30 minutes after all the added ammonium sulphate had dissolved. Precipitated protein was collected by centrifugation at 20,000 x g for 15 minutes at 3°. The supernatant solution was discarded. The precipitated protein was dissolved in 200 ml of potassium phosphate buffer, pH 7.5, containing 1 mM EDTA.

Heat treatment of thioredoxin and thioredoxin reductase. Since ammonium sulphate appeared to stabilise thioredoxin reductase when exposed to higher temperatures (c.f. Williams et al., 1967), the redissolved ammonium sulphate precipitate was used without dialysis. The stirred protein solution, in a stainless steel beaker, was heated in a water bath and maintained at the particular temperature for 5 minutes, after which it was rapidly cooled in ice water. Precipitated protein was centrifuged at 20,000 x g for 15 minutes at 3°. The supernatant solution was decanted and stored at 0°. The precipitate was washed with two 50 ml portions of 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The washings were combined with the original heat-treated supernatant solution and the residue was discarded. The effect of heat treatment on

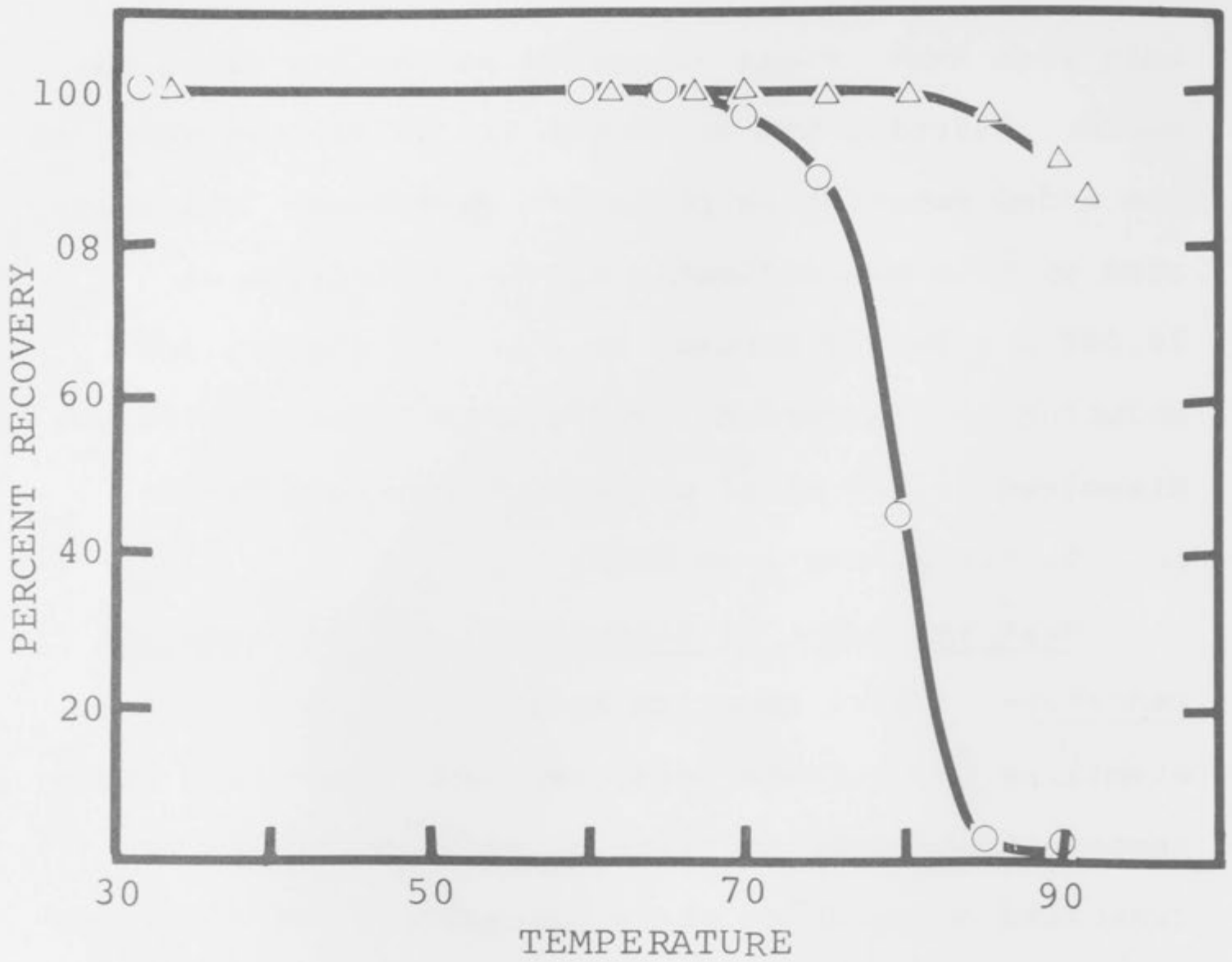


FIG. IV.1. Effect of temperature on thioredoxin and thioredoxin reductase.
Redissolved ammonium sulphate precipitate was heated to, and maintained at, the temperatures shown for a period of five minutes. Samples were then centrifuged and activity determined on aliquots of the supernatant as described in the Methods section. Results are expressed as a percentage of initial activity. O—O, thioredoxin reductase; Δ—Δ thioredoxin.

thioredoxin and thioredoxin reductase under these conditions is shown in Fig. IV.1. For routine preparative use, extracts were heated to 65° for 5 minutes to provide maximal recovery to both activities.

Ammonium sulphate was added to the combined supernatant solutions as previously described, to give 0.9 saturation. Precipitated protein was collected by centrifugation (20,000 x g for 15 minutes at 3°) and the supernatant discarded. The precipitate was transferred with the aid of a spatula to a thick walled (0.5 mm in dry state) dialysis tube and rinsed in with a minimal volume of 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The sac was tied under pressure to prevent excessive increase in volume during dialysis for 12-24 hours against three 5 litre portions of 0.05 M potassium phosphate buffer containing 1 mM EDTA. Losses of thioredoxin occur if this procedure is performed with thin walled dialysis sacs.

Gel filtration of thioredoxin and thioredoxin reductase on Sephadex G-100. The dialysed protein solution from the heat treatment (containing 6-10 g

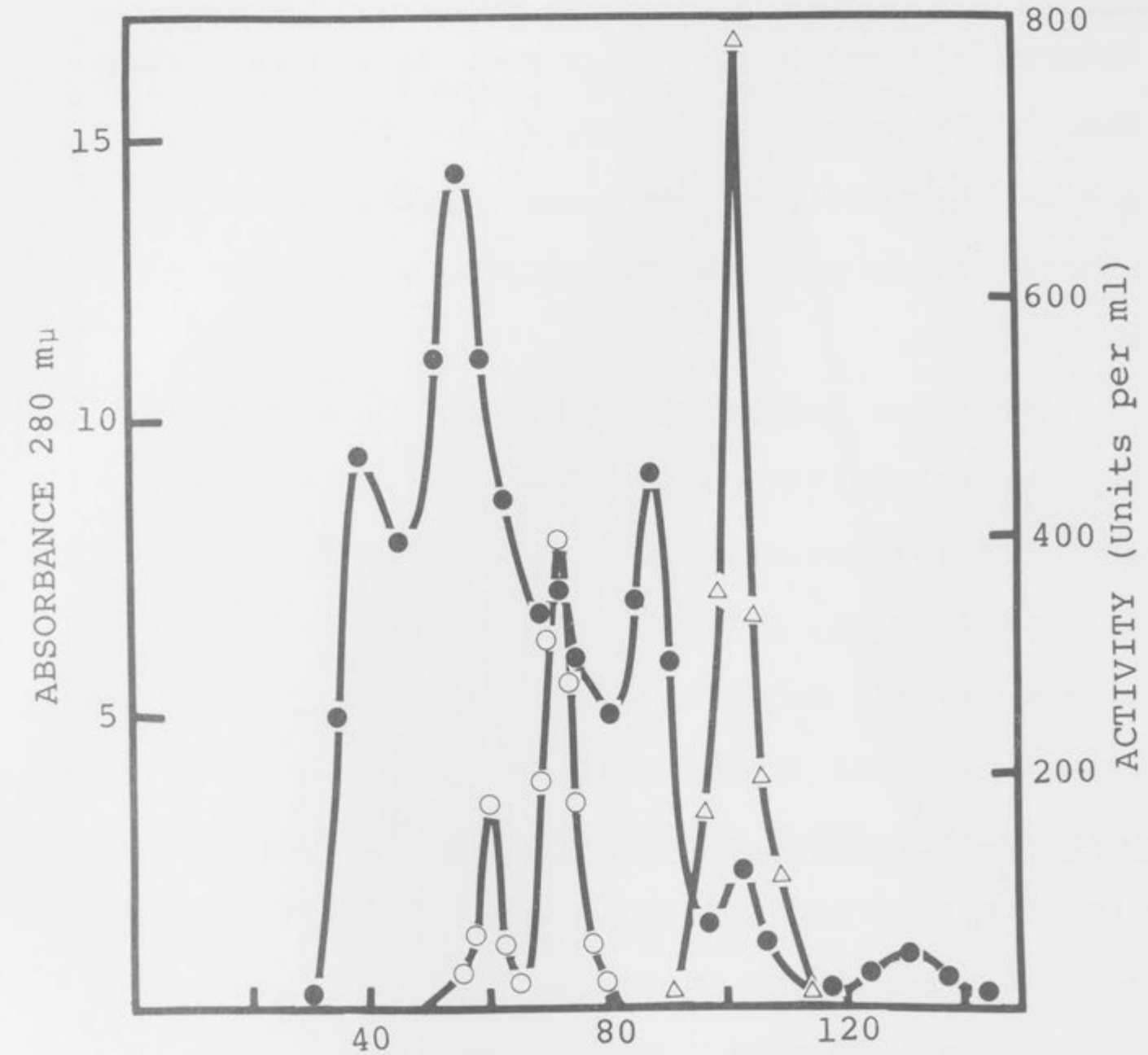


FIG. IV.2. Gel filtration of thioredoxin and thioredoxin reductase on Sephadex G-100. The column (150 x 5 cm) was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5 containing 1 mM EDTA), which was also used to elute the fractions.

●—● Absorbance at 280 mμ;

○—○ Thioredoxin reductase activity;

△—△ Thioredoxin activity.

