STRUCTURAL STUDIES ON PYRUVATE KINASE

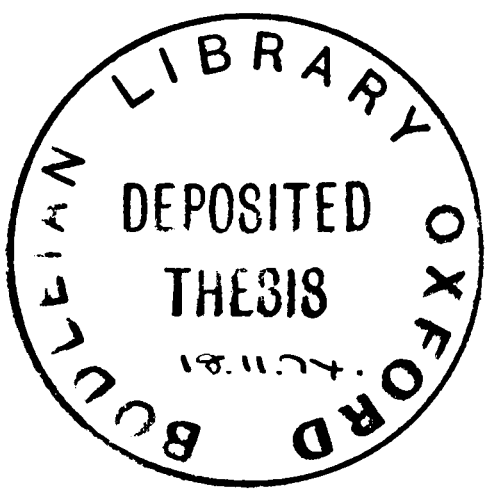
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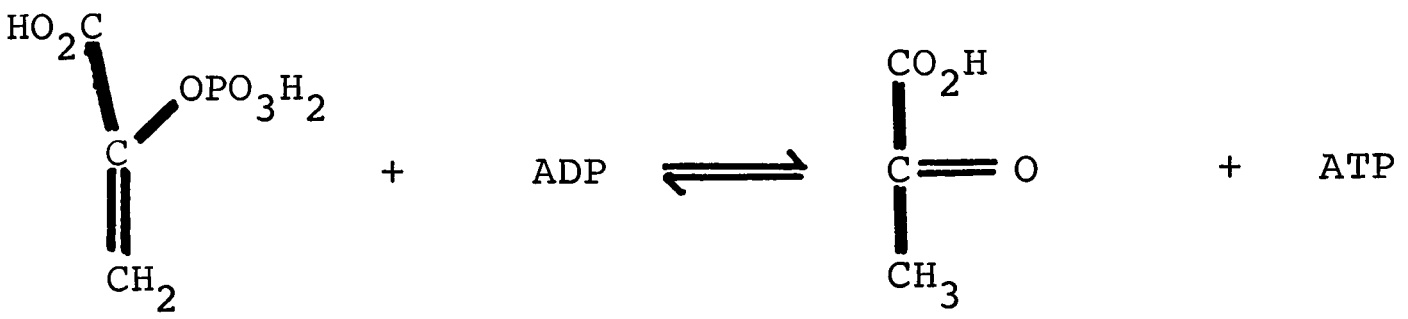
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CHAPTER 1

INTRODUCTION

a) Background to the Present Work

The enzyme pyruvate kinase (E.C. 2.7.1.40) catalyses the following step in glycolysis:



Phosphoenolpyruvate Pyruvate

A divalent metal ion (Mg^{2+}) and a monovalent cation (K^{+}) are essential for activity. The equilibrium constant for the reaction is of the order of 10^{3} at pH 7, and the reversal of this step is not considered to occur to a significant degree *in vivo*.

Pyruvate kinase from brewers' yeast (*Saccharomyces cerevisiae*) had previously been selected for study in this laboratory since it exhibits co-operative kinetics with respect to the substrate phosphoenolpyruvate and is activated by the glycolytic intermediate fructose diphosphate (Hess *et al*, 1966b; Hunsley and Suelter, 1969b), that is, it exhibits the homotropic and heterotropic interactions characteristic of an allosteric enzyme (Monod *et al*, 1963). The intention behind the present investigation was to study the binding of ligands by the enzyme and conformational changes induced by the ligands to attempt to gain an insight into the molecular mechanism of an allosteric enzyme. Yeast pyruvate kinase seemed to be a particularly suitable choice since the enzyme from rabbit muscle, which does

not exhibit homotropic interactions in the dependence of enzymic activity on phosphoenolpyruvate concentration nor heterotropic activation by fructose diphosphate, had already been studied extensively. Magnetic resonance techniques appeared to be a particularly suitable tool for these investigations, both because the results with the rabbit muscle enzyme were available for comparison, and because magnetic resonance techniques are potentially sensitive to perturbations of low energy, and allosteric interactions may require only a small coupling energy.

Ashton (1971) had developed a method for the preparation of pyruvate kinase from S. cerevisiae in this laboratory. She had found that the method devised by Haeckel et al. (1968) for the preparation of the enzyme from S. carlsbergensis was unsuitable with S. cerevisiae, as the enzyme from the latter source was cold labile, whilst that from the former was not (Hess, personal communication). Hunsley and Suelter (1969a) had devised a method for the preparation of pyruvate kinase from S. cerevisiae; they had also found the enzyme to be cold-labile, and had stabilised it during preparation at 4°C by the use of solutions containing 50% (v/v) glycerol. Ashton's preparation was more convenient since it was performed at room temperature and was relatively rapid. She reported (1971) that the enzyme thus obtained was homogenous by electrophoresis, although it showed evidence of both dissociation (which was enhanced at 4°C) and aggregation in sedimentation velocity experiments. The enzyme could be stabilised in 100 mM fructose diphosphate at 4°C, and this solvent was used for sedimentation equilibrium experiments which established that the protein was monodisperse with a molecular weight of 162,000. This was in close agreement with a molecular weight of 168,000 obtained by

Kuczenski and Suelter (1970b) for pyruvate kinase prepared from S. cerevisiae by the method of Hunsley and Suelter (1969a). The molecular weight of the enzyme from S. carlsbergensis was reported to be 191,000 (Bischofberger et al, 1971). Ashton (1971) found a subunit molecular weight of 20,000 for the maleylated enzyme in 6 M guanidinium chloride by sedimentation equilibrium and osmotic pressure, and a single band for the reduced protein in SDS gel electrophoresis with a mobility corresponding to a molecular weight of 19,000. However, Kuczenski and Suelter (1970b) found the subunit molecular weight of pyruvate kinase from S. cerevisiae to be 42,000 to 45,000 by sedimentation equilibrium in 6 M guanidinium chloride plus 1% 2-mercaptoethanol. Ashton (1971) claimed that this solvent did not fully dissociate the enzyme, since she obtained a polydisperse solution in this solvent. Bischofberger et al. (1971) found that the pyruvate kinase from S. carlsbergensis contained four subunits of molecular weight 45,000-51,000 in a variety of solvents, (although in an earlier paper, Bischofberger et al. 1970, they had reported a subunit molecular weight of 62,000). The specific activity of the enzyme from all three sources was of the order of 200 U/mg, but the value of the specific extinction coefficient, $E^{0.1\%}_{1\ cm}$, at 280 nm was 0.65 $ml.mg^{-1}.cm^{-1}$ for both preparations from S. cerevisiae, (Hunsley and Suelter, 1969a; Ashton, 1971) whilst that for the pyruvate kinase from S. carlsbergensis was 0.76 (Haeckel et al, 1968). It therefore seemed that there were significant differences between pyruvate kinases from S. cerevisiae and S. carlsbergensis, but that both preparations from the former source had similar properties, except that there was disagreement over the molecular weight of the subunits.

b) The Role of Pyruvate Kinase in the Regulation of Metabolism

Observations of metabolite levels in glycolysing cells have demonstrated that the pyruvate kinase reaction is significantly displaced from equilibrium, which would suggest that it is a rate-limiting step in this pathway (the evidence is reviewed by Newsholme and Start, 1973). However, the maximum activity of pyruvate kinase present in the tissues is at least an order of magnitude greater than the lowest enzymic activities in the glycolytic pathway, which argues against a role as a control enzyme in glycolysis. The concentrations of phosphoenolpyruvate in the cell are below the K_m of the enzyme for this substrate (e.g. 10-20 μM in muscle compared with 100 μM; Newsholme and Start, 1973), and *in vivo* concentrations of ATP should be sufficient to cause some inhibition (K_i for the muscle enzyme is about 3.5 mM; Boyer, 1962). Although the enzyme may be working below its maximum capacity under these conditions, the activity is probably not regulated by these factors because ATP levels are maintained comparatively constant in the cell, and there is no significant change in phosphoenolpyruvate concentration when the rate of glycolysis of blowfly flight muscle is increased one hundred fold (Newsholme and Start, 1973). Hess *et al*. (1966b) proposed that pyruvate kinase was rate-limiting in glycolysis in yeast since a cross-over point was observed between phosphoenolpyruvate and pyruvate in oscillating glycolysis generated in intact cells (Hommes, 1964) and cell-free extracts (Hess *et al*. 1966a). A cross-over point at pyruvate kinase was also observed in glycolytic oscillations in bovine heart preparations (Chance *et al*, 1965). However, the glycolytic oscillations are primarily generated by phosphofructokinase and are propagated through the

rest of the pathway by coupling with co-factors. The cross-over point at pyruvate kinase may therefore be caused by oscillation in the concentration of the other substrate ADP, which is 180^{o} out of phase with the variations in the concentration of phosphoenolpyruvate (Hess and Boiteux, 1971). Further, it is not clear whether oscillations occur in physiological conditions.

Inhibition of rabbit muscle pyruvate kinase by creatine phosphate has recently been reported (Kemp, 1973), and since the concentration of this metabolite in muscle at rest is of the order of 20 mM, whilst it drops sharply in muscular work, and the K_i is about 2 mM, the effect could well be of physiological significance.

Control of glycolysis by Ca^{2+} inhibition of pyruvate kinase has been proposed (e.g. Gevers and Krebs, 1966), but there is no convincing evidence of relevance in vivo, and Vaughan et al. (1973) have discounted the possibility of such an effect in muscle, where it could have functioned to co-ordinate glycolysis and muscular contraction.

Thus, in summary, there is at present no definitive evidence for a role for pyruvate kinase in the regulation of glycolysis itself, and certainly, since the control of this enzyme could only affect the concentrations of the intermediates back to phosphofructokinase and not the rate of glucose uptake, there would seem to be little that could be gained by such control.

On the other hand, there is considerable evidence to show that the control of pyruvate kinase activity occurs in gluconeogenesis, when the conversion of pyruvate to phosphoenolpyruvate is effected by the enzymes pyruvate carboxylase and

phosphoenolpyruvate carboxykinase:

$$\text{Pyruvate} + CO_2 + \text{ATP} \longrightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

$$\text{oxaloacetate} + \text{ITP} \longrightarrow \text{phosphoenolpyruvate} + \text{IDP} + CO_2.$$

Thus in the yeasts Rhodoturula Glutinis and Candida Utilis, pyruvate kinase, which is not activated by fructose diphosphate, is induced by growth on glucose, but is repressed when gluconeogenesis occurs, in growth on aspartate for example (Fernandez et al. 1967; Gancedo et al. 1967). In S. cerevisiae, however, although the content of the fructose diphosphate-sensitive pyruvate kinase during growth on a gluconeogenic substrate is about one-third of that in growth supported by glycolysis, the maximum activity of the enzyme is still high in relation to the rate of gluconeogenesis (Barwell and Hess, 1971). These authors also demonstrated that the concentration of fructose diphosphate in gluconeogenesis is about 100-fold lower than in glycolysis, so that, at the low concentration of phosphoenolpyruvate present in the cell, the enzyme would have been inhibited. Further, the phosphoenolpyruvate concentration was lower in glycolysis, when the flux through pyruvate kinase was higher, than in gluconeogenesis, thus indicating the control of this step. There is also a need for inhibition of pyruvate kinase in the gluconeogenic tissues of mammals during gluconeogenesis, as the activity of the enzyme is an order of magnitude greater than those of pyruvate carboxylase and phosphoenolpyruvate carboxykinase, the rate-limiting enzymes of gluconeogenesis (Scrutton and Utter, 1968). Significantly, there appears to be a correlation between the presence in mammalian tissues of at least one of the isoenzymes of pyruvate kinase which are activated by fructose diphosphate and the presence of phosphoenolpyruvate carboxykinase (Carbonell

et al. 1973). These authors consider that the inhibition of pyruvate kinase by phenylalanine (e.g. Rozengurt et al. 1970) is non-physiological, whilst the inhibition of the liver and adipose tissue isoenzymes by alanine could be related to the flux of alanine from muscle to liver for gluconeogenesis in starvation.

c) Properties of Pyruvate Kinase

The literature on the properties of pyruvate kinase has recently been reviewed by Kayne (1973), and previously by Boyer (1962), so an extensive bibliography will not be given here. A substantial proportion of the work has been on the rabbit muscle enzyme, since it is readily prepared (Bücher and Pfleiderer, 1955) and is stable, whereas the enzymes from sources other than mammalian muscle have often proved to be unstable (see Chapter 6).

(i) Molecular Weight and Subunits

Rabbit muscle pyruvate kinase has a native molecular weight of 237,000 (Warner, 1958) and is composed of four subunits of molecular weight 57,000 (Steinmetz and Deal, 1966). The number of tryptic peptides is that expected for 4 identical subunits (Cottam et al, 1969), and it is now established that there are four sites per mole active in binding substrates and co-factors, and not just two sites as previously thought (see Kayne, 1973, for discussion). The uncertainty about the molecular weight of pyruvate kinase from yeast has already been referred to, and will be the subject of further discussion in this thesis. A Table of molecular weights for the enzymes from other sources is given in Chapter 6 (Table 6.2).

(ii) Enzyme Kinetics

The catalytic properties of pyruvate kinase have been studied in most detail with the rabbit muscle enzyme (Boyer, 1962). The specificity for nucleotide diphosphate substrates is quite broad for the rabbit muscle enzyme, and several trinucleotides in addition to ATP are effective in the inhibition of the yeast enzyme (Haeckel et al. 1968). A number of analogues of phosphoenolpyruvate bind to the rabbit muscle enzyme with an apparent K_m close to that of the natural substrate and act as poor substrates, e.g. phosphoenol-α-ketobutyrate, phosphoenol-α-ketovalerate, phosphoenol-α-ketocaproate, phosphoenol-3-bromopyruvate and phosphoenol-3-fluoropyruvate (Kayne, 1973). D-phospholactate and phosphoglycolate bind to the enzyme but are not substrates (Nowak and Mildvan, 1970). There is no evidence for the formation of a stable phosphorylated enzyme; no incorporation of phosphate from phosphoenolpyruvate or ATP into the protein can be detected, and there is no conversion of phosphoenolpyruvate into pyruvate in the absence of nucleotide substrates (Boyer, 1962). Partial reactions which have been observed are: the enzyme bound enolisation of pyruvate, without phosphate transfer, in the presence of K^+ and ATP or inorganic phosphate (Rose, 1960), and phosphorylation of fluoride and hydroxylamine by ATP with bicarbonate - which is a competitive inhibitor with respect to phosphoenolpyruvate - as a co-factor

Like most kinases, the enzyme requires a divalent metal cation as a co-factor, and Mn^{2+} can be substituted for the physiological activator Mg^{2+}, whilst Ca^{2+} inhibits by competition at the metal binding site (Mildvan and Cohn, 1965). Pyruvate kinase was the first enzyme for which an absolute requirement for activation by a monovalent cation was demonstrated (Boyer,

1962). Potassium is the most effective activator, and the degree to which other monovalent cations can substitute for it depends on their ionic size relative to K^+. Ammonium ions can activate both the yeast (Hess and Haeckel, 1967) and the rabbit muscle enzymes (Kayne, 1971). Na^+ is not an activator for either enzyme, and Tl^+ is known to activate the rabbit muscle enzyme (Kayne, 1971).

Fructose diphosphate is a heterotropic activator for those enzymes which exhibit co-operative homotropic kinetics with respect to phosphoenolpyruvate, e.g. yeast pyruvate kinase (Hess et al. 1966b) and the isoenzymes of pyruvate kinase from mammalian tissues other than muscle. These enzymes are, in the nomenclature of Monod et al. (1965), 'K-systems', i.e. the effector alters the apparent affinity of the enzyme for the substrate and not the maximal velocity.

Inhibition by phenylalanine appears to be a property shared by all the mammalian isoenzymes of pyruvate kinase, although no physiological role is proposed. Inhibition of the isoenzymes sensitive to fructose diphosphate by alanine has been mentioned previously in relation to the regulation of gluconeogenesis.

(iii) Mechanism of the Enzyme

Some evidence as to the mechanism of the pyruvate kinase reaction comes from enzyme kinetics. Mildvan and Cohn (1966) reported that there was random-order binding of phosphoenol-pyruvate, ADP and Mn^{2+} or ADP.Mn to the rabbit muscle enzyme. This interpretation of their results was challenged by Cleland (1967), who claimed that only ADP.Mn, and not ADP and Mn^{2+} separately, was a substrate for the enzyme. Mildvan did not accept Cleland's interpretation (Mildvan et al. 1971) and cited preliminary experiments to show that the rabbit muscle enzyme

catalysed the reaction equally well if presented with the ADP.Ni complex, or if it was allowed to bind Ni^{2+} and then presented with ADP.Ni^{2+} was used because it dissociates only slowly from its complexes, and rapid reaction techniques were used to determine the extent of the reaction in 4 ms (about 1 turnover time), during which the Ni^{2+} complexes would not have significantly re-equilibrated. Ainsworth and MacFarlane (1973) claimed to have shown that ADP.Mg was not a substrate, and that there was equilibrium random order binding of phosphoenolpyruvate, Mg^{2+} and ADP, and of pyruvate and ATP.Mg. They also confirmed that inhibition by ATP.Mg was competitive with both phosphoenolpyruvate and ADP (cf. Boyer, 1962), and they have evidence that a dead-end complex of pyruvate, Mg^{2+} and ADP is formed. In contrast, MacFarlane and Ainsworth (1972) found that, for the yeast enzyme in the presence of fructose diphosphate (i.e. in conditions where the kinetics are hyperbolic and not sigmoidal), there was binding of the substrates in the order phosphoenolpyruvate, ADP and Mg^{2+}, and release in the order pyruvate then ATP.Mg. Pyruvate formed a dead-end complex with the enzyme, and ATP.Mg was a competitive inhibitor with respect to phosphoenolpyruvate.

It was competitive inhibition by ATP and the lack of any evidence for a phosphorylated enzyme which had led to the overlapping ATP-phosphoenolpyruvate site model, i.e. the proposal of a common site for the transferable phosphate (Boyer, 1962), and this mechanism is still assumed to be correct. MacFarlane and Ainsworth (1972) suggested that the separate binding of ADP and Mg^{2+} enabled the metal to bridge the phosphate groups of the two substrates, and thus render unnecessary a later shift of the metal from the α- and β- phosphates to the β- and γ- phosphates

of ATP, such as would be required if the ADP.Mg complex were the true substrate. On the basis of isotope exchange kinetics, Robinson and Rose (1972) reported that enzyme-bound ATP plus pyruvate could revert to substrates with exchange of the phosphoenolpyruvate methylene protons with protons from the solvent, and that product release was rate-limiting in the forward direction, whilst the release of phosphoenolpyruvate and ADP was rate-limiting in the reverse direction. This would appear to be in conflict with Ainsworth and MacFarlane's (1973) report of equilibrium random-order kinetics which require the catalytic step to be rate-limiting; however, their conclusion is based on the linearity of their double reciprocal plots, which is said not to be a sensitive test for equilibrium kinetics. Robinson and Rose (1972) also demonstrated that phosphate transfer precedes protonation of enolpyruvate, which seemed probable from the nature of the partial reactions. Rose (1970) determined that the direction of addition of the proton to enolpyruvate was to the 'si-face' designated by a counterclockwise sequence of phosphate, carboxyl and vinyl groups at C-2. This si-face would also seem to be exposed to the solvent, since reduction of pyruvate by $NaBH_4$ in the presence of pyruvate kinase gives predominantly D-lactate (Phillips and Kosicki, 1970).

(iv) Functional Groups at the Active Site

Attempts have been made to identify functional groups at the active site of pyruvate kinase by chemical modification studies, but only limited information has been obtained. The thiol groups of rabbit muscle pyruvate kinase are not particularly reactive, and there is no 1:1 stoichiometry between substitution and inactivation. In the presence of phosphoenolpyruvate, K^+

and Mg^{2+}, 4 thiol groups per mole react with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), but inactivation does not occur unless the substrate and metals are removed, when a disulphide interchange occurs (Flashner et al. 1972). Kinetic and proton relaxation rate enhancement studies of the disulphide enzyme (Flashner et al. 1973) have shown that binding of Mn^{2+} is unaffected by the modification, and a ternary enzyme-metal-phosphoenolpyruvate complex is detected, although the binding of phosphoenolpyruvate is weaker by a factor of ten. The ATP-activated de-tritiation of pyruvate is inhibited, but phosphate-activated detritiation is preserved, and hence it was proposed that the disulphide formation affects mainly the nucleoside binding site.