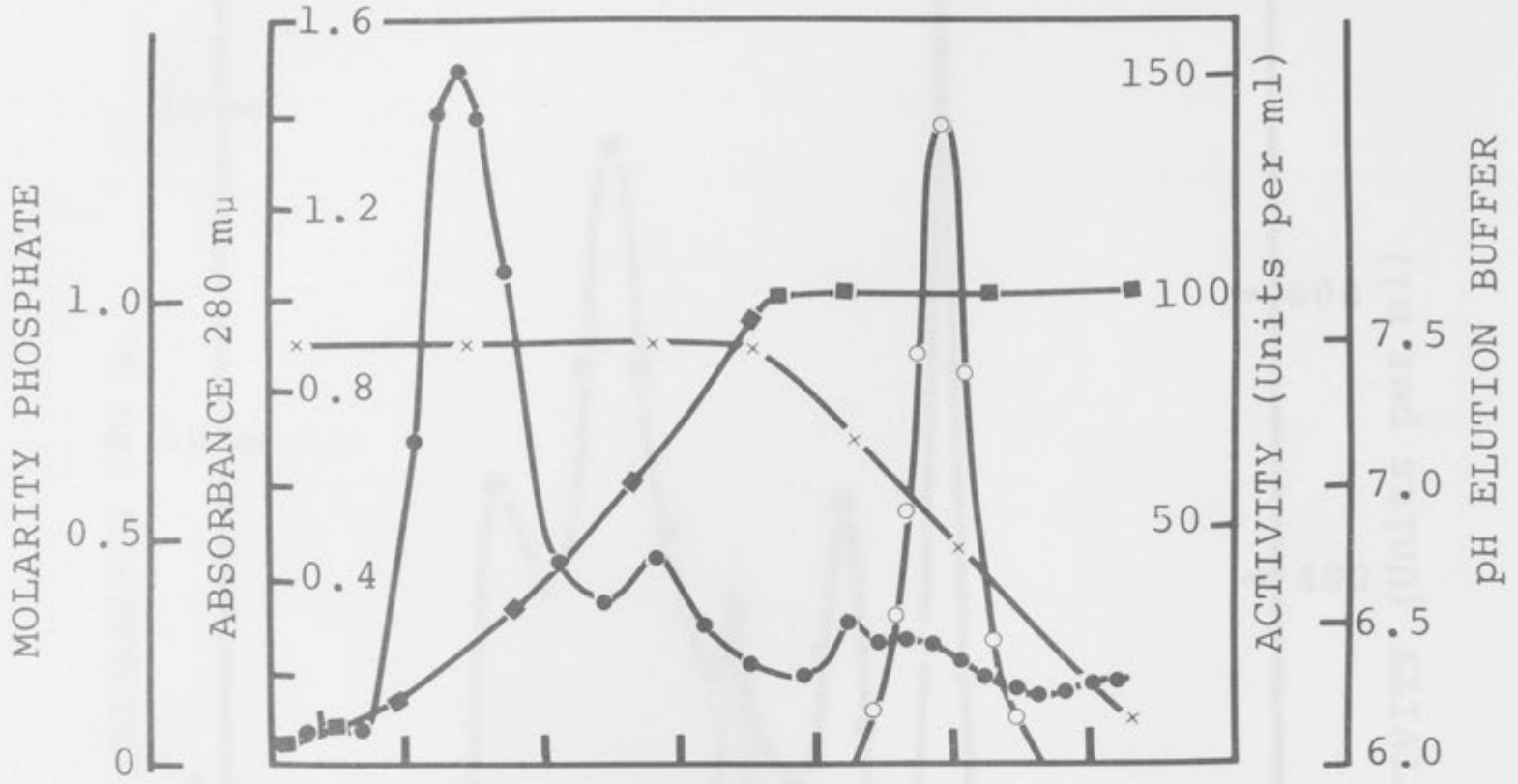


FIG. IV.3. DEAE-Sephadex chromatography of thioredoxin reductase.
The sample (0.2-2.0 g in 100-300 ml) was applied in 0.05 M potassium phosphate (pH 7.5, containing 1 mM EDTA) to a 10 x 4 cm column and a major peak of inactive protein was eluted with a linear phosphate gradient from 0.05 M to 1 M, pH 7.5. Thioredoxin reductase activity (O—O) was eluted with a linear gradient from pH 7.5 to 6.0, 1 M potassium phosphate. Peak activity was eluted at pH 6.8. ●—●, absorbance 280 mμ; □—□, molarity of phosphate; X—X, pH of eluted fractions.

in 80-115 ml) was applied to a column of Sephadex G-100. The elution profiles of protein and activity are shown in Fig. IV.2. Thioredoxin of E. coli or L. leichmannii may be used as substrate to detect thioredoxin reductase activity, but only thioredoxin reductase of L. leichmannii can utilise the thioredoxin of this organism as substrate. It will be noted that there are two peaks of thioredoxin reductase activity.

Further purification of thioredoxin reductase. DEAE-Sephadex A-50 chromatography. The reductase cannot be eluted from this ion exchanger with phosphate buffer at pH 7.5 at concentrations up to 3 M. If, however, the pH of the eluting buffer is lowered the protein is eluted at a concentration of 1 M phosphate at pH 6.5. Plots showing the elution of protein, activity, phosphate concentration and pH are shown in Fig. IV.3.

Elution of thioredoxin reductase from DEAE-Sephadex with acetate buffer, pH 4.5, was investigated and produces reductase of a high specific activity. However, polyacrylamide-gel electrophoresis of extracts purified in this manner indicate the presence of multiple bands and two peaks of activity.

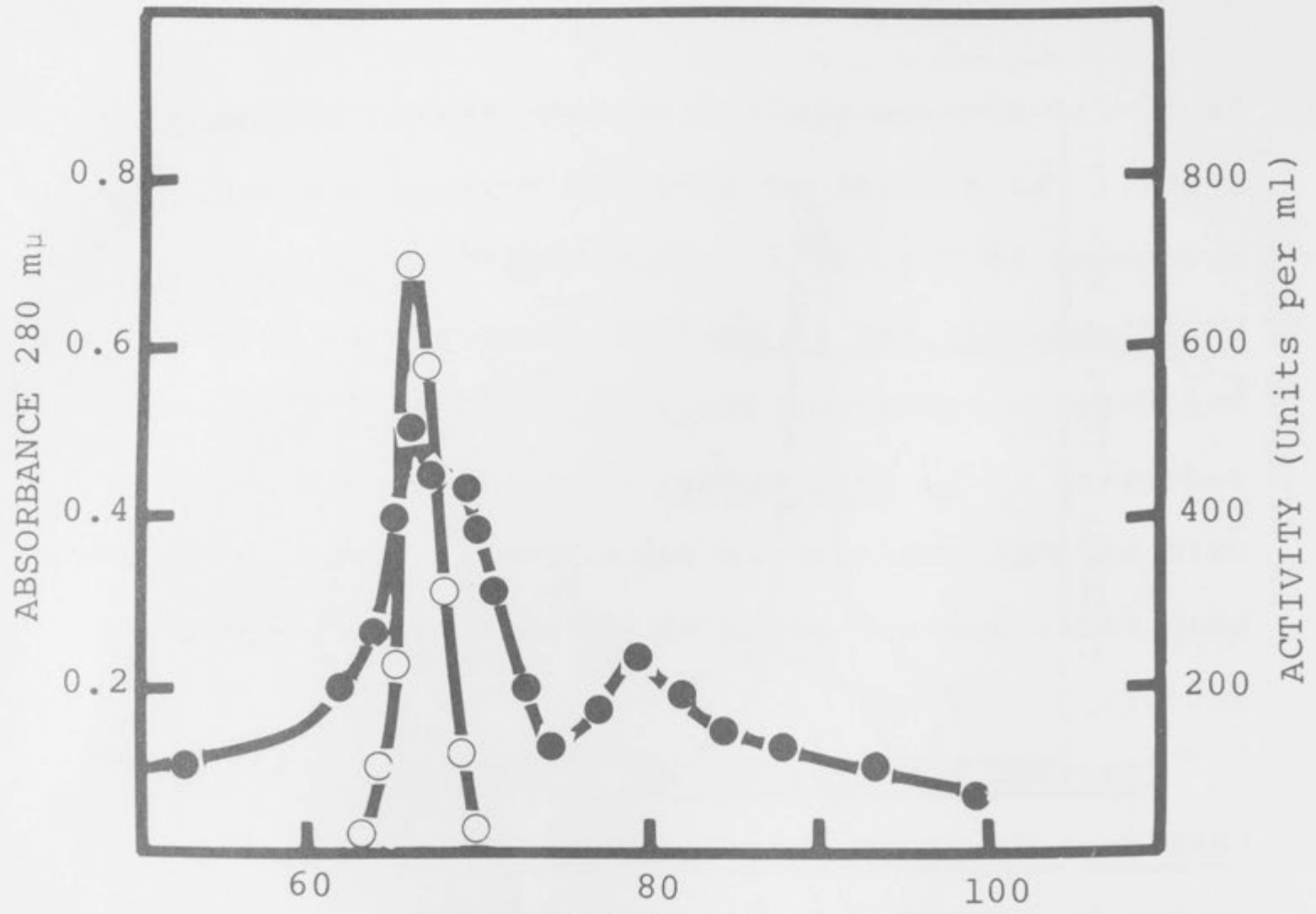
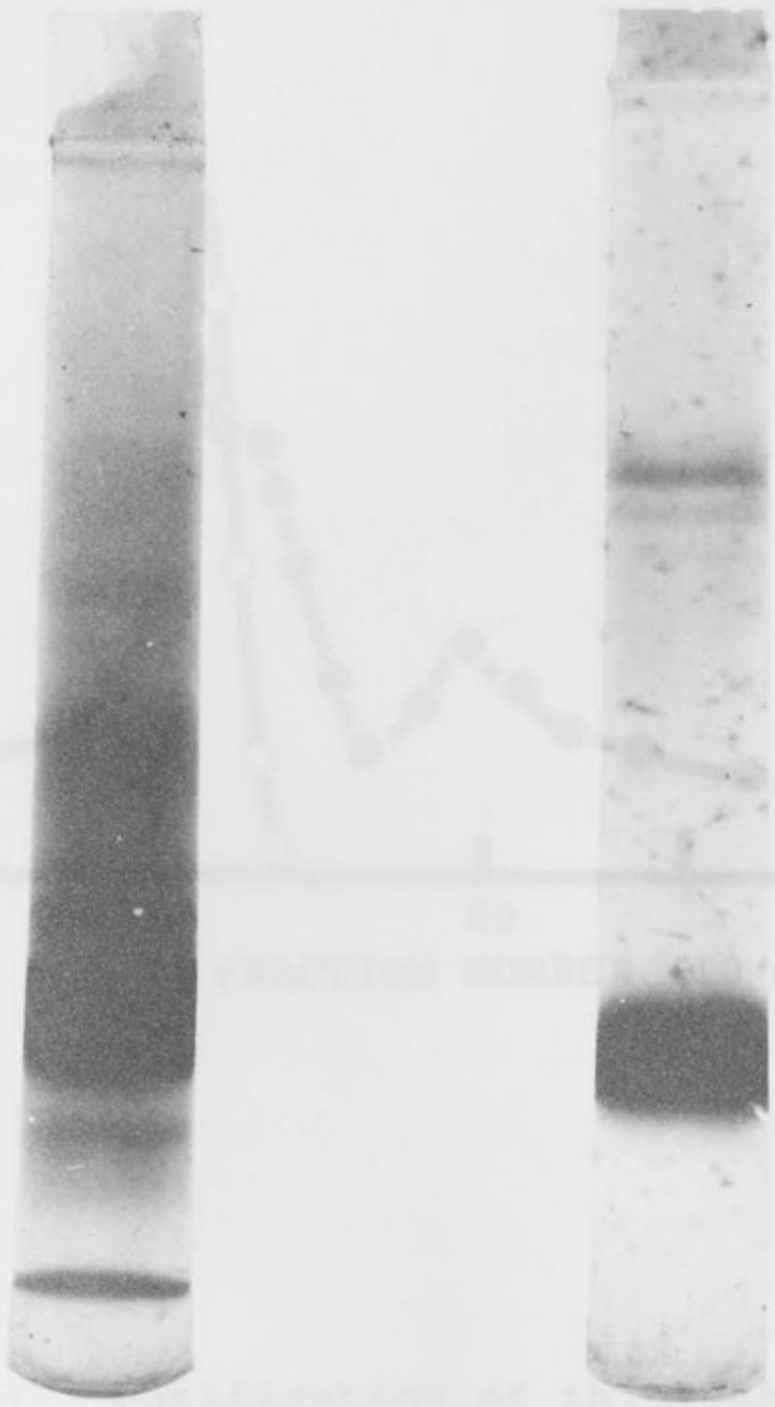