Rabbit muscle pyruvate kinase is 90% inactivated by the binding of 2-4 moles of pyridoxal phosphate per mole (Johnson and Deal, 1970), but the authors consider that the lysine residues involved are not at the active site since substrates do not protect the enzyme. Hollenberg et al. (1971) also studied modification of lysine residues with 2,4,6-trinitro-benzene-1-sulphonate; they observed 4 reactive lysine groups per mole, substitution of which led to 95% inactivation, and both ADP and ATP protected the enzyme against reaction. Flashner et al. (1973) reported that the binding of Mn^{2+} and phosphoenol-pyruvate by the modified enzyme is normal, but no enzyme-metal-ADP complex was detected. Again, ATP-activated detritiation of pyruvate was inhibited, whilst phosphate-activated exchange was not. Therefore, lysine may be situated at the nucleoside part of the nucleotide binding site.

4 lysine groups per mole of yeast pyruvate kinase are trinitrophenylated by 2,4,6-trinitrobenzene-1-sulphonate (Roschlau and Hess, 1972b) to give 75% inhibition. However,

although ADP, Mg^{2+} or ADP plus Mg^{2+}, but not fructose diphosphate or phosphoenolpyruvate, protected the enzyme, the $K_{0.5}$ for ADP was unaffected, whilst $K_{0.5}$ and the Hill coefficient for phosphoenolpyruvate were. Wieker and Hess (1972) studied the reaction of the thiol groups of pyruvate kinase from S. carlsbergensis with DTNB, and proposed that there is a thiol group at the active site, modification of which completely inactivates the enzyme, but which can be protected by phosphoenolpyruvate in the presence of fructose diphosphate. Evidence for a separate "allosteric" site for fructose diphosphate was based on the claim that substitution of another thiol group which was protected by this ligand led to alterations in the allosteric properties of the enzyme.

Recently, Davidoff et al. (1973) reported that 3,5-dimethylpyrazole-1-carboxamide was a competitive inhibitor at the metal binding site on a short time scale, but over a longer period, inactivated the enzyme by reaction with a lysine group (to give homoarginine) at a rate which was greater than the rate of reaction with other lysine residues, and which was reduced by the presence of Mn^{2+}.

Mildvan and Cohn (1965) found that the pH dependence of the binding of Mn^{2+} by rabbit muscle pyruvate kinase was consistent with the presence of two groups of pK 6.8 and 7.8 respectively at the metal site. One of these groups could well be an imidazole group; histidine has been proved to be a co-ordinating ligand in several metalloproteins (Mildvan, 1970).

(v) Magnetic Resonance Studies

Rabbit muscle pyruvate kinase was studied in Cohn's early work in the development of the use of magnetic resonance techniques for the elucidation of the structure of enzyme-

substrate complexes (e.g. Mildvan and Cohn, 1965, 1966). By the use of the proton relaxation rate enhancement effect of Mn^{2+} (described in Chapter 2) Mildvan and Cohn (1970) aimed to classify the co-ordination scheme of the ternary enzyme-substrate-metal complexes of enzymes which required divalent metal ions for activity into three groups:

(i) Type I; substrate bridge, E-S-M ($\varepsilon_b < \varepsilon_{EMS}$)

(ii) Type II; metal bridge, E-M-S or  ($\varepsilon_b > \varepsilon_{EMS}$)

(iii) Type III; enzyme bridge, S-E-M ($\varepsilon_b = \varepsilon_{EMS}$)

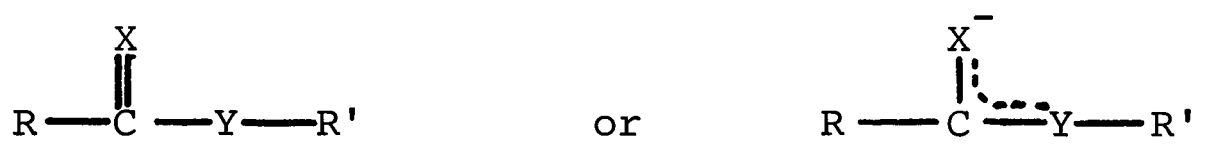
On this basis, the ternary complexes of pyruvate kinase with phosphoenolpyruvate, ADP, pyruvate or ATP were classified as Type II. The original analysis of the results (Mildvan and Cohn, 1966) was by graphical techniques; a later development was the use of computerised fitting techniques to solve the complex equations involved (Reed et al. 1970); when the original results were re-examined, it was found that the titrations with the nucleotides could be fitted equally well assuming either $\varepsilon_b = \varepsilon_{EMS}$ or $\varepsilon_b > \varepsilon_{EMS}$ (Reed and Cohn, 1973), and so proton relaxation rate enhancement could not be used to determine the co-ordination of the complex. (The uncertainty about the value of the enhancement parameter was coupled with uncertainty about the values of the dissociation constants for the ternary nucleotide complexes). Reed and Cohn (1973) also reported that the presence of nucleotides had no discernible effect on the ESR spectrum of Mn^{2+} bound to the enzyme (although the degree of formation of the ternary complex in these experiments was not known because of the uncertainty in the dissociation constants). This absence of evidence for co-ordination to the nucleotides may seem surprising since it had frequently been assumed that the role of the divalent metal ion was to co-ordinate to the β-

phosphate of the nucleotide and the transferrable phosphate. In part, this view followed from the opinion that the metal complex of ADP was the true substrate for pyruvate kinase (e.g. Cleland, 1967), and also because studies of the ^{31}P nuclear resonances of ATP have demonstrated that not only does the β-phosphate atom exhibit greater electronic shielding than the α- and γ-atoms, which should therefore be more susceptible to nucleophilic attack, but that divalent metal ions preferentially chelate to the β- and γ-phosphate groups and cause further electronic deshielding of the phosphate atoms, so that nucleophilic attack at the γ-phosphate group should be further aided (Cohn and Hughes, 1962). Ainsworth and MacFarlane (1973), whose results have already been mentioned, concurred with the view that the metal ion was chelated between the phosphate groups of ADP and phosphoenolpyruvate. It is perhaps worth noting that the map of the active site of creatine kinase obtained from distance measurements made with nuclear magnetic resonance and spin-label techniques (Cohn et al, 1971) excludes the co-ordination of the divalent metal to the transferrable phosphate group. Reed and Cohn (1973) and James et al. (1973) have recently reported extensive studies of the ternary complexes of enzyme and metal with phosphoenolpyruvate, pyruvate or substrate analogues by electron spin resonance and proton relaxation rate enhancement. The electron spin resonance spectrum of the Mn(II) ion bound to the enzyme - which is broadened relative to that of the free metal, but is still isotropic - undergoes a marked change to an anisotropic form when pyruvate binds to the enzyme, indicating that the co-ordination sphere of the metal is highly distorted in this ternary complex. This anisotropic spectrum is unaffected by whether the monovalent cation present

is non-activating (e.g. $(CH_3)_4N^+$) or activating, i.e. K^+, but addition of enolisation cofactors such as ATP, phosphate or arsenate produces minor changes. The spectrum in the ternary complex with phosphoenolpyruvate indicates a highly anisotropic structure in the presence of K^+, but in the presence of other monovalent cations, such as $(CH_3)_4N^+$, the spectrum appears to be a combination of an anisotropic and an isotropic form. James et al. (1973) present evidence from proton relaxation enhancement measurements which suggests that these two spectra are from two conformations in equilibrium with one another which are resolved by electron spin resonance but are averaged over the longer time scale of the nuclear magnetic resonance measurements. Studies of the temperature and frequency dependence of the proton relaxation rate enhancement for the ternary enzyme-Mn-phosphoenolpyruvate complex in the presence of K^+ led to a value of 0.2-0.5 for the number of water molecules in the first co-ordination sphere of the bound metal. Reuben and Cohn (1970) had shown that, in the binary pyruvate kinase-Mn complex, 3 water molecules remained in the first co-ordination sphere. Since phosphoenolpyruvate could contribute at most a further two ligands to the metal ion, it is probable that the reduction of the number of water molecules to less than one is in whole, or in part, caused by a conformational change on the formation of the ternary complex; the magnetic resonance measurements do not distinguish between these possibilities. Several co-ordination schemes that would give an apparent value of less than one water molecule in the measurements were proposed; one was that the 6th ligand was a hydroxide ion which acted as the base catalyst at the active site. Robinson and Rose (1972) had also suggested that a metal hydroxide was the base catalyst for the tritium exchange partial reaction, since the rate

depended on the electronegativity of the activating cation. Nowak and Mildvan (1972) determined the distances between Mn and the hydrogen and phosphorous nuclei of phosphoenolpyruvate, phospholactate and phosphoglycolate; these were consistent with the co-ordination of the metal to the phosphate groups, but there are doubts about the significance of these distances now that it is known that, in the absence of K^+, nuclear magnetic resonance measurements with these molecules are averages of two conformations.

Reuben and Kayne (1971) studied the effect of Mn(II) on the nuclear magnetic resonance spectrum of $^{205}Tl(I)$ when bound to pyruvate kinase in place of K^+. They found that the Mn-Tl distance of 8.2 Å decreased to 4.7 Å in the presence of phosphoenolpyruvate, suggesting a substantial conformational change, and showing that the monovalent cation is also close to the active site. Suelter (1970) has suggested that a potential site for keto-enol tautomerism, as contained in the structures:



with X = O, N or C and Y = O or N,

is a characteristic shared by substrates or probable intermediates of enzymes known to be activated by monovalent cations. Since certain chelating agents for such ions contain similar groupings, he concludes that the cation is co-ordinated to the substrate at the active sites of such enzymes.

(vi) Conformational States

Conformational changes of rabbit muscle pyruvate kinase induced by ligands have been demonstrated by a number of other techniques. Suelter and Melander (1963) detected an ultraviolet

difference spectrum upon binding divalent metal ions. Difference spectra were also obtained on binding monovalent cations and phosphoenolpyruvate, and on lowering the temperature of the solution (Kayne and Suelter, 1965). These ligands and temperature changes also cause alterations in the circular dichroism spectrum between 260 and 290 nm (Wildes et al. 1971). K^+ and Mn^{2+} quench tryptophan fluorescence and increase fluorescence polarisation; the latter effect is also given by low temperatures (Suelter, 1967). Kayne and Suelter (1968) observed a small change in sedimentation velocity in the presence of K^+ and Mn^{2+}, but no significant changes in optical rotatory dispersion. Reuben and Cohn (1970) also obtained evidence for a temperature-dependent transition in the binding of Mn^{2+}.

Kuczenski and Suelter (1971a) demonstrated small changes (2-3%) in the tryptophan fluorescence of pyruvate kinase from yeast when mono- or di-valent cations were bound. A larger change (12% quenching) was obtained when the allosteric effector fructose diphosphate was bound. The enhancement by fructose diphosphate of cold inactivation of the enzyme from S. cerevisiae (Kuczenski and Suelter, 1970a) is further evidence for a conformational change induced exclusively by the effector.

d) Allosteric Enzymes

The mechanisms by which allosteric effectors control the activity of enzymes has stimulated considerable interest since Monod, Changeux and Jacob (1963) first defined the phenomenon. It should be noted that the description "allosteric" is commonly applied to any enzyme exhibiting co-operative interactions, although strictly it should be reserved for those enzymes - a minority of the former cases - where the interacting ligand sites are known to be separated sufficiently to preclude direct contact

interactions between the ligands. The hypotheses of the mechanisms of allosteric enzymes (which have been reviewed by Koshland and Neet, 1968; Koshland, 1969 and 1970; and Whitehead, 1970) can be classified as either equilibrium or kinetic. The former type, embracing the theories of Monod et al (1965) and of Koshland (the sequential hypothesis, Koshland et al. 1966; the general hypothesis, Haber and Koshland, 1967), propose that co-operativity, manifested as sigmoidal functions of enzyme velocity versus ligand concentration, arises from co-operative binding phenomena with the binding steps being at - or close to - equilibrium, and the rate of catalysis being limiting. The kinetic hypotheses (e.g. Rabin, 1967; Ferdinand, 1966; Frieden, 1964) postulate that co-operative interactions arise in multi-step reaction pathways where the rate constants are in certain relationships to one another. The equilibrium hypotheses have attracted more attention, since there has not been a demonstration of co-operative interactions explicable only by a kinetic mechanism, whereas there are many instances of co-operative interactions in direct studies of the binding of ligands to allosteric enzymes. However, the experimental problems of defining a suitable equilibrium mechanism for any particular case can be enormous. Obviously, the ease of choosing between mechanisms increases with the number of restrictions placed on them; thus the theory of Monod et al. (1965), with restriction to two conformational states of the protomers in pre-existing equilibrium and to symmetry of the oligomer, and the sequential model of Koshland et al. (1965), with obligatory linkage between ligand binding and an induced conformational change, both lead to precise predictions about conformational and ligand-binding equilibria. For example, only the symmetry model of Monod et al. allows a reduction to Hill-type kinetics

(i.e. the Hill coefficient = the number of protomers), although of course, this need not be so for the model to be valid. Again, in the sequential model of Koshland et al., the fractional conformational change must be the same as the fractional ligand saturation curve, whilst the observation of negative homotropic interactions (as distinct from heterogeneity of binding sites) is not compatible with the symmetry model. Unfortunately, both hypotheses can be criticised as too idealistic, but whilst it is possible theoretically to analyse more realistic cases, the amount of experimental information required to test such models increases. One particularly problematical restriction is that of the small number of conformational states (normally two in the models of Monod et al. and Koshland et al.). Weber (1972) has criticised this as inherently unlikely given the structure of proteins, and has shown that it is unnecessary; linkage between the binding of different ligands can occur when they separately stabilise different conformations, and together a hybrid conformation. Although he criticises the model of Koshland et al. for postulating a one to one relationship between ligand binding and structural changes, which he considers contrary to the principles of equilibrium thermodynamics, his approach is perhaps close to the general formulation of Haber and Koshland (1967). However, where these authors describe their conformational equilibria in terms involving 'tautomerisation energy' for the conformational change, Weber considers that a description based on explicit free energies of association of the protomers is more useful because (a) tautomerisations, characterised by some structural detail that happens to be observable, involving one protomer alone, cannot be separated in a simple way from those involving two neighbouring protomers, a distinction that is essential for the recognition of changes

in inter-subunit interactions, and (b) it is unlikely that the free energies of tautomerisation can be precisely determined experimentally; in practice they are given values obtained by curve-fitting, which may not give a unique or accurate solution, whereas the association constants of the protomers in the free and liganded states may be experimentally accessible and lead directly to values for the changes in subunit interactions.

Whitehead (1970) proposed an experimental test for mechanisms involving only two conformational states. He also predicted, as did Koshland (1969), that there might be different conformations of a protomer, induced by ligands, which were nevertheless equivalent at the points of interaction with the other protomers so that the conformations were equivalent in mediating co-operative interactions: i.e. put another way, not all the observed ligand-induced changes in the conformation of a protein need be relevant to inter-subunit interactions, which is similar to Weber's argument. Cornish-Bowden and Koshland (1970) have pointed out that if symmetry is not conserved in the oligomer, then the geometry of the oligomer determines the number of inter-subunit interactions possible, and thus the details of the mechanism; in these cases, simplicity of the hypothesis requires restriction to one set of averaged interaction constants between the protomers, but this may be somewhat arbitrary; for example, in the common tetrahedral geometry for oligomers, there are three different types of inter-protomer bond in the simplest, isologous case, and the occurrence of dimers as intermediates in the dissociation of tetrameric proteins testifies that these interactions are often of different strengths.

It can thus be seen that, experimentally, the determination

of an allosteric mechanism for any given enzyme may be a very complex problem, and it is not possible dogmatically to propose a suitable approach. Obviously it is necessary to define the number and types of protomers in the protein and the number and types of ligand binding site which they bear. A determination of the binding curves of all the ligands of the molecule, and the detection of the linkages between them, defines the interactions occurring and the magnitude of some of the free-energy changes in the system. The correlations of ligand binding and any conformational changes are essential in determining the mechanism, but for the reasons given above, it is possible that these may be misleading if interpreted incautiously. The usefulness of determining the effects of the ligands on the dissociation constants of the oligomer has already been referred to. Ultimately the mechanism is only fully described when all the possible conformations and the free energies of all the possible equilibria are defined. This may only be feasible when the three-dimensional atomic structure of the main conformations has been determined.

CHAPTER 2

MATERIALS AND METHODS

a) Materials

Fructose diphosphate (tetracyclohexylammonium or sodium salt), phosphoenolpyruvate (potassium salt), ADP (di- or tri-sodium salts), NADH (disodium salt) and rabbit muscle lactate dehydrogenase (as a precipitate in $(NH_4)_2SO_4$) were obtained from Boehringer Corp. (London) Ltd., London, W.5. U.K.; Tris (reagent grade), sodium dodecyl sulphate (SDS) and pyruvate from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; cacodylic acid from Serva Feinbiochemica GmbH, Heidelberg, Federal Republic of Germany; 5,5'dithiobis-(2-nitrobenzoate) (DTNB) from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A., and from BDH Chemicals Ltd., Poole, Dorset, U.K.; iodo[1-^{14}C]acetamide from The Radiochemical Centre, Amersham, Bucks., U.K.; the spin-labels N-(1-oxyl 2,2,6,-tetramethyl-4-piperidinyl)-iodoacetamide and 4-isothiocyanate-2,2,6,6,tetramethyl piperidinoxyl from Synvar, Palo Alto, California, U.S.A.; the liquid-scintillation fluid, 'Aquasol' from New England Nuclear Corp., Boston, Mass., U.S.A.; DEAE-cellulose (DE52), CM-cellulose (CM52) and cellulose phosphate (P11) from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. and Sephadex from Pharmacia, Uppsala, Sweden. All other chemicals were BDH AnalaR grade, except that 'Aristar'$(NH_4)_2SO_4$ was used when the purified enzyme was precipitated, and 'Biochemical reagent' grade guanidinium chloride was used for the sedimentation equilibrium studies. Dialysis sacs were prepared from Visking tubing that had been boiled for 5 min in 1% $NaHCO_3$ 1% EDTA, washed several times in distilled water, and stored in

distilled water at 4°C.

b) Preparation of Solutions

In the description of buffers used in this work, the component named first was the one present at the indicated concentration, and the second component was the one used to adjust the pH to the required value. An EIL model 23A direct-reading pH meter with a glass electrode was used for the pH measurements.

For some solutions of phosphoenolpyruvate, pyruvate, ADP or ATP, it was necessary to know the molarity with greater accuracy than could be obtained by weighing out the amount calculated from the manufacturer's nominal composition. The molarities of these solutions were measured either from their optical densities (absorption maxima and extinction coefficients are given in Dawson et al. 1969 and Pon and Bondar, 1953), or alternatively, in the case of phosphoenolpyruvate and ADP, by allowing the linked pyruvate kinase-lactate dehydrogenase reaction to go to completion in the presence of excess NADH (for other details, see this Chapter section g).

Solutions of pyruvate kinase were prepared from the $(NH_4)_2SO_4$ precipitate, as which the enzyme was usually stored. The precipitate was dissolved in a minimal volume of the required buffer, and the residual $(NH_4)_2SO_4$ was generally removed by passing the solution through a column (10 ml) of Sephadex G-25 (coarse grade) which had been swollen and equilibrated with the same buffer. The centre of the void volume peak was collected manually by monitoring the absorption of the eluate at 254 nm with an LKB "Uvicord I" (LKB, Uppsala, Sweden). For hydrodynamic and sedimentation equilibrium measurements on the native enzyme, equilibration with the buffer was completed by a further 4 hrs

dialysis. Concentrations of pyruvate kinase were determined from measurements of the absorbance at 280 nm with a Gilford-modified Beckmann DU or a Unicam SP500 spectrophotometer, and were converted to mgs/ml using an extinction coefficient of $E^{1\%}_{1cm}$ at 280 nm = 6.5 $ml.mg^{-1}.cm^{-1}$ (Chapter 5; Ashton, 1971; Hunsley and Suelter, 1969a).

For the preparation of subunits of the enzyme with the thiol groups blocked by reaction with N-ethylmaleimide, the procedure used was essentially that described by Ashton (1971). The enzyme was dissolved in 0.5M phosphate-K^+, pH 7.0, and dialysed against this buffer for 2 h. Sufficient N-ethyl-maleimide was then added to give approximately a 50-fold molar excess over the enzyme. The reaction was allowed to proceed for 2h at room temperature, during which time the protein precipitated. This suspension was then dialysed against two changes of 6M guanidinium chloride (10 ml) for a total of 24 h; the precipitate completely dissolved in this solvent. Solutions of pyruvate kinase denatured in 6M guanidinium chloride - 1% 2-mercaptoethanol were prepared by dialysis for at least 24 h against two changes of this solvent.

c) Preparation of Ion-exchange Columns

Precycling with acid and alkali was not necessary for the pre-swollen varieties of CM- and DEAE- cellulose which were used. However, precycling by the method recommended by Whatmann was used on occasions to clean material contaminated with irreversibly-bound protein. For the CM- and DEAE-cellulose columns required in the preparative method of Ashton (this Chapter, section d), the ion-exchanger was suspended in about 1 litre of the column buffer at molar concentration and was then washed several times with buffer at the concentration required

in the preparation. Fines were removed in the supernatant after the ion-exchanger had settled at each equilibration step.