FIG. IV.4. Purification of thioredoxin reductase by preparative polyacrylamide-gel electrophoresis.

The buffer system described by Thelander (1967) was used. The sample applied consisted of 20 mg of protein purified by DEAE-Sephadex chromatography. ●—●, absorbance 280 mμ; O—O, thioredoxin reductase activity.



A B

FIG. IV.5. Polyacrylamide-gel electrophoresis of thioredoxin reductase.

A. Before preparative electrophoresis.

B. After preparative polyacrylamide-gel electrophoresis.

The buffer system of Thelander was used.

As the two peaks of activity may result from aggregation-disaggregation effects at the lower pH, this method of ion exchange chromatography has been discontinued.

Preparative polyacrylamide-gel electrophoresis of thioredoxin reductase. Thioredoxin reductase purified by DEAE-Sephadex chromatography was electrophoresed in the buffer system of Thelander (1967) with the results shown in Fig. IV.4. A single peak of activity was observed in fractions eluted from the preparative apparatus which corresponded to the largest protein peak. Although this peak of activity did not appear to be completely separated from inactive material, the fraction exhibiting maximum activity was almost pure as shown by polyacrylamide-gel electrophoresis. Fig. IV.5 shows protein before and after preparative electrophoresis. The small amount of low mobility material present after electrophoresis appears to constitute less than 5% of the applied protein sample. No faster migrating species was observed. This pure preparation exhibited a specific activity of 1,700. Eighty percent of the protein and 69% of the activity applied was recovered. The purification of thioredoxin reductase is summarised in Table IV.2.

TABLE IV.2. Purification of thioredoxin reductase

Procedure	Vol.	Total Units	Specific Activity	Yield %	Purification
Cell-free extract	1240	121,000	4.9	100	-
Streptomycin sulphate	1360	91,000	4.2	75	-
Heat treated	118	72,000	6.7	60	1.4
Gel filtration					
Peak I		14,000	12	12	2.5
Peak II		69,000	65	57	13
DEAE-Sephadex	132	49,000	1,200	40	250
Electrophoresis	10	7,000	1,700	22*	350

*Recovery in tube of peak activity only; overall recovery for this step 69%.

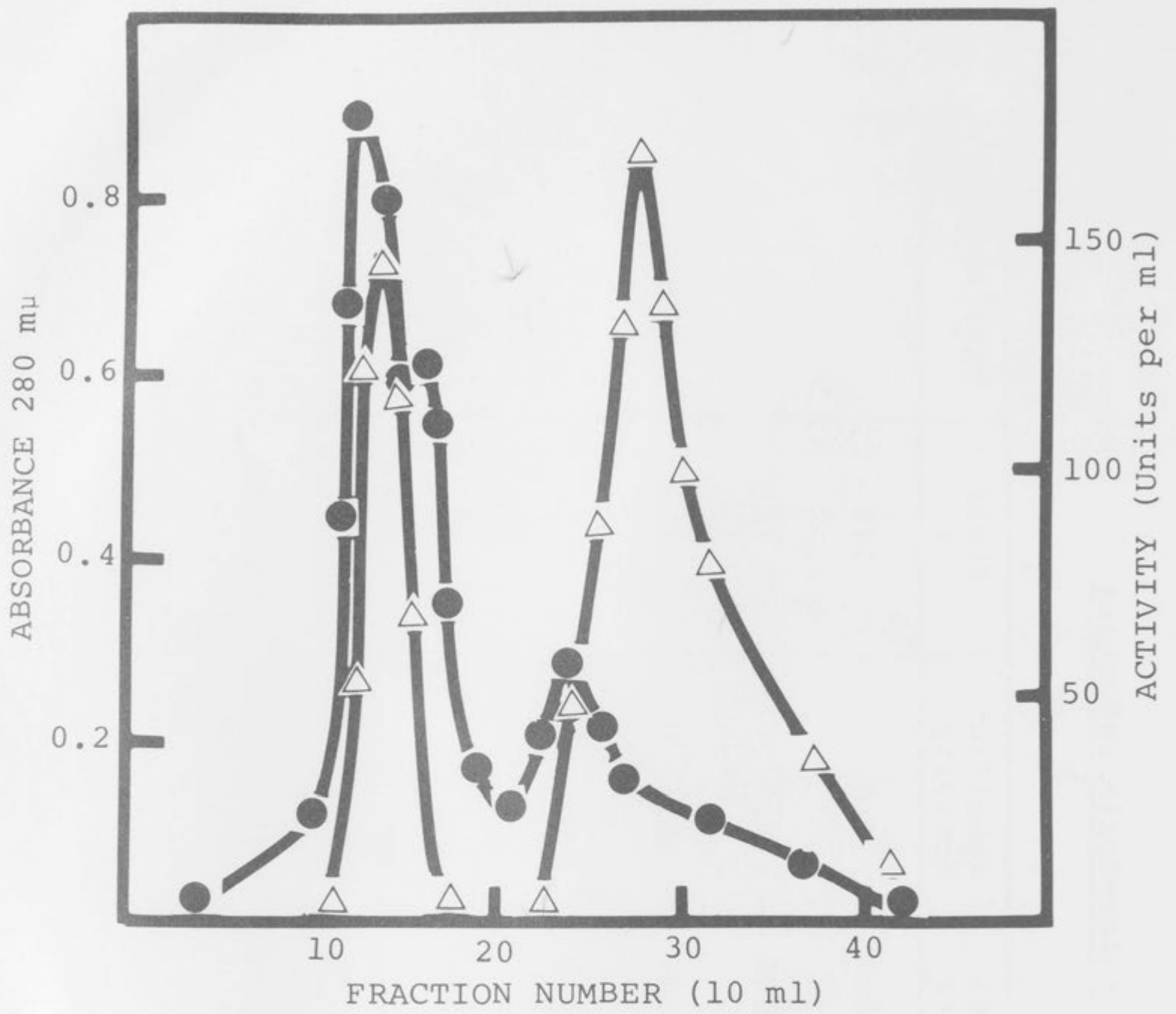


FIG. IV.6. DEAE-Sephadex A-50 chromatography of thioredoxin.

The sample (6-200 mg in 50-300 ml) was applied in 0.05 M potassium phosphate (pH 7.5 containing 1 mM EDTA) to a 10 x 4 cm column. Protein was eluted with a linear phosphate gradient from 0.05 M to 2.0 M, pH 7.5. ●—●, absorbance 280 mμ; Δ—Δ, thioredoxin activity.

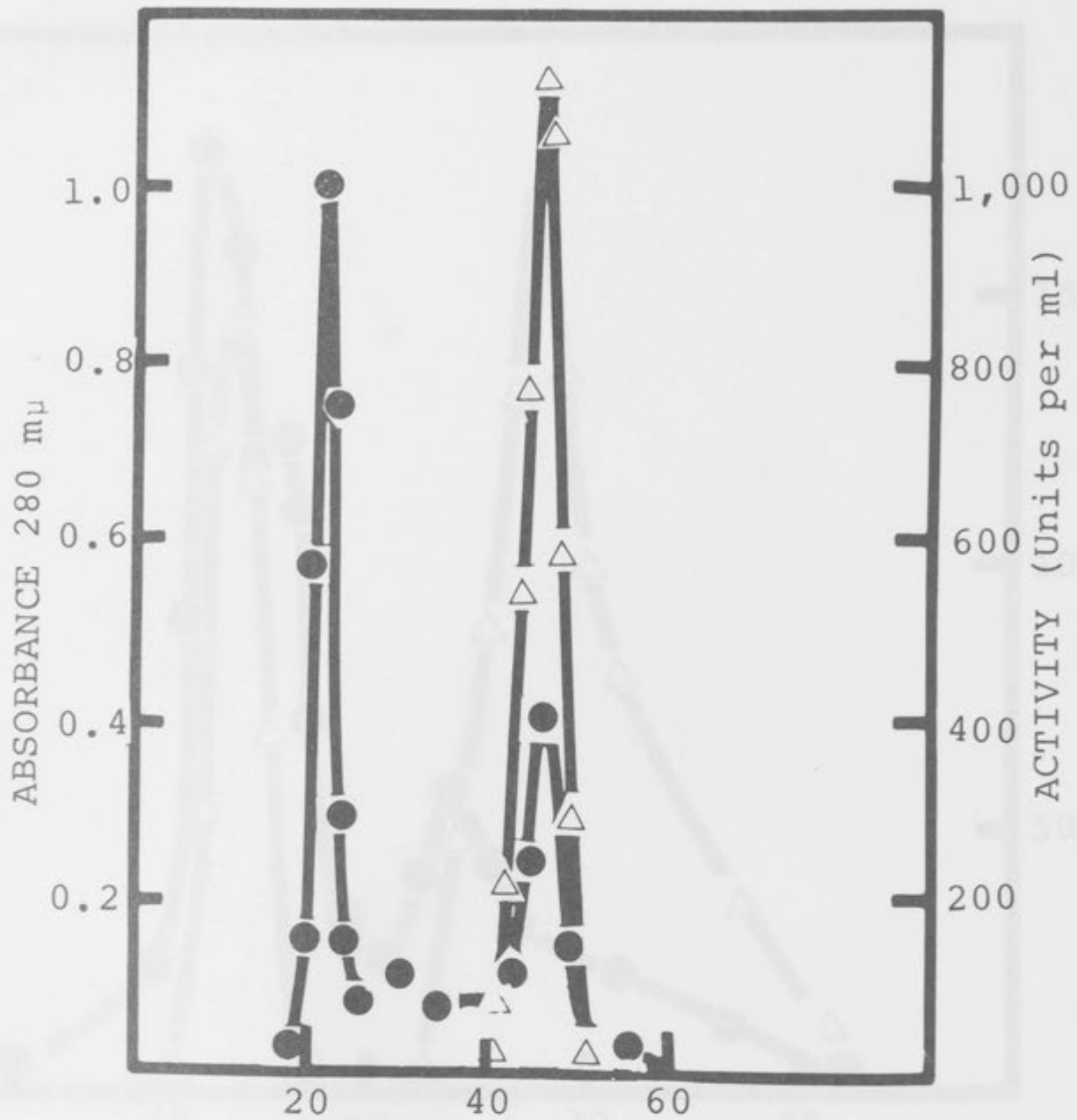


FIG. IV.7. Gel filtration of thioredoxin on Sephadex G-50.

The 150 x 2 cm column was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5 containing 1 mM EDTA) which was also used to elute the fractions

●—● Absorbance at 280 mμ;

Δ—Δ Thioredoxin activity.

Further purification of thioredoxin

DEAE-Sephadex A-50 chromatography. The combined active fractions from gel filtration on Sephadex G-100 were further purified on DEAE-Sephadex as shown in Fig. IV.6. Two peaks of activity were always observed, the first (Peak I) being eluted at a phosphate concentration of 0.2-0.4 M phosphate, and the second, larger peak (Peak II) of activity was eluted above a concentration of 0.6 M phosphate. As Peak II constitutes up to 80% of the activity applied, it has been used for all of the investigations to follow. Following dialysis in thick-walled sacs against 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, Peak II thioredoxin was concentrated in a Diaflow pressure cell.

Gel filtration of thioredoxin on Sephadex G-50. After concentration, the combined active fractions from DEAE-Sephadex chromatography (about 10-30 mg protein in 3 ml) were dialysed against 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. Elution profiles of absorbance at 280 mμ and thioredoxin activity are shown in Fig. IV.7. Active fractions were combined and concentrated on the Diaflow apparatus.

FIG. IV.8. Polyacrylamide-gel electrophoresis of thioredoxin.

TEA-TES buffer was used. Sample consisted of 80 µg of thioredoxin from the Sephadex G-50 filtration and concentrated by ultrafiltration.

Polyacrylamide-gel electrophoresis. Electrophoresis of the concentrated fractions indicate a high degree of purity as shown in Fig. IV.8. The purification of thioredoxin is summarised in Table IV.3.

Molecular weight of thioredoxin and thioredoxin reductase. Comparison of the elution volumes of these proteins with those of proteins of known molecular weight (c.f. Fig. III.6), indicated a molecular weight of 12,000-14,000 for thioredoxin and 70,000-80,000 and 35,000-40,000 for the first and second species of thioredoxin reductase eluted, respectively.

The species of thioredoxin eluted first from DEAE-Sephadex has an identical elution volume on Sephadex G-50 to the major thioredoxin species (Peak II). It is unlikely, therefore, that the two species represent aggregate and monomer forms of the same enzyme. Void volume and elution volume in this experiment were 34 ml and 54 ml, respectively. Insertion of these values into the relationship given by Determann (1966)

$$\text{Log } M = 5.415 - 0.864 \; (V_e/V_o)$$

provides a value for the molecular weight of about 12,000.

TABLE IV.3. Purification of thioredoxin

Procedure	Vol.	Total Units	Specific Activity U/mg	Yield %	Overall Purification
Cell-free extract	1100	136,000	5.8		
Streptomycin sulphate	1270	152,500	6.8	112	1.2
Heat treated	101.5	102,500	10.8	75	1.9
Gel filtration	400	35,000	190	26	33*
DEAE-Sephadex chromatography					
Peak I	155	6,750	87.6	5	15
Peak II	485	25,400	569	18	98
Gel filtration	20	28,600	7,000	20	1,200
Concentration Diaflow	1.41	27,400	5,900	20	1,020

*In this particular preparation, recovery of activity at this step was unusually low. Normally, recovery over this step is in the range 80-90%.

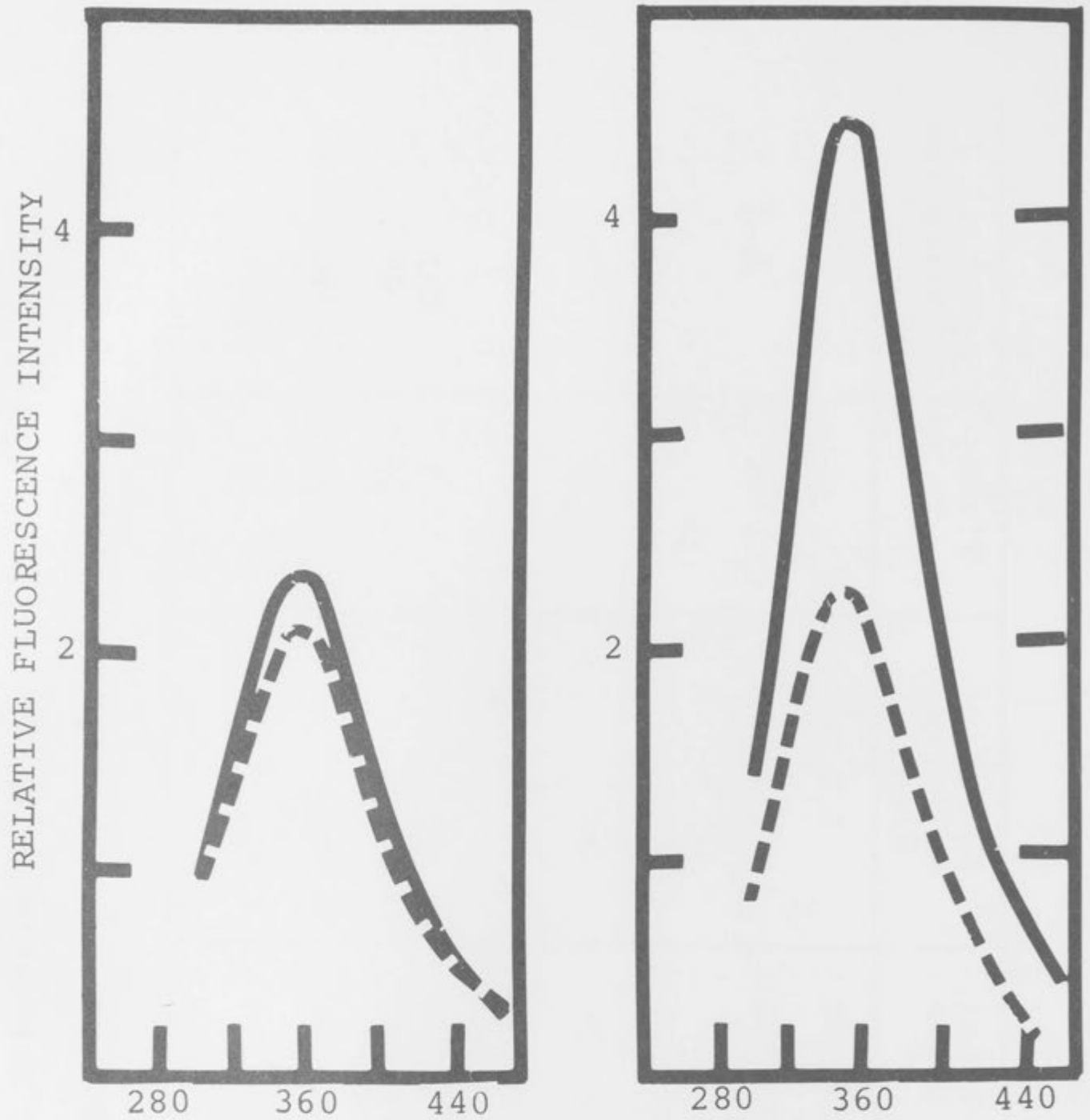


FIG. IV.9. Fluorescence emission spectra of the oxidised (----) and reduced (——) forms of thioredoxin.
Excitation was at 280 mμ. Thioredoxin (2.25 mμmoles) in 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. After obtaining the oxidised spectrum, the thioredoxin was reduced by the addition of dithiothreitol (0.01 ml, 0.5 M). L. leichmannii thioredoxin (A) exhibits only a slight change compared to the two-fold increase in E. coli thioredoxin fluorescence (B) (cf 2.5-fold, Stryer et al., 1967). Emission maximum was not altered. The curves, A and B, have not been corrected for variation with wavelength in the sensitivity of the recording system, nor has the monochromator been calibrated for exact wavelengths of emission.