A different procedure was used to prepare the large quantities of DEAE-cellulose required for the chromatography of pyruvate kinase in 50% 10mM phosphate-Na^+, pH 7.5, 50% glycerol. The ion-exchanger was suspended in about 2 litres of 0.5M phosphate-Na^+, pH 7.5. After 10 min, this was removed by filtration and its pH adjusted back to 7.5 with phosphoric acid. The DEAE-cellulose was resuspended in the solution, and the procedure repeated until the pH of the filtrate was 7.5. Then the ion-exchanger was washed several times with 10mM phosphate-Na^+, pH 7.5, and finally it was washed twice with the column buffer before packing the column.

Precycling is not recommended for cellulose phosphate, although it is supplied dried. It was swollen overnight in 1M phosphate-Na^+, pH 6.5, and its pH then adjusted to 6.5 and its equilibration completed by the method described above for DEAE-cellulose.

Once packed, columns were generally used for several preparations. After each preparation, they were regenerated by the elution of bound protein with the appropriate column buffer containing 1M NaCl, and re-equilibrated with 2 to 3 column volumes of the column buffer.

d) Preparation of Yeast Pyruvate Kinase - Method of Ashton (1971)

A suspension of brewer's yeast (Saccharomyces cerevisiae from Morrell's Brewery, Oxford) was centrifuged (1,000g for 10 mins) to obtain about 1 kg of yeast, which was then cytolysed by stirring with 70 ml of toluene at 37°C for 1 h, or until the evolution of carbon dioxide ceased. After the addition of 200 ml of 25% glycerol, the suspension was left to stand for

1 h at 4°C. The pH of the cytolysate was then taken to 7.5 by the addition of 4% ammonium hydroxide, and the suspension was left overnight at room temperature. Next morning, the cell debris was removed by centrifugation at 35,000g for 10 mins. The volume of the supernatant, which contained the pyruvate kinase activity, was measured and an aliquot retained for assay. Solid $(NH_4)_2SO_4$ (AnalaR) was added to give a final concentration of 55% of (25°C) saturation; the solution was stirred for 1 h, and 90% of the pyruvate kinase activity was recovered in the precipitate which was collected by centrifugation at 35,000g for 10 min. This precipitate was re-suspended in 100 ml of 30% saturated $(NH_4)_2SO_4$ solution (pH 6.0) and stirred for 1 h. The insoluble material was removed by centrifugation (35,000 g, 10 min), and solid $(NH_4)_2SO_4$ added to the supernatant to give a final concentration of 65% saturation. The precipitate, containing pyruvate kinase, was collected by centrifugation (35,000 g, 10 min) and stored at 4°C for up to 6 weeks before further purification. Up to this point, the preparation is essentially similar to that of Haeckel et al. (1968).

One quarter (about 10g, wet weight) of the crude precipitate containing pyruvate kinase was dissolved in the minimum possible volume of 0.005M potassium acetate, pH 6.2. This solution was pumped on to a column of CM-cellulose (15 x 5 cm) and the column was eluted at 3 ml/min. with the same buffer. Fractions of the eluate were assayed for pyruvate kinase activity, which emerged in the first protein peak. The pyruvate kinase was collected by centrifugation after precipitation by the addition of two volumes of a saturated solution of $(NH_4)_2SO_4$ to one volume of the protein solution. This precipitate was dissolved in the minimum possible volume of 0.05M Tris-HCl (pH 7.2) and the

solution applied to a column of DEAE-cellulose (15 x 5 cm) equilibrated with the same buffer. Pyruvate kinase eluted as the first protein fraction from the column. Fractions were assayed for pyruvate kinase activity using the assay of Haeckel et al. (1968) (this Chapter, section g), and the specific activity calculated with the concentrations determined from the absorbance at 280 nm. The fractions with the highest specific activity (greater than 180 U/mg) were pooled, and the pyruvate kinase collected by precipitation with a saturated solution of $(NH_4)_2SO_4$ and centrifugation. The enzyme was stored at 4^oC as this precipitate.

e) Preparation of Pyruvate Kinase from Freeze-thawed Yeast

3.5 kg (wet weight) of Saccharomyces cerevisiae were collected from Morrell's Brewery, Oxford, immediately after fermentation of the beer had ceased. The yeast was washed twice by suspension in 3 litres of 5mM-EDTA, 10mM $MgCl_2$, 40mM $NaHCO_3$ at 4^oC, and centrifugation (1,000g, 10 min), and the yeast paste stored overnight at 4^oC.

The yeast was added, in small amounts, to 5 litres of toluene at -20^oC. The temperature of the mixture was kept below -10^oC by frequent additions of solid CO_2. After 6 h at -10^o, the toluene was decanted, and the slurry allowed to melt at 4^oC. When the temperature of the yeast had risen above 0^oC, 2.5 litres of 0.1M tris-HCl (pH 8.0)-0.1M KCl-1mM EDTA-2mM $MgCl_2$ at 4^oC were added. After a further period at 4^oC, about 4.5 litres of cell supernatant were separated from cell debris by centrifugation (6,300 g for 20 min). In this step, and all subsequent steps of the preparation, the temperature was not allowed to rise above 5^oC. Pyruvate kinase activity was precipitated from the supernatant by the addition of $(NH_4)_2SO_4$

(350g/l of solution), and stirring for 1 h. The precipitate was collected by centrifugation (6,300g for 45 min) and the supernatant was assayed. If more than 80% of the enzyme activity had not precipitated, more $(NH_4)_2SO_4$ was added. The precipitated pyruvate kinase was dissolved in 600 ml of 0.1M tris-HCl (pH 7.5)-2mM $MgCl_2$-1mM EDTA plus $(NH_4)_2SO_4$ (114g) by stirring for 1h. Undissolved protein was removed by centrifugation (35,000g for 10 min), $(NH_4)_2SO_4$ (20g/100ml of supernatant) added, and the precipitate collected, after 1h, by centrifugation as before.

This precipitate was then suspended in the minimum possible volume (about 50 ml) of distilled water, and dialysed against several 1 litre changes of 50% 10mM phosphate-Na (pH 7.5) 50% (v/v) glycerol. The protein went into solution as the $(NH_4)_2SO_4$ dialysed out, and the solution was concentrated as water diffused out of the dialysis sac faster than glycerol diffused in. The protein solution was then ready for chromatography as described below.

Further details of this preparation are given in Chapter 4.

f) Column Chromatography of Pyruvate Kinase

This method is adapted from that given in Hunsley and Suelter (1969a). The protein solution was loaded on to a 2.3 litre column (70 x 6.5 cm) of DEAE cellulose, equilibrated with 50% 10mM phosphate-Na, pH 7.5, 50% (v/v) glycerol in a 4^{o}C cold-room, and eluted with the same buffer at 100 ml/h. Fractions were collected every 20 min, and the elution profile monitored by its absorbance at 254 nm with an LKB "Uvicord I". Fractions containing absorbing material were measured at 280 nm, and assayed for pyruvate kinase activity. A typical elution profile is shown in Fig. 4.3. The central two-thirds of the

pyruvate kinase peak was collected and pooled to give a solution containing pyruvate kinase at a specific activity between 150 and 180 U/mg (the protein concentrations being based on an assumed $E_{1cm}^{0.1\%}$ at 280 nm = 1 ml.mg^{-1}.cm^{-1}). The solution was taken to pH 6.5 by the addition of dilute phosphoric acid, and loaded on to a 200 ml column (10 x 5 cm) of cellulose phosphate equilibrated with 50% 10mM phosphate-Na, pH 6.5, 50% (v/v) glycerol. The column was eluted at 60 ml/h, first with 500 ml of the same buffer, and then with a linear gradient from 0 to 500 mM $(NH_4)_2SO_4$. The optical density of the eluate at 254 nm was monitored with an LKB "Uvicord I", and 20 ml fractions were collected. The optical density at 280 nm and the pyruvate kinase activity were measured on fractions containing absorbing material. An elution profile is given in Fig. 4.4, and further details in Chapter 4.

The pyruvate kinase was recovered from the column buffer by dialysis against a saturated solution of "Aristar" $(NH_4)_2SO_4$ in 0.1M tris-HCl (pH 7.2)-2mM $MgCl_2$-1mM EDTA, and the precipitate collected by centrifugation.

g) Enzyme Assays

Two assay systems were used, the first for assays connected with experiments described in Chapter 3, and the second for most of the experiments in Chapters 4 and 5. Both were based on the continuous assay of the production of pyruvate by the consumption of NADH in the presence of excess lactate dehydrogenase. The absorbance of NADH at 340 nm was monitored with a Gilford-modified Beckmann D.U. spectrophotometer equipped with a thermostatted cell holder and a chart recorder.

(1) This assay system was described by Haeckel et al. (1968). To 1 ml of assay medium, containing 0.05M phosphate-

potassium (pH 6.0)-30mM $MgSO_4$-5mM phosphoenolpyruvate-5mM ADP-0.15mM NADH-1mM fructose diphosphate in a reduced-volume 1cm cuvette, was added 0.02ml of a suspension of lactate dehydrogenase (1mg/ml in $(NH_4)_2SO_4$ solution). The reaction was started by the addition of 0.02 to 0.05 mls of pyruvate kinase diluted in 50% (v/v) glycerol-water. The consumption of NADH was linear with time, and the reaction rate was measured over at least two minutes at 25°C.