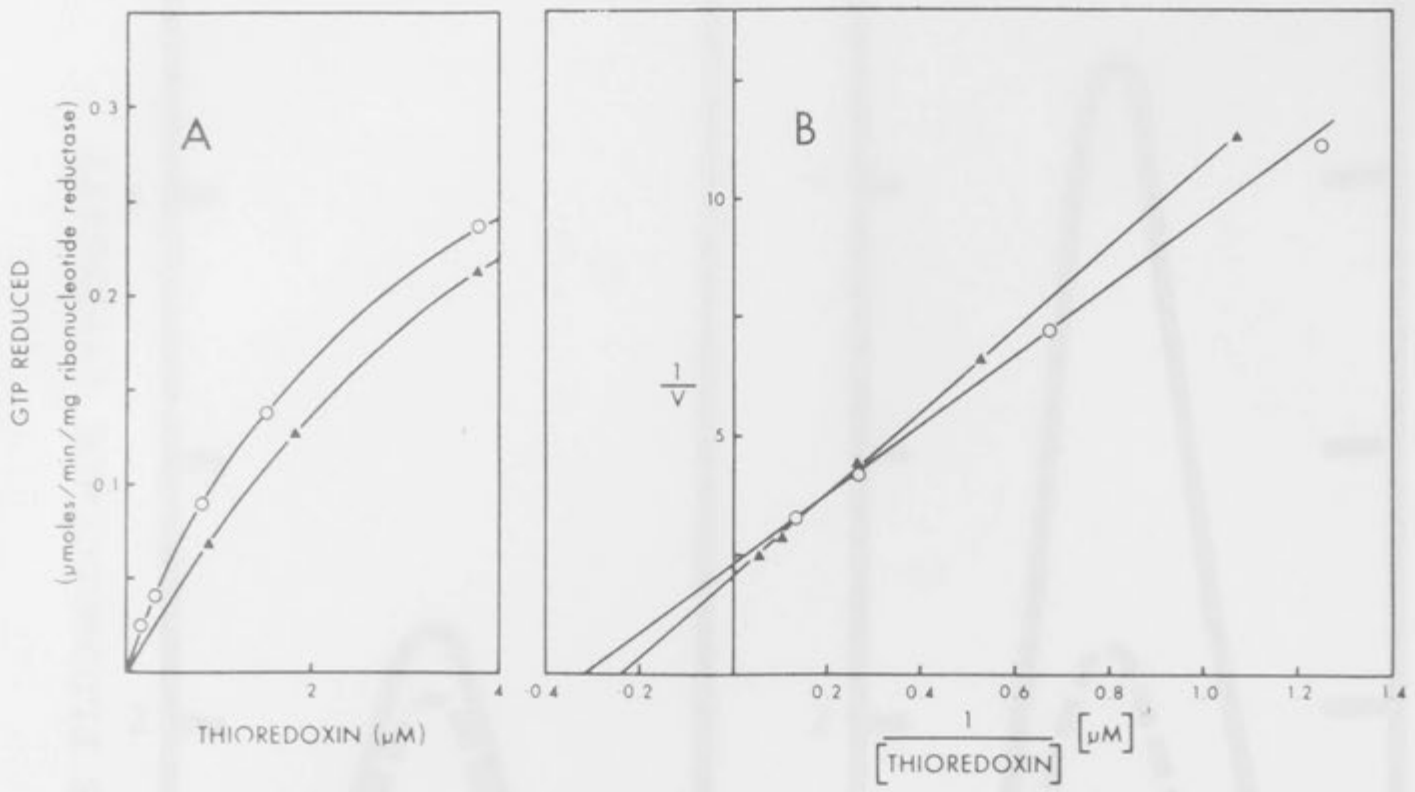


FIG. IV.10. The rate of GTP reduction as a function of thioredoxin concentration. The rate of the ribonucleotide reductase reaction was assayed at 37° by measuring NADPH oxidation at 340 mμ on a Cary Model 14 recording spectrophotometer. The experimental cuvette contained 100 μmoles of potassium phosphate buffer, pH 7.5, 2 μmoles of EDTA, 1 μmole GTP, 100 mμmoles NADPH, 15 μg of L. leichmannii ribonucleoside triphosphate reductase, 5 or 10 μg of thioredoxin reductase, 0.16-18.8 μM thioredoxin and 2 mμmoles 5'-deoxyadenosylcobalamin in a total volume of 0.5 ml. The reaction was started by the addition of the cobamide coenzyme after a 3 min preincubation. The content of the reference cuvette was identical except for the omission of GTP. O—O, thioredoxin system of L. leichmannii; Δ—Δ, thioredoxin system of E. coli.

Amino-acid analysis of thioredoxin. The amino-acid analysis is shown in Table IV.4, together with that of E. coli thioredoxin. Tryptophan was not determined. A molecular weight of 12,000 was used to calculate the number of residues per mole. No corrections were made for possible losses during the estimation.

Fluorescence of thioredoxin. The emission spectrum of L. leichmannii thioredoxin was determined on both the oxidised and reduced forms of the protein with excitation at 280 mμ (Stryer et al., 1967). The increase in emission of fluorescence when the thioredoxin was reduced with dithiothreitol is shown in Fig. IV.9. Fig. IV.9A shows the emission spectra of oxidised and reduced thioredoxin of L. leichmannii, and Fig. IV.9B that of E. coli. Although the difference between the reduced and oxidised spectra of the latter protein is not so large as that reported by Stryer et al. (1967), it is still considerably greater than that observed with the L. leichmannii enzyme.

The effect of increasing thiol concentration on the rate of ribonucleotide reduction. Fig. IV.10 shows the rates of GTP reduction by ribonucleoside triphosphate reductase by the coupled system indicated

TABLE IV.4. Amino-acid analysis of thioredoxin

The values expressed were calculated on the assumption that there was one residue of histidine per 12,000 m.w.

Amino Acid	L. leichmannii	E. coli*
Lysine	7.30 (7)	9.55 (10)
Histidine	1.0 (1)	0.92 (1)
Arginine	2.94 (3)	1.00 (1)
Aspartic Acid	16.70 (17)	15.00 (15)
Threonine	9.95 (10)	5.50 (6)
Serine	2.50 (3)	2.58 (3)
Glutamic Acid	15.50 (16)	8.00 (8)
Proline	5.30 (5)	5.10 (5)
Glycine	6.25 (6)	8.50 (9)
Alanine	3.50 (3)	11.60 (12)
Half Cystine	1.47 (2)	1.65 (2)
Valine	6.8 (7)	4.95 (5)
Methionine	3.71 (4)	0.93 (1)
Isoleucine	5.57 (6)	7.90 (8-9)
Leucine	8.6 (9)	12.60 (13)
Tyrosine	1.55 (2)	1.86 (2)
Phenylalanine	1.62 (2)	3.82 (4)
Tryptophan	-	2 (2)
TOTAL	103	107-108

*Figures published by Holmgren and Reichard (1967).

Values in parentheses are the nearest integers.

by the following equations :

$$\text{NADPH} + \text{Thioredoxin-S}_2 \xrightarrow[\text{Reductase}]{\text{Thioredoxin}} \text{NADPH} + \text{Thioredoxin-SH}_2 \quad (1)$$

$$\text{Thioredoxin(SH)}_2 + \text{GTP} \xrightarrow[\text{Coenzyme } B_{12}]{\text{Ribonucleotide reductase}} \text{Thioredoxin-S}_2 + \text{dGTP} \quad (2)$$

Various non-saturating concentrations of thioredoxin from either L. leichmannii or E. coli were used. Each thioredoxin was tested in the presence of excess thioredoxin reductase from the same organism. Under these conditions the reduction of thioredoxin by NADPH (1) is much faster than reduction of GTP by ribonucleotide reductase (2), so that practically all of the thioredoxin was in the reduced form. It is therefore possible to determine the apparent K_m for reduced thioredoxin in the reduction of ribonucleotides. As shown in Fig. IV.10B, the system exhibited simple Michaelis-Menten (1913) kinetics with thioredoxin from either source. The $K_{m(app)}$ for L. leichmannii thioredoxin was 3×10^{-6} M and for E. coli thioredoxin 4×10^{-6} M. The slight difference in the apparent V_{max} obtained under the experimental conditions employed is probably not significant.

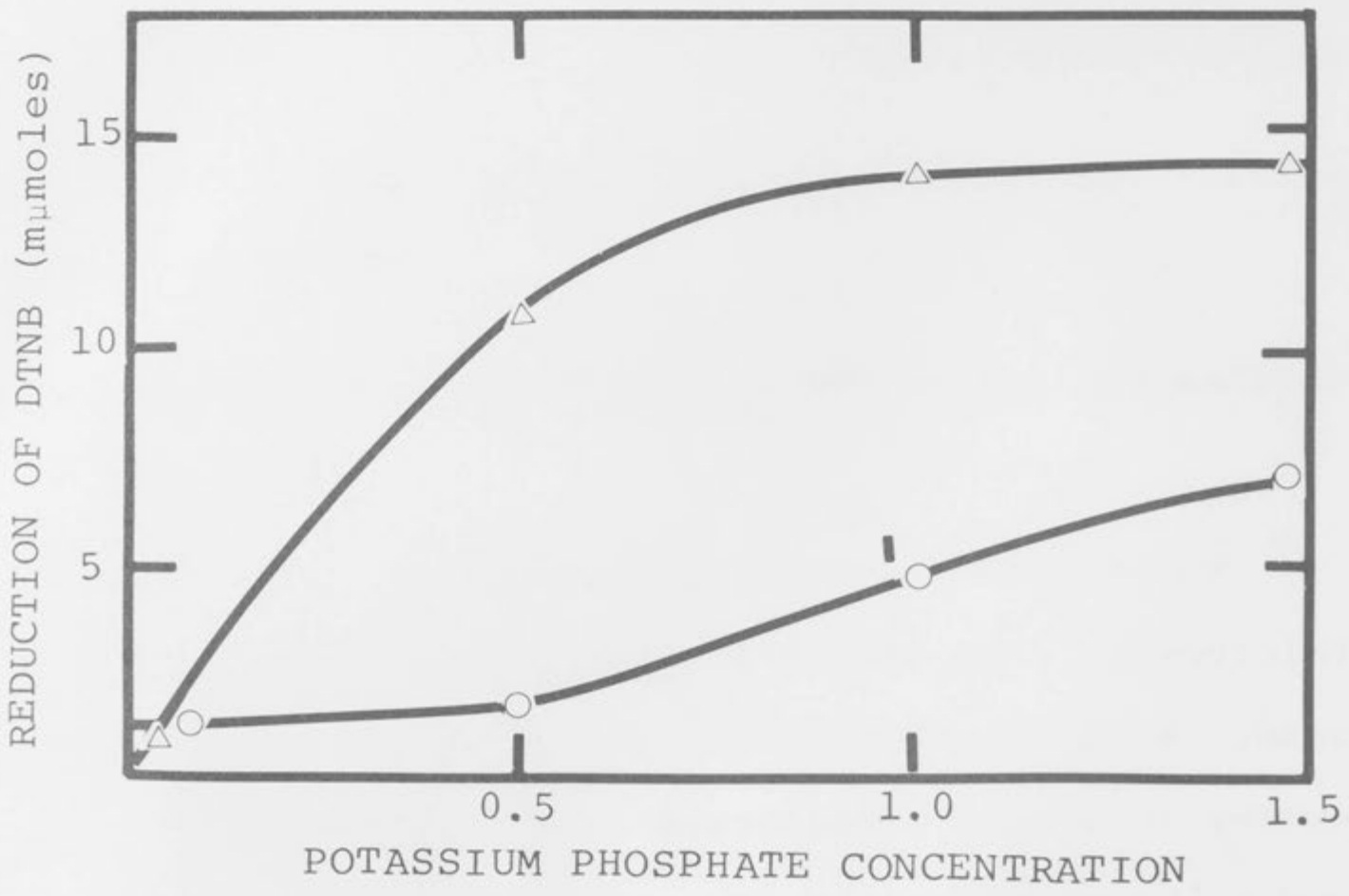


FIG. IV.11. The effect of alkali metal cations on the reduction of DTNB by thioredoxin and thioredoxin reductase.
Standard assay was used with the buffer concentrations shown. Cuvettes contained 5 μg of thioredoxin reductase and 100 mμmoles of NADPH. Thioredoxin (0.2 mμmoles) was omitted from the reference cuvette. DTNB reduction in presence of the complete system ▲—▲; DTNB reduction in the absence of thioredoxin O—O.

DTNB reduction by the thioredoxin systems of *E. coli* and *L. leichmannii*. The activities of the thioredoxin system of each organism are compared in Table IV.5. The results shown in experiment 3 demonstrate that the thioredoxin reductase of *L. leichmannii* can reduce thioredoxin of *E. coli*. Quantitative comparison of the actual rates is not valid as the levels of thioredoxin used may not have been saturating. On the other hand, the thioredoxin reductase of *E. coli*, while showing high activity with its natural substrate (experiment 1), reduces *L. leichmannii* thioredoxin only to a negligible extent. Thus the two thioredoxin reductases exhibit a marked difference in substrate specificity. The significance of the difference in rates of the two complete systems (experiments 1 and 2) cannot be determined from this experiment, as the reductases were of unknown molarity and substrate concentrations may have been non-saturating.

Effect of potassium phosphate on reduction of DTNB by the thioredoxin system of *L. leichmannii*. Increasing potassium phosphate concentrations up to 1.5 M enhance the reduction of DTNB in the system (Fig. IV.11). In addition, the blank rate observed in the absence of

TABLE IV.5. DTNB reduction by the thioredoxin systems of *E. coli* and *L. leichmannii*

Exp.	*E. coli*		*L. leichmannii*		
	Thioredoxin (mμmoles)	Thioredoxin reductase (μg)	Thioredoxin (mμmoles)	Thioredoxin reductase (μg)	DTNB reduction (mμmoles/min)
1	0.4	5			87
2			0.4	5	131
3	0.4			5	58
4		5	0.4		2

The reduction of DTNB was measured at 412 mμ on a Cary Model 15 recording spectrophotometer. The experimental cuvette contained 200 μmoles of potassium phosphate buffer, pH 7.5, 10 μmoles of EDTA, 80 mμmoles of DTNB, 100 mμmoles of NADPH, thioredoxin and thioredoxin reductase as indicated in a total volume of 1.0 ml. The reference cuvette contained an identical mixture, except for the omission of either thioredoxin or thioredoxin reductase.

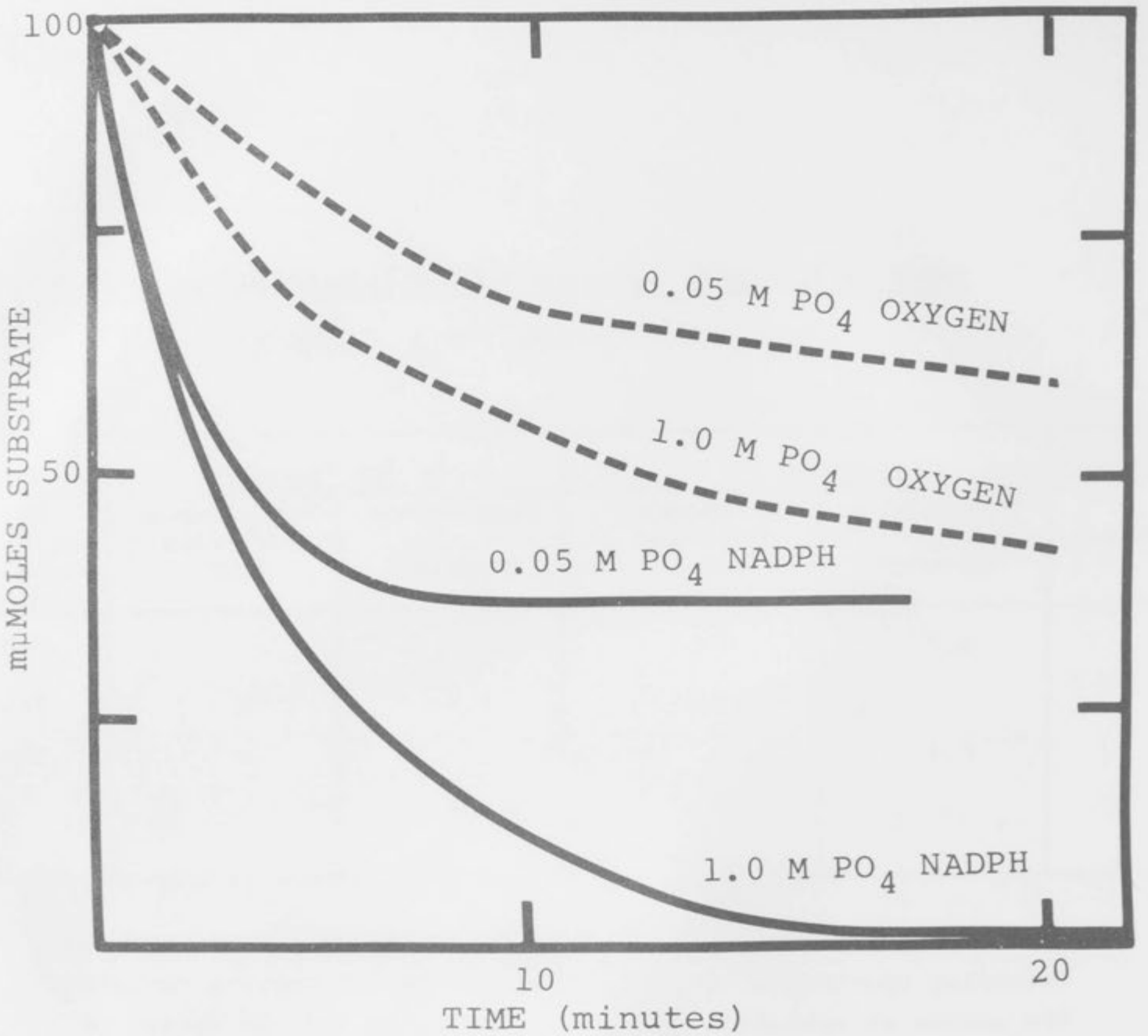


FIG. IV.12. Consumption of NADPH and oxygen in the presence of thioredoxin and thioredoxin reductase.
Absorbance was measured at 340 mμ on a Cary 14 recording spectrophotometer and oxygen consumption measured on an identical solution with the oxygen electrode. Both experiments were performed at 25°. Reaction mixtures contained phosphate buffer in the concentrations shown, together with 50 μg thioredoxin reductase and 100 mμmoles of NADPH per ml.

thioredoxin, increases four-fold between 0.05 M and 1.5 M potassium phosphate. Similar stimulatory effects were observed at high concentrations of sodium acetate and ammonium sulphate (max. 1.5 M).

NADPH oxidase activity of thioredoxin reductase. Consumption of NADPH by thioredoxin reductase in the absence of thioredoxin was significant even in the purest preparations of the reductase, up to 0.36 μmoles of NADPH being consumed per min per mg of protein. Attempts to separate this activity from thioredoxin reductase preparations by chromatography on hydroxy-apatite or by preparative electrophoresis were unsuccessful. These preliminary investigations suggest that consumption of NADPH in the presence and in the absence of thioredoxin is catalysed by the same protein.

This rapid disappearance of NADPH in the presence of the reductase, especially at high concentrations of potassium phosphate (Fig. IV.12), suggested that dissolved oxygen functions as a hydrogen acceptor for NADPH. This was substantiated by the finding that oxygen was consumed from the reaction mixture during NADPH disappearance, this oxygen consumption being dependent on the addition of NADPH (see Table IV.6). At both phosphate buffer concentrations used (0.05 M and 1.0 M) total oxygen consumption was lower than

TABLE IV.6. Oxygen consumption by thioredoxin reductase

The reaction chamber contained 125 µg of thioredoxin reductase (specific activity 1,200), 125 or 2,500 µmoles of potassium phosphate buffer, pH 7.5, and the additions as indicated in a total volume of 2.3-2.5 ml.

Experiment 1 : 0.05 M potassium phosphate

	Additions	Initial velocity mµmoles oxygen consumed/minute
a	None	-
b	NADPH (250 mµmoles)	5
c	NADPH + Catalase*	3
d	NADPH + G6P + G6PD†	5

Experiment 2 : 1.0 M potassium phosphate

	Additions	Initial velocity mµmoles oxygen consumed/minute
a	None	-
b	NADPH (250 mµmoles)	16
c	NADPH + Catalase*	11
d	G6P + G6PD + NADP†	2.5

*100 µg catalase used.

†Glucose-6-phosphate 250 mµmoles and Glucose-6-phosphate dehydrogenase 50 µg.

expected for the usual equation for NADPH oxidase

$$H^+ + NADPH + O_2 \longrightarrow H_2O_2 + NADP^+ \qquad (3)$$

As shown in Fig. IV.12, the NADPH consumption was approximately twice the number of equivalents of oxygen consumed. This apparent discrepancy may be explained if heavy metal ions present in the phosphate buffer catalyse the rapid decomposition of H_2O_2

$$2H_2O_2 \xrightarrow{Fe^{2+}\ Cu^{2+}} 2H_2O + O_2 \qquad (4)$$

Under these conditions, the total oxygen consumption would be half that predicted by the oxidase equation (3). Addition of catalase to the reaction mixture after the reaction had proceeded for 20 minutes liberated oxygen equivalent to between 25 and 40% of the oxygen consumed in the presence of 0.05 M potassium phosphate, but negligible amounts (<5%) in the presence of 1.0 M phosphate.

At low phosphate concentration, both NADPH and oxygen consumption cease after about 10 to 15 minutes (Fig. IV.12), despite the presence of NADPH and oxygen. The reaction continues for a longer period at high phosphate concentrations or in the presence of catalase. These observations suggest that inactivat-

ion of the enzymes, perhaps by peroxide, causes cessation of the reaction.

Discussion

The ability of thioredoxin and thioredoxin reductase, isolated as described in the previous section, to replace the corresponding two proteins from *E. coli* in ribonucleoside triphosphate reduction with a slightly lower $K_{m(app)}$ provides strong evidence that these two proteins, together with NADPH, constitute the physiological hydrogen donor system for this reaction. The activity of *E. coli* thioredoxin with *L. leichmannii* thioredoxin reductase also indicates some similarity between the two substrates. However, it is not surprising that thioredoxin of *L. leichmannii* does not serve as a substrate for *E. coli* thioredoxin reductase. The different chromatographic behaviour of thioredoxin from the two sources on ion-exchange columns and the difference in the ratio of acidic to basic amino acid residues indicate that *L. leichmannii* thioredoxin is by far the more negatively charged. Furthermore, the fluorescence data indicates that the amino-acid sequences at the reactive disulphide are different in the two proteins.