(2) The second assay method was derived from that described by Hunsley and Suelter (1969a). The assay medium contained 0.1M cacodylate-potassium (pH 6.2)-0.1M KCl-25mM $MgSO_4$-0.5mM EDTA-5mM ADP-5mM phosphoenolpyruvate-1mM fructose diphosphate-0.15mM NADH. The procedure was as above, except that the lactate dehydrogenase used had been dialysed against 50% (v/v) glycerol-50% 0.1M cacodylate-potassium (pH 6.2). Enzyme activities are expressed in International units (μ moles of product formed per minute).

h) Determination of Molecular Weights by Sedimentation Equilibrium

The equilibrium distribution of an uncharged solute in a two-component solution in a gravitational field generated by rotation is given by the equation:-

$$\frac{1}{c}\cdot\frac{dc}{dr} = \frac{M^o\ (1 - \bar{v}\ \rho)\ \omega^2 r}{RT\left(1 + c.\frac{\partial \ln\gamma}{\partial c}\right)} = \frac{M^{app}.(1 - \bar{v}\rho)\omega^2 r}{RT} \qquad (2.1)$$

where c is the concentration of the solute at any point; r is the radial position in the gravitational field; M^o is the molecular weight of the solute; γ is the activity coefficient; M^{app} is the apparent molecular weight obtained at any finite concentration; $\bar{v}$ is the partial specific volume of the solute;

ρ is the density of the solution, and ω is the angular velocity of the rotation. Casassa and Eisenberg (1964) have shown that this equation is true for a three component solution, such as a charged protein in a solution containing low molecular weight electrolytes, with the following restrictions:- (i) that the pH of the solution is not far from the isoelectric point of the protein, so that the total charge on the molecule is small; (ii) that the ionic strength of the solution is high (typically O.1M); (iii) that the solution is at dialysis equilibrium with the solvent against which the concentration distribution is determined, and (iv) that the molecular weight of the solute obtained is that of the component defined by the concentration determination used in measuring $\bar{v}$. Usually, the concentration dependence of the molecular weight is not described by an activity coefficient, but by a virial expansion, of which only the first term is significant at protein concentrations of less than 10 mgs/ml. For the weight-average molecular weight ($\bar{M}_w = \Sigma_i\ c_i.M_i/\Sigma_i c_i$):-

$$\bar{M}_w^{app} = \frac{\bar{M}_w^o}{1 + 2B.\bar{M}_w^o.\bar{c}} \qquad (2.2)$$

where $\bar{c}$ is the mean concentration of the solution, and B is the second virial coefficient. For the z-average molecular weight ($\bar{M}_z = \Sigma_i\ c_i.M_i^2/\Sigma_i\ c_i.M_i$), Van Holde and Cohen (1964) showed that non-ideality is approximately represented by equation 2.2 if $\bar{c}$ is replaced by twice the mean concentration.

Equation2l was used in two other forms for the analysis of the results presented here; where 'c' and 'r' were obtained from photographs taken with interference optics, the form

$$\frac{d(\ln c)}{dr^2} = \frac{\bar{M}_w^{app}(1 - \bar{v}\rho)\omega^2}{2RT} \qquad (2.3)$$

was used, with $d(\ln c)/dr^2$ being determined from the slope of a graph of ln c v. r^2; where dc/dr and r were obtained from photographs taken with Schlieren optics, the form

$$\frac{d \ln \left(\frac{1}{r}\cdot\frac{dc}{dr}\right)}{dr^2} = \frac{\bar{M}_z^{app}(1 - \bar{v}\rho)\omega^2}{2RT} \qquad (2.4)$$

(Creeth and Pain, 1967) was used, with the differential on the left hand side again being determined graphically. It is important to note that equation 2.3 evaluates the weight-average molecular weight for a heterogeneous solute, whilst equation 2.4 gives the z-average molecular weight.

The two types of sedimentation equilibrium experiment routinely used were the "conventional" low-speed equilibrium run and the Chervenka high-speed method (Chervenka, 1970). In the low-speed experiments - that is, where the rotor speed is chosen so that the concentration of the sedimenting solute at the meniscus is finite - 3mm solution columns were used for the determination of the sub-unit molecular weights, but 2mm columns were used for the native enzyme in order to reduce the time taken to reach equilibrium. (The time to reach 99.9% of equilibrium may be estimated from the equation $t = 0.7 \times (\text{column length})^2/D$, where D is the diffusion coefficient, Van Holde, 1967). Approximately 24 h were necessary for equilibrium to be attained. Either a double-sector 12mm centrifuge cell, or a 12mm 6-channel Yphantis cell with three solution columns of different initial concentration, was used. Fluorocarbon oil was omitted, and the diffusate from the dialysis was used for the solvent column. For the calibration of the interference fringe pattern in units directly proportional to the solute

concentration, the zero-order (or white light) fringe method of Richards and Schachmann (1959) was used to locate the hinge point. Photographs of the initial distribution were taken with the interference optical system by white light and by the green Mercury line; the distribution at equilibrium was similarly photographed, and the Schlieren image was also photographed. A microcomparator (Precision Grinding Limited) was used for the measurement of the photographs by the procedures described in Van Holde (1967) and Chervenka (1969). The radius of the hinge point was determined by comparison of the zero-order fringe in the white light photographs of the initial and equilibrium distributions of solute, and this position was checked with the corresponding green light pictures. The initial solute concentration had been determined either by measurement of the difference in the refractive index between the solution and the solvent against which it had been dialysed with a Brice-Phoenix differential refractometer (Phoenix Precision Instrument Co. Philadelphia, P.A., U.S.A.), or by a separate centrifuge run in which the concentration was measured as the displacement of interference fringes across a boundary formed between the solution and the solvent in a synthetic boundary cell. The difference in refractive index (Δn) is related to the solute concentration in interference fringes (J_o) by the relationship:

$$J_o = \Delta n.l/\lambda \qquad (2.5)$$

where l is the thickness of the solution column in the centrifuge cell and λ is the wavelength of the green mercury line (546 nm). After measurement of the refractive index difference, sufficient 1,3-butanediol was added to the solvent to equalise the refractive indices of the solution and solvent so that the zero-order white

light fringe would fall within the fringes photographed. With solutions in guanidinium chloride, the adjustment cannot be made with 1,3-butanediol, and must be made with solid guanidinium chloride. However, slight evaporation of the solvent introduces such large errors into measurements of refractive index on these solutions that it was found preferable to forego the adjustment of refractive index, to use a higher order achromatic fringe to find the hinge point, and to determine J_o from a synthetic boundary experiment.

The long-column, high-speed equilibrium method of Chervenka (1970) has two advantages over the low-speed method:- (i) Since most of the solution column is depleted of solute, the interference fringe pattern has a zero-concentration calibration point, thus obviating the need for a measurement of the initial concentration and a determination of the hinge point; (ii) The formation of the solution column by the layering of the solvent over a small volume of the solution greatly accelerates the approach to equilibrium, which may be reached in 4h or less. However, the accuracy of the method is less than that of the low-speed method (cf. the similar method of Yphantis, discussed in Yphantis, 1964), and heterogeneity is more readily overlooked. 20 to 40 µl of a protein solution of concentration 1 to 3 mg/ml were added to the solution side of the 12mm double sector synthetic boundary cell used for these experiments, and 0.45 ml of solvent was added to the other side. The run was abandoned if a sharp boundary did not form as the rotor was accelerated, since the time taken to reach equilibrium is very much longer if solute is carried up the long solution column by mixing.

The partial specific volume used for calculation of the molecular weight of the native enzyme was 0.734 ml/g,

(Bischofberger et al. 1970; Hunsley and Suelter, 1969a). The value used for the subunits in solutions of 6M guanidinium chloride was 0.724 ml/g (Ashton and Peacocke, 1971). The densities of the solutions were measured by pycnometry for the evaluation of $(1-\bar{v}\rho)$.

i) Hydrodynamic Measurements

(i) Sedimentation Velocity

A Beckmann-Spinco Model E analytical ultracentrifuge was used for sedimentation velocity experiments. The rate of migration of a macromolecule in a gravitational field is described by the equation:

$$\frac{dr}{dt} = r.\omega^2.s \qquad (2.6)$$

where 's' is the sedimentation coefficient, expressed in Svedbergs (i.e. units of 10^{-13} seconds). 's' can be shown to be given by:

$$s = M.(1-\bar{v}\rho)/N.f \qquad (2.7)$$

where N is Avogadro's number and f is the molecular frictional coefficient. Experimentally, the sedimentation coefficient is determined from the rate of migration of the boundary between solvent and the sedimenting solute. Although the exact point on the boundary which should be measured is the second moment, it is conventional and sufficiently accurate in this context to measure the peak on the Schlieren image.

Photographs of the sedimenting boundary were taken at eight minute intervals. The rotor thermostat (the RTIC unit) was set, after the rotor reached speed, to maintain the temperature indicated at that time. The sedimentation coefficient was calculated from the gradient of the regression line through a

plot of ln(r) against time. Observed sedimentation coefficients were corrected to the standard conditions of water at 20°C as solvent by means of the equation:-

$$s_{20,w} = s_{T,s} \times \frac{\eta_s}{\eta_w} \times \frac{\eta_{T,w}}{\eta_{20,w}} \times \frac{(1-\bar{v}\rho_w)}{(1-\bar{v}\rho_s)} \qquad (2.8)$$

where η_s/η_w is the relative viscosity of the solvent, and $\eta_{T,w}/\eta_{20,w}$ is the viscosity of water at temperature T relative to 20°C. The sedimentation coefficient was concentration-dependent; the value at infinite protein dilution, $s^o_{20,w}$, was determined by a linear extrapolation of values $s_{20,w}$ against the initial concentrations of the respective protein solutions. The viscosity values needed for equation2.8 were obtained from published tables (e.g. Svedberg and Pedersen, 1940; Handbook of Biochemistry, 1970). Densities were measured by pycometry.

(ii) Diffusion Coefficient

The diffusion coefficient of a solute may be shown to be related to a molecular parameter, the frictional coefficient, which also enters into the expression for sedimentation velocity:

$$D = \frac{R.T}{N.f} \qquad (2.9)$$

where 'N' is Avogadro's number, and 'f' the molecular frictional coefficient. The frictional coefficient can be eliminated between equations 2.7 and 2.9 to give the following expression, known as the Svedberg equation:

$$\frac{s}{D} = \frac{M(1 - \bar{v}.\rho)}{RT} \qquad (2.10)$$

Thus the combination of two hydrodynamic measurements on a solution of a macromolecule gives a value for the molecular weight. If the solute is heterogeneous, the result is between a number-average and a weight-average molecular weight. D has often

been determined from measurements of the rate of diffusion of a sharp boundary formed between a solution and its solvent in a centrifuge cell, but the method described here is the intensity fluctuation spectroscopy of scattered light. The following account of the theory of this method is based on those of Foord et al. (1970) and Ford (1972). (The first of these references describes the application of this technique to the measurement of the diffusion coefficients of several proteins). Reference is made here solely to the application of the method to the determination of the translational diffusion coefficient of a rigid globular particle.