The kinetic double reciprocal plots of velocity of ribonucleotide reduction as a function of reduced

thioredoxin concentration are clearly linear for the thioredoxin isolated from either organism. This result differs from the findings of Beck *et al.* (1966) who reported non-linear kinetics for this reaction with *E. coli* thioredoxin as substrate. In view of the fit of the data to the computed lines, we conclude that the kinetics of this reaction are unireactant under the experimental conditions used.

The increased rate of reduction of DTNB by the thioredoxin system in the presence of high phosphate concentrations possibly reflects a change in the conformation of the reduced protein which facilitates exposure of the sulphydryl groups. A similar effect was observed with ribonucleotide reductase (c.f. Chapter III). As will be discussed later, the presence of heavy-metal ion contamination in the phosphate will catalyse H_2O_2 destruction, a factor which may play a part in the enhanced reaction at high phosphate concentrations.

The elucidation of methods for purification of these proteins to electrophoretic purity of greater than 90% will permit further studies on chemical structure. The material of low mobility observed on the analytical polyacrylamide-gel electrophoresis of purified thioredoxin reductase is produced during the

analytical electrophoresis. The absence of proteins of intermediate mobility which were present in the sample subjected to preparative electrophoresis indicates that the material of low mobility was not present in the fractions eluted from the preparative gel. The formation of this type of aggregate in the Tris-glycine buffer system has been discussed in Chapter II.

Thioredoxin reductase. Detection of activity in crude extracts was hampered by the presence of the oxidase activity. A major difference in the isolation of thioredoxin reductase from the two organisms is the inability of the L. leichmannii reductase to separate from thioredoxin during acid precipitation. This difficulty possibly arises from the lower isoelectric point of L. leichmannii thioredoxin reductase indicated by the observed behaviour on DEAE-Sephadex.

The elution of two peaks of thioredoxin reductase activity from Sephadex G-100 with different elution volumes indicates the presence of species of differing molecular weight. The molecular sizes corresponding with these elution volumes are compatible with the view that the first and second peaks represent dimer and monomer, respectively. Thelander (1968) has shown that thioredoxin reductase of E. coli consists of two

identical subunits which are bound by non-covalent linkage, and which each possess a catalytic site that includes one molecule of FAD and one oxidisible disulphide bridge. Denaturing conditions (urea, guanidine) are required to disaggregate the dimer and cause dissociation from FAD. If the two species of the reductase from L. leichmannii extracts also represent dimer and monomer forms, it would seem that inter-protomer bonding is much weaker than in the corresponding protein from E. coli. Despite the apparent ease of disaggregation, addition of FAD or FMN to either of the forms did not increase their activity, an observation indicating that dissociation of the flavin is absent or slight.

If the monomer reductase isolated from Sephadex G-100 chromatography is subjected to ion-exchange chromatography at pH 7.5, a single, homogeneous species can be purified by preparative polyacrylamide-gel electrophoresis. However, when the monomer is chromatographed on DEAE-Sephadex at pH 4.5, material of high specific activity is obtained, but preparative polyacrylamide-gel electrophoresis of this material separates two peaks of activity, the elution patterns of which are compatible with monomer and dimer forms.

This may mean that exposure to acid conditions promotes aggregation.

The preliminary investigation of purified thioredoxin reductase indicates that this enzyme can catalyse the oxidation of NADPH in the absence of thioredoxin albeit at a lower rate. In the reaction occuring under these conditions, oxygen is consumed and hydrogen peroxide is formed, as indicated by the evolution of oxygen in the presence of catalase. Especially at low potassium phosphate concentrations, the enzyme appears to be subject to inactivation by peroxide. This inactivation is less marked in the presence of high potassium phosphate concentrations, perhaps because of heavy-metal ion contaminants of the phosphate that catalyse the rapid destruction of enzymically-formed peroxide.

Thioredoxin. The molecular weight of this protein (12,000) corresponds closely to that of the protein isolated from E. coli. The small size of this protein was emphasised by the losses sustained during dialysis in thin-walled sacs.

Amino-acid analysis of thioredoxin indicates that for molecular weight 12,000, the molecule contains only two half cystine residues. As would be expected from the behaviour on DEAE-Sephadex chromatography,

the L. leichmannii thioredoxin contains more acidic and fewer basic amino acids than its E. coli counterpart. The sequence of the catalytic site of E. coli thioredoxin is

31	32	33	34	35	36	37
Trp	- Cys	- Gly	- Pro	- Cys	- Lys	- Homoserine

(Holmgren and Reichard, 1967). The proximity of tryptophan to the active disulphide bridge is manifest in a marked (2 to 2.5-fold) change in intensity of fluorescence emission on conversion of the oxidised protein to its reduced form (Stryer et al., 1967). In the case of L. leichmannii thioredoxin, there is only a small increase (0.15-0.2 fold) in fluorescence on reduction. This probably indicates that in this protein a tryptophan residue is not adjacent to either residue of the disulphide bridge. These preliminary indications of differences between the thioredoxins isolated from two bacteria are in contrast to the similarity of the primary amino-acid sequence of many proteins (e.g. cytochrome C) isolated from widely different sources.

Summary

Thioredoxin and thioredoxin reductase, the two proteins coupling NADPH to ribonucleotide reduction

in L. leichmannii, have been isolated and purified. Limited cross reactions occur between thioredoxin and thioredoxin reductase isolated from L. leichmannii and from E. coli, but the specificity of E. coli thioredoxin reductase for its native substrate indicates the dissimilarity of the two protein substrates. This has been substantiated by the comparison of the amino acid content of the two proteins and by their respective changes in fluorescence intensity on reduction.

Thioredoxin reductase from L. leichmanni is able to oxidise NADPH in the absence of added hydrogen acceptor, during which time oxygen is consumed from the reaction mixture. This oxidase activity is present even in the purest preparations, and both activities appear to be present on the same protein.

Reduced thioredoxin, the natural substrate for ribonucleoside triphosphate reductase, exhibits unireactant kinetics for this reaction under the conditions investigated.

REFERENCES

Andrews, P. (1964), Biochem. J., 91, 222.

Andrews, P. (1965), Biochem. J., 96, 595.

Asahi, T., Bandurski, R.S., and Wilson, L.G. (1961), J. Biol. Chem., 240, 1830.

Atkinson, D.E. (1966), Ann. Rev. Biochem., 35, 85.

Atkinson, D.E. (1968), Biochemistry, 7, 4030.

Baginsky, M.L., and Huennekens, F.M. (1966), Biochem. Biophys. Res. Commun., 23, 600.

Baldwin, R.L. (1954), J. Am. Chem. Soc., 76, 402.

Baldwin, R.L. (1959), J. Phys. Chem., 63, 1570.

Bandurski, R.S., Wilson, L.G., and Asahi, T. (1960), J. Am. Chem. Soc., 82, 3218.

Bates, R.G., and Hetzer, H.B. (1961), J. Phys. Chem., 65, 667.

Beck, W.S. (1967), J. Biol. Chem., 242, 3148.

Beck, W.S., Goulian, M., Larsson, A., and Reichard, P. (1966), J. Biol. Chem., 241, 2177.

Bell, K., McKenzie, H.A., and Shaw, D.C. (1968), Biochim. Biophys. Acta, 154, 284.

Benesch, R., and Benesch, R.E. (1962), Meth. Biochem. Analysis, 10, 43.

Bethell, M.R., Fellenberg, F.V., Jones, M.E., and Levine, L. (1968a), Biochemistry, 7, 4315.

Bethell, M.R., Smith, K.E., White, J.S., and Jones, M.E. (1968b), Proc. Natl. Acad. Sci., U.S., 60, 1442.

Bjerrum, J., and Nielsen, E.J. (1948), Acta Chem. Scand., 2, 297.

Black, S., Harte, E.C., Hudson, B., and Wartofsky, L. (1960), J. Biol. Chem., 235, 2910.

Blakley, R.L. (1965), J. Biol. Chem., 240, 2173.

Blakley, R.L. (1966a), Fedn. Proc. Fedn. Am. Socs. Exp. Biol., 25, 1633.

Blakley, R.L. (1966b), J. Biol. Chem., 241, 176.

Blakley, R.L., and Barker, H.A. (1964), Biochem. Biophys. Res. Commun., 16, 391.

Blakley, R.L., Ghambeer, R.K., Nixon, P.F., and Vitols, E. (1965), Biochem. Biophys. Res. Commun., 20, 439.

Blakley, R.L., and Vitols, E. (1968), Ann. Rev. Biochem., 37, 201.

Blomendahl, H. (1967) In : Electrophoresis : Theory, Methods and Applications, Vol. II, ed. Milan Bier, Academic Press, New York and London, p.379.

Boyer, P.D. (1954), J. Am. Chem. Soc., 76, 4331.

Bray, G.A. (1960), Analyt. Biochem., 1, 279.

Brewer, J.M. (1967), Science, 156, 256.

Brown, N.C., Eliasson, R., Reichard, P., and Thelander, L. (1968), Biochem. Biophys. Res. Commun., 30, 522.

Brown, N.C., Larsson, A., and Reichard, P. (1967), J. Biol. Chem., 242, 4272.

Cecil, R., and Ogston, A.G. (1951), J. Scient. Instrum., 28, 253.

Changeux, J.-P., Gerhart, J.C., and Schachman, H.K. (1968), Biochemistry, 7, 531.

Changeux, J.-P., and Rubin, M.M. (1968), Biochemistry, 7, 553.

Clarke, K., and Rothwell, K. (1960), J. Chem. Soc., 1885.

Colman, R.F., and Black, S. (1965), J. Biol. Chem., 240, 1796.

Cowles, J.R., and Evans, H.J. (1968), Arch. Biochem. Biophys., 127, 770.

Creeth, J.M., and Pain, R.H. (1967), Prog. in Biophys., 17, 219.

Crestfield, A.M., Moore, S., and Stein, W.H. (1963), J. Biol. Chem., 238, 622.

Datta, S.P., and Grzybowski, A.K. (1958), Trans. Faraday Soc., 54, 1179.

Davis, B.J. (1964), Ann. N.Y. Acad. Sci., 121, 404.

Determann, H. (1966), Protides of Biol. Fluids, 14, 563.

Dinning, J.S., and Spell, W.H. (1959), J. Am. Chem. Soc., 81, 3804.

Downing, M., and Schweigert, B.S. (1956), J. Biol. Chem., 220, 521.