When light is scattered from a solution of macromolecules, the frequency of the light is Doppler-shifted by the Brownian motion of the molecules. Shifts are of the order of 10^3 Hz relative to the light frequency of about 5×10^{14} Hz, but this is detectable if the line width of the incident light is sufficiently narrow, as is the case with a laser source. One method of analysing the frequency-shifted light is to use optical beating techniques to bring the spectrum of scattered light to audio- or radio- frequencies. There are several methods by which this may be achieved, but the apparatus in this laboratory depended on the self-beating phenomenon, where the light scattered from the solution is focussed on the surface of a photomultiplier, and the different components present beat against one another.

Although the power spectrum of the photomultiplier signal may be analysed, the information present in the spectrum is also present in the distribution in time of photon detection events at the anode of the photomultiplier. The probability of photon detection is proportional to the intensity of the light, so that

the fluctuations in intensity caused by the beating of the Doppler-shifted frequencies are shown by bunching of the train of pulses at high intensities, and thinning out at low intensities. The frequencies present in these intensity fluctuations could be found by comparing the train of pulses with itself at a later time; the "bunchings" will match up one cycle later if a given frequency is present. If the signal at time 't' is multiplied by the signal at time $(t+\tau)$, the time-averaged value of the product as a function of τ is referred to as the auto-correlation function $G^{(2)}\tau$. This function contains the information present in the power spectrum, since it is its Fourier transform. The relationship between $G^{(2)}\tau$ and the diffusion coefficient of a macromolecule in the scattering solution is derived by consideration of the transient, microscopic fluctuations of the concentration (and therefore of the refractive index) of the solution (Ford, 1972). In this derivation, it is assumed that there are spontaneous random changes in the local concentration of the solution which do not contribute to the observed correlation function, since their time-average will be zero. However, the decay of these random fluctuations is a systematic change, an approach towards equilibrium at a rate determined by the diffusion coefficient, so that a correlation coefficient defined as the product of the concentration at a given point at time t=0, and the concentration at the same point at a later time $t = \tau$, will decay with a time constant related to D. Since it is microscopic fluctuations of concentration which are responsible for the scattering of light from solutions, the latter correlation function can be related to that defined previously for the intensity of scattering, and the following expression is obtained:

$$G^{(2)}\tau = I_\theta^2 \cdot \left[1 + \exp.(-2DK^2\tau)\right] = I_\theta^2 . g^{(2)}\tau. \qquad (2.11)$$

'I_θ' is the result derived for the integrated intensity of light scattered by small molecules at angle θ (Tanford, 1961). i.e.

$$I_\theta = \frac{4\pi^2 kTn^2 c\left(\frac{\partial n}{\partial c}\right)^2 \bar{v} \sin^2\phi}{\lambda^4 (-\partial\mu/\partial c)} \cdot \left[\frac{l.A.I_o}{r^2}\right] \qquad (2.12)$$

with 'λ' = wavelength of incident light, and 'I_o' its integrated intensity; 'n' = refractive index of the solution; 'ϕ' = the angle between the plane of polarisation of the incident light and the direction of scattering; 'l' = pathlength of light in the solution; 'A' = the area of the detector, and 'r' the distance between the solution and the detector. K is also familiar as the scattering vector in conventional light scattering, i.e.:

$$K = \frac{4\pi n . \sin(\theta/2)}{\lambda} \qquad (2.13$$

where 'θ' is the scattering angle.

In the "Malvern" digital auto-correlator used for these experiments, the process of calculating the auto-correlation function is simplified by the process known as "clipping". The signal is collected for an interval of time - the sample time, t - and the number of photons arriving in that time is counted. A "clipping level" is selected - ideally the mean number of photons arriving per sample time - and if the number of photons in the sample is greater than the clipping level, a 'l' is recorded, whereas if the number is equal to, or less than, the clipping level, a 'O' is recorded. The 'l' or 'O' is then used to multiply each of the clipped signals from the previous 'i' sample times, which are contained in the 'i' channels of a "shift register", and the products are added into the 'i' channels of the store. The contents of the shift register are

moved along one channel to accommodate the latest sample, and the whole process is repeated for the next sample. Thus 'i' discrete values of the product at τ = t, 2t,.....it are accumulated in the storage register, and the experiment is continued until sufficient counts have been recorded to give the required statistical accuracy. It will be noted that the process of multiplication has been reduced to binary multiplication. Although this appears to discard a large amount of the available information, there is, in fact, little loss in statistical accuracy. The contents of each channel are recorded at the end of the experiment, and are divided by the product of the total photon count and the total clipped count to give the normalised auto-correlation function, $g^{(2)}\tau$. Equation 2.11 should contain further terms which take account of the finite length of the sample time and the finite sizes of the sample volume and the detector, but the exponent is not modified. Thus a plot of $\log.(g^{(2)}\tau-1)$ against τ will have a slope of $-2.K^2.D$. The observed diffusion coefficient is corrected to 20^{o} and water as solvent:

$$D_{20,w} = D_{T,s} \times \frac{\eta_s}{\eta_w} \times \frac{\eta_{T,w}}{\eta_{20,w}} \times \frac{293}{T(^{o}K)} \qquad (2.14)$$

If concentration-dependence is observed, the diffusion coefficient should be extrapolated to zero solute concentration.

Enzyme solutions used in these experiments were clarified by centrifugation for 60 min at 40,000 rpm in an MSE Superspeed centrifuge using a 3 x 5 ml swing-out rotor. After centrifugation, 1 ml of solution from near the middle of the centrifuge tube was transferred to a scrupulously clean spectrofluorimeter cell by means of a pasteur pipette attached by rubber tubing to a syringe. The pipette was mounted rigidly on a jack which allowed it to be

lowered to the desired level in the tube without significant disturbance of the tube contents.

The light source used was a 30 mW Scientific Cook He-Ne laser, and for the most dilute protein solution studied (1 mg/ml), the scattered photon count rate was 3,400/s. Sampling times of either 10 or 5 μs were selected, and 10^8 sample times were accumulated in the 24 channels of a "Malvern" auto-correlator (Precision Devices and Systems, Malvern). The theoretical statistical inaccuracy in $g^{(2)}\tau$ was less than 1%, and linearity of the plots of $\log(g^{(2)}\tau-1)$ against τ indicated that the scattering from dust was negligible. The temperature was maintained at 19.6°C by thermostatting the refractive index matching bath surrounding the cell.

j) Proton Relaxation Enhancement Studies

When placed in a magnetic field, an assembly of nuclei such as protons with a spin quantum number of a ½, will distribute between two energy levels which correspond to the individual nuclear magnetic moments having a component either parallel or antiparallel to the external field. Although the energy difference between these levels is small compared with kT in magnetic fields of the strength available for nuclear magnetic resonance (NMR) spectrometers, it is sufficient for there to be a slight excess of nuclei in the lower-energy, or ground, state so that the assembly as a whole possesses a net magnetic moment (M_o) in the direction of the field, with no net component in the plane perpendicular to this ($M_{xy} = 0$). Absorption of electromagnetic radiation of the appropriate frequency (in the radio frequency range) by the nuclei causes an increase in the population of the excited state (Fig. 2.1). After irradiation has ceased, the rate of return of the assembly to the ground-state

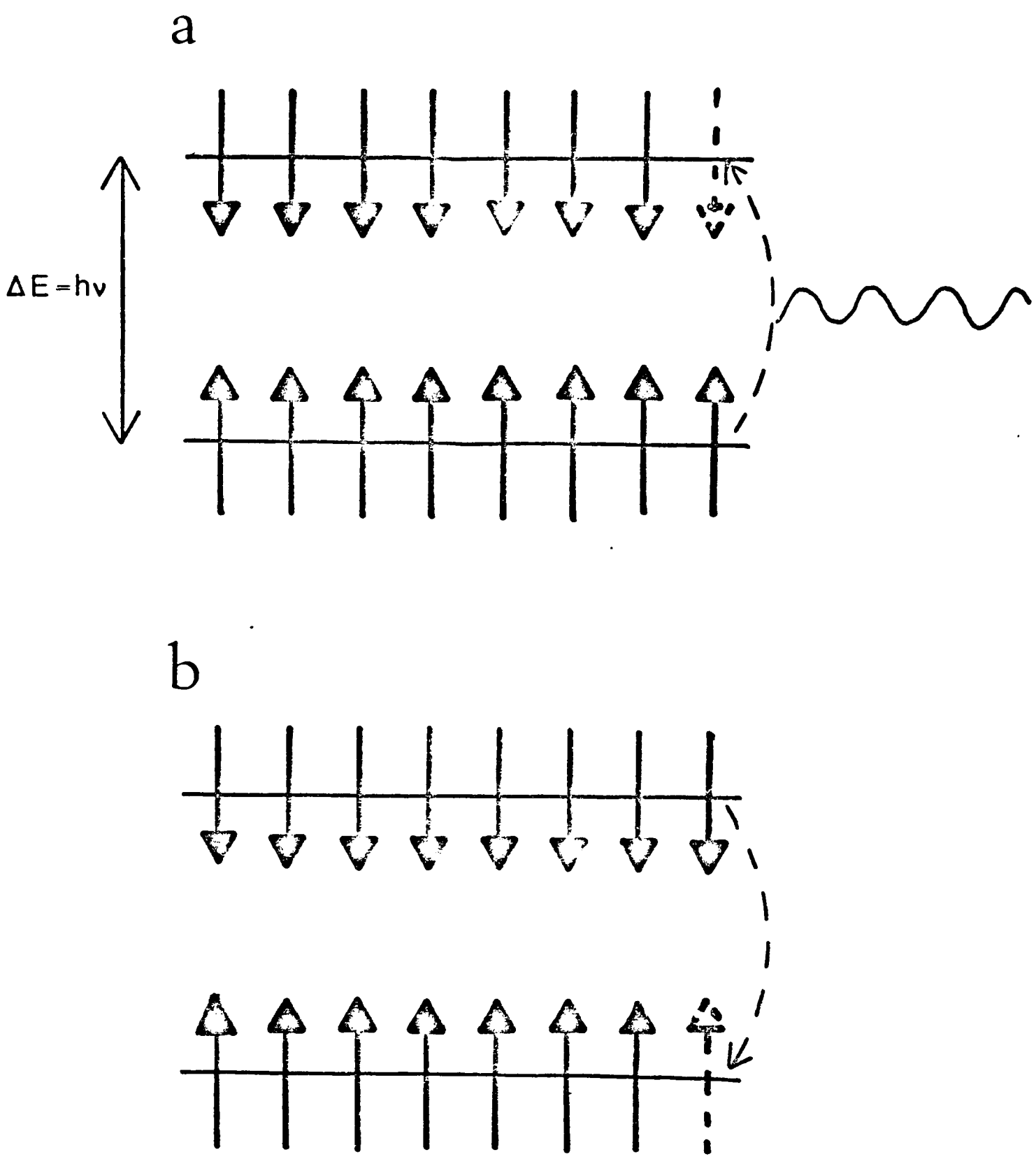


FIG. 2.1 Spin Lattice Relaxation. The arrows represent individual nuclear spins. (a) Increase in the population of the excited state by absorption of radio-frequency radiation (b) Return to the original distribution by spin-lattice relaxation.