Ellman, G.L. (1959), Arch. Biochem. Biophys., 82, 70.

Fantes, K.H., and Furminger, I.G.S. (1967), Nature, 215, 750.

Fellenberg, R.V., Bethell, M.R., Jones, M.E., and Levine, L. (1968), Biochemistry, 7, 4322.

Frieden, C. (1964), J. Biol. Chem., 239, 3522.

Frieden, C. (1967), J. Biol. Chem., 242, 4045.

Fruchter, R.G., and Crestfield, A.M. (1967), J. Biol. Chem., 242, 5807.

Fujita, H. (1959), J. Phys. Chem., 63, 1092.

Gerhart, J.C. (1964), Brookhaven Symp. Biol., 17, 232.

Gerhart, J.C., and Holoubek, H. (1967), J. Biol. Chem., 242, 2886.

Gerhart, J.C., and Pardee, A.B. (1962), J. Biol. Chem., 237, 891.

Gerhart, J.C., and Schachman, H.K. (1965), Biochemistry, 4, 1054.

Gerhart, J.C., and Schachman, H.K. (1968), Biochemistry, 7, 538.

Ghambeer, R.K., and Blakley, R.L. (1966), J. Biol. Chem., 241, 4710.

Goldberg, R.J. (1953), J. Phys. Chem., 57, 194.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S., and Singh, R.M.M. (1966), Biochemistry, 5, 467.

Gordon, A.H., and Louis, L.N. (1967), Analyt. Biochem., 21, 190.

Gornall, A.C., Bardawill, C.J., and David, M.M. (1949), J. Biol. Chem., 177, 751.

Goulian, M., and Beck, W.S. (1966), J. Biol. Chem., 241, 4233.

Haber, J.E., and Koshland, D.E. (1967), Proc. Natl. Acad. Sci., U.S., 58, 2087.

Hammarsten, E., Reichard, P., and Saluste, E. (1950), J. Biol. Chem., 183, 105.

Herve, G.L., and Stark, G.R. (1967), Biochemistry, 6, 3743.

Hirs, C.H.W. (1956), J. Biol. Chem., 219, 611.

Hirs, C.H.W., Moore, S., and Stein, W.H. (1956), J. Biol. Chem., 219, 623.

Hogenkamp, H.P.C., Ghambeer, R.K., Brownson, C., and Blakley, R.L. (1967), Biochem. J., 103, 5c.

Holmgren, A., Reichard, P., and Thelander, L. (1965), Proc. Natl. Acad. Sci., U.S., 54, 830.

Holmgren, A., and Reichard, P. (1967), Europ. J. Biochem., 2, 187.

Holmgren, A. (1968), Europ. J. Biochem., 5, 359.

Hummel, J.P., and Dreyer, W.J. (1962), Biochim. Biophys. Acta, 63, 530.

Ingram, L., Tombs, M.P., and Hurst, A. (1967), Analyt. Biochem., 20, 24.

Johnston, J.P., and Ogston, A.G. (1946), Trans. Faraday Soc., 42, 789.

Jovin, T., Chrambach, A., and Naughton, M.A. (1964), Analyt. Biochem., 9, 351.

Kaufman, B.T. (1966), Proc. Natl. Acad. Sci., U.S., 56, 695.

Kawahara, K., and Tanford, C. (1966), J. Biol. Chem., 241, 3228.

Kirtley, M.E., and Koshland, D.E. (1967), J. Biol. Chem., 242, 4192.

Kitay, E., McNutt, W.S., and Snell, E.E. (1950), J. Bacteriol., 59, 727.

Klein, S.M., and Sagers, R.D. (1967), J. Biol. Chem., 242, 297.

Koshland, D.E., and Neet, K.E. (1968), Ann. Rev. Biochem., 37, 359.

Koshland, D.E., Nemethy, G., and Filmer, D. (1966), Biochemistry, 5, 365.

Larsson, A., and Neilands, J.B. (1966), Biochem. Biophys. Res. Commun., 25, 222.

Larsson, A., and Reichard, P. (1967), Progr. Nucleic Acid Res., 7, 303.

Laurent, T.C., Moore, E.C., and Reichard, P. (1964), J. Biol. Chem., 239, 3436.

Leach, A.A., and O'Shea, P.C. (1965), J. Chromat., 17, 245.

McClintock, D.K., and Marcus, G. (1968), J. Biol. Chem., 243, 2855.

McClintock, D.K., and Marcus, G. (1969), J. Biol. Chem., 244, 36.

Manson, L.A. (1960), J. Biol. Chem., 235, 2955.

Margoliash, E. (1962), J. Biol. Chem., 237, 2161.

Margolis, J., and Kenrick, K.G. (1967), Nature, 214, 1334.

Massey, V., and Williams, C.H. (1965), J. Biol. Chem., 240, 4470.

Michaelis, L., and Menten, M.L. (1913), Biochem. Z., 49, 333.

Mickel, B.L., and Andrews, A.C. (1955), J. Am. Chem. Soc., 77, 5291.

Mitchell, W.M. (1967), Biochim. Biophys. Acta, 147, 171.

Monod, J., and Jacob, F. (1961), Cold Spring Harb. Symp. Quant. Biol., 26, 389.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), J. Mol. Biol., 12, 88.

Moore, E.C. (1967), Biochem. Biophys. Res. Commun., 29, 264.

Moore, E.C., and Hurlbert, R.B. (1966), J. Biol. Chem., 241, 4802.

Moore, E.C., and Reichard, P. (1964), J. Biol. Chem., 239, 3453.

Moore, E.C., Reichard, P., and Thelander, L. (1964), J. Biol. Chem., 239, 3445.

Morris, C.J.O.R. (1966), Protides of Biol. Fluids, 14, 543.

Morrison, J.F. (1965), Aust. J. Sci., 27, 317.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1967), Biochemistry, 6, 2449.

Nierlich, D.P., and Magasanik, B. (1965), J. Biol. Chem., 240, 358.

Nishihara, M., Chrambach, A., and Aposhian, H.V. (1967), Biochemistry, 6, 1877.

Oncley, J.L., Scatchard, G., and Brown, A. (1947), J. Phys. Colloid Chem. (now J. Phys. Chem.), 51, 184.

Ornstein, L. (1964), Ann. N.Y. Acad. Sci., 121, 321.

Orr, M.D., and Vitols, E. (1966), Biochem. Biophys. Res. Commun., 25, 109.

Oster, G. (1954), Nature, 173, 300.

Oster, G.K., Oster, G., and Prati, G. (1957), J. Am. Chem. Soc., 79, 595.

Perkins, J., and Bertino, J.R. (1964), Biochem. Biophys. Res. Commun., 15, 121.

Perkins, J.P., and Bertino, J.R. (1965), Biochemistry, 4, 847.

Perrin, D.D. (1965) In : Dissociation Constants of Organic Bases in Aqueous Solution, Butterworths, London.

Phelps, R.A., and Putnam, F.W. (1960) In : The Plasma Proteins, Vol. 1, ed. Putnam, F.W., New York, Academic Press Inc., p.171.

Raftery, M.A., and Cole, R.D. (1966), J. Biol. Chem., 241, 3457.

Raymond, S., and Weintraub, L. (1959), Science, 130, 711.

Reyes, P., and Huennekens, F.M. (1967), Biochem. Biophys. Res. Commun., 28, 833.

Rose, I.A., and Schweigert, B.S. (1953), J. Biol. Chem., 202, 635.

Sanwal, B.D., and Cook, R.A. (1966), Biochemistry, 5, 886.

Schachman, H.K. (1957) In : Methods in Enzymology, eds. Colowick, S.P. and Kaplan, N.O. New York, N.Y., Academic Press, Vol. IV, p.32.

Schachman, H.K. (1963), Cold Spring Harb. Symp. Quant. Biol., 28, 409.

Schachman, H.K., and Edelstein, S.J. (1966), Biochemistry, 5, 2681.

Shive, W., Sibley, M.E., and Rogers, L.L. (1951), J. Am. Chem. Soc., 73, 867.

Shuster, L., and Schrier, B.K. (1967), Analyt. Biochem., 19, 280.

Signer, R., and Gross, H. (1934), Helv. Chim. Acta, 17, 726.

Snoswell, A.M. (1966), Biochemistry, 5, 1660.

Spackman, D.H., Stein, W.H., and Moore S. (1958), Anal. Chem., 30, 1190.

Stryer, L., Holmgren, A., and Reichard, P. (1967), Biochemistry, 6, 1016.

Svedberg, T., and Pedersen, K.O. (1940) In : The Ultracentrifuge, London, Oxford University Press (Johnston Reprint Corp., New York, N.Y.), p.22.

Sweeny, J.R., and Fisher, J.R. (1968), Biochemistry, 7, 561.

Tanford, C. (1961), Physical Chemistry of Macromolecules, John Wiley & Sons, Inc., New York, p.147.

Thelander, L. (1967), J. Biol. Chem., 242, 852.

Thelander, L. (1968), Europ. J. Biochem., 4, 407.

Umbarger, H.E. (1956), Science, 123, 848.

Van Holde, K.E. (1960), J. Phys. Chem., 64, 1582.

Vitols, E., Brownson, C., Gardiner, W., and Blakley, R.L. (1967a), J. Biol. Chem., 242, 3035.

Vitols, E., Hogenkamp, H.P.C., Brownson, C., Blakley, R.L., and Connellan, J. (1967b), Biochem. J., 104, 58c.

Warner, R.C. (1954) In : The Proteins, Vol. 2, part A, eds., Neurath, H. and Bailey, K., New York, N.Y., Academic Press, p.435.

Weber, K. (1968a), J. Biol. Chem., 243, 543.

Weber, K. (1968b), Nature, 218, 1116.

Wiley, D.C., and Lipscomb, W.N. (1968), Nature, 218, 1119.

Williams, C.H., Zanetti, G., Arscott, L.D., and McAllister, J.K. (1967), J. Biol. Chem., 242, 5226.

Williams, D.E., and Reisfeld, R.A. (1964), Ann. N.Y. Acad. Sci., 121, 373.

Winzor, D.J., and Scheraga, H.A. (1963), Biochemistry, 2, 1263.

Worcel, A., Goldman, D.S., and Cleland, W.W. (1965), J. Biol. Chem., 240, 3399.

Wu, Y.U., and Scheraga, H.A. (1962), Biochemistry, 1, 698.

Yates, R.A., and Pardee, A.B. (1956), J. Biol. Chem., 221, 757.

Yphantis, D.A. (1964), Biochemistry, 3, 297.

Zanetti, G., Williams, C.H., and Massey, V., J. Biol. Chem., 243, 4013.