distribution (Fig. 2.1) is governed by the "spin-lattice relaxation time", T_1. This is defined from the rate of recovery of the component of the net magnetic moment in the direction of the external field (M_z) to its equilibrium value, it being assumed that the process is exponential so that:

$$\frac{d\,M_z}{dt} = - \frac{M_{z(t)} - M_o}{T_1} \qquad (2.15)$$

(Bloch, 1946). NMR spectrometers are insensitive to the intensity of magnetisation in the z-direction, but Carr and Purcell (1954) devised methods for measuring relaxation times using short, intense pulses of radiation and the phenomenon of the "spin echo" (Hahn, 1950).

Spin-lattice relaxation occurs by transitions between the energy levels brought about by that component, with the same frequency as that required for absorption, of the fluctuating magnetic fields which arise from the thermal motion of the surrounding nuclear magnetic dipoles. The addition of a paramagnetic ion such as Mn^{2+} to an aqueous solution increases the relaxation rate of the water protons because the magnetic moment of the unpaired electron is about a thousand times greater than that of a nucleus, and so its contribution to magnetic 'noise' is correspondingly greater. The Solomon-Bloembergen equation for T_1 in the presence of a paramagnetic ion may be written in an abbreviated form as:

$$\frac{1}{T_1} = \frac{f(\tau_c)}{r^6} + f(\tau_e) \qquad (2.16)$$

τ_c is the correlation time for the modulation of the dipolar interaction between the nucleus and the ion at distance 'r', and is given by:

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m} \qquad (2.17)$$

where τ_r is the correlation time for the rotational motion of the nucleus relative to the ion, τ_s is the electron spin relaxation time and τ_m is the lifetime of a nucleus in the co-ordination sphere of the ion. Similarly, τ_e is the correlation time for the modulation of the scalar interaction that depends on the electron spin density at the nucleus, and is also a composite term:

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_m} \qquad (2.18)$$

For the Mn^{2+} -aquo complex, the rate of exchange ($1/\tau_m$) of water protons between the first co-ordination sphere and the bulk solvent is fast compared with T_1, and the contribution of the paramagnetic ion to relaxation outside this sphere is relatively negligible, so that the observed relaxation time of the bulk water ($T_{1,obs}$) is given by the weighted average of the paramagnetic contribution to the relaxation rate ($1/T_{1,p}$) and the normal diamagnetic relaxation rate in the absence of the ion ($1/T_{1,(O)}$); i.e.

$$\frac{1}{T_{1,obs}} = \frac{1}{T_{1,p}} + \frac{1}{T_{1(O)}} = \frac{p.q}{T_{1,M}} + \frac{1}{T_{1(O)}} \qquad (2.19)$$

where $1/T_{1,p}$ has been approximated by $p.q/T_{1,M}$, with p the mole fraction of Mn^{++}, q the number of water molecules in the co-ordination sphere, and $T_{1,M}$ the relaxation time of protons in the first co-ordination sphere.

The presence of a macromolecule (indicated by the superscript *) to which Mn^{++} can bind allows the possibility of two contributions to $T_{1,p}$ - that from relaxation in the co-ordination sphere of free Mn^{2+} (indicated by the subscript 'f'), and that from relaxation in the co-ordination sphere of Mn^{2+} bound to the macromolecule (indicated by 'b'). Thus:

$$\frac{1}{T^*_{1,p}} = \frac{p_f \cdot q}{(T^*_{1,M})_f} + \frac{p_b \cdot q^*}{(T^*_{1,M})_b} \qquad (2.20)$$

Experimentally, $1/T^*_{1,p}$ may be found to be greater than $1/T_{1,p}$ for a given total concentration of Mn^{2+}, and a proton relaxation rate enhancement factor is defined as:

$$\varepsilon^* = \frac{\frac{1}{T^*_{1,p}}}{\frac{1}{T_{1,p}}} = \frac{\frac{1}{T^*_{1,obs}} - \frac{1}{T^*_{1,(O)}}}{\frac{1}{T_{1,obs}} - \frac{1}{T_{1,(O)}}} \qquad (2.21)$$

Combination of equations 2.19, 2.20 and 2.21 gives the relationship:

$$\varepsilon^* = \frac{[M]_b}{[M]_t} \cdot \varepsilon_b + \frac{[M]_f}{[M]_t} \cdot \varepsilon_f \qquad (2.22)$$

where $[M]_t$ represents the total molar concentration of Mn^{2+}. Here $\varepsilon_f = (T_{1,M})_f/(T^*_{1,M})_f$, and is assumed to be unity, so it follows that:

$$\varepsilon_b = \frac{q^* \cdot (T_{1,M})_f}{q \cdot (T^*_{1,M})_b} . \qquad (2.23)$$

Further, since the scalar contribution to 2.16 is small, ε_b may be expressed as:

$$\varepsilon_b = \frac{q^* \cdot (r)^6 \cdot f(\tau^*_c)_b}{q \cdot (r^*)^6 \cdot f(\tau_c)} \qquad (2.24)$$

Since q* must be less than q for binding to occur, the implication of $\varepsilon_b > 1$ is that $(\tau^*_c)_b > \tau_c$. This can arise because the rotational correlation time, which dominates τ_c for free Mn^{2+} ($\tau_r \approx 3 \times 10^{-11}$ sec), increases when the ion is bound to the macromolecule (which is tumbling more slowly, $\tau_r \approx 10^{-7}$-10^{-8} s) and may no longer be the dominant term in the expression for τ_c (cf. τ_s and τ_M for the Mn-aquo complex are both $\sim 10^{-8}$s). In principle, ε_b could contain information about the structure and dynamics of the ion-macromolecule complex (i.e. about q*, r*,

τ_M, τ_r and τ_s), but the difficulties in making an unambiguous analysis have been reviewed by Dwek (1972 and 1973).

However, enhancements can also be used empirically in much the same way as other spectroscopic parameters, and Mn^{2+} is particularly suitable for the study of enzymes which require Mg^{2+} for activity since its chemistry resembles that of the physiological activator. Thus it is possible to use equation 2.22 to analyse for the amount of Mn^{2+} bound to the enzyme, provided that the value of ε_b can be determined. Further, if the binding of ligands to the enzyme causes changes at the binding site for the metal, either because of direct co-ordination to the Mn^{2+}, or because of a conformational change in the enzyme, then this may be reflected in a change in the characteristic enhancement of the complex.

In the analysis of the binding of phosphoenolpyruvate and fructose diphosphate to pyruvate kinase, the following equilibria were potentially involved:-

$$K_D = (n_M \cdot [E]_f \cdot [M]_f) / [EM] . \quad (2.25)$$

$$K_{ES} = (n_s \cdot [E]_f \cdot [S]_f) / [ES] . \quad (2.26)$$

$$K_{MS} = ([M]_f \cdot [S]_f) / [MS] . \quad (2.27)$$

$$K'_{EMS} = (n_s \cdot [E]_f \cdot [MS]) / [EMS] . \quad (2.28)$$

$$K''_{EMS} = ([EM] \cdot [S]_f) / [EMS] . \quad (2.29)$$

$$K'''_{EMS} = ([ES] \cdot [M]_f) / [EMS] \quad (2.30)$$

Equation 2.22 was extended to include all possible species containing bound Mn^{2+} thus:-

$$\varepsilon^* = \frac{[M]_f}{[M]_t} + \varepsilon_b \cdot \frac{[EM]}{[M]_t} + \varepsilon_{MS} \cdot \frac{[MS]}{[M]_t} + \varepsilon_{EMS} \cdot \frac{[EMS]}{[M]_t} \quad (2.31)$$

(This nomenclature is related to that used by Mildvan and Cohn, 1966, in the following way:- $K_{ES} \equiv K_s$; $K_{MS} \equiv K_1$; $K'_{EMS} \equiv K_2$; $K''_{EMS} \equiv K_3$; $K'''_{EMS} \equiv K'_a$; $\varepsilon_{MS} \equiv \varepsilon_a$; $\varepsilon_{EMS} \equiv \varepsilon_t$.)

The analysis generally involved selecting the appropriate terms from equations 2.25 to 2.30 and combining them with the conservation equations (e.g.: $[M]_t = [M]_f + [MS] + [EM] + [EMS]$). Under appropriate circumstances equations 2.25 to 2.30 are not all independent, but in the case of an allosteric enzyme, for which there is the possibility that there are sites for M spatially distinct from sites for MS for example, it does not necessarily follow that EMS defined by equation 2.28 is the same set of species involved in equation 2.30.

The spin-lattice relaxation rates of the protons of water in the various solutions were measured by the null method using a spin-echo spectrometer which has been described by Bell (1967) and which was operated at 20°C and 35 MHz for the experiments described in Chapter 3 and at 20 MHz for those described in Chapter 5. Samples were made up in 0.08M KCl, 0.02M Tris-HCl (pH 7.2). Approximately 0.1 ml was required for accurate measurement.

ε_b was determined from the titration of a constant concentration of $Mn\,Cl_2$ with varying concentrations of yeast pyruvate kinase - an "E titration". Combination of equations 2.22 and 2.25 gives:

$$\frac{1}{(\varepsilon^* - 1)} = \frac{1}{n_M \cdot [E]_f} \cdot \frac{K_D}{(\varepsilon_b - 1)} + \frac{1}{(\varepsilon_b - 1)} \qquad (2.32)$$

Mildvan and Cohn (1963) proposed that ε_b could be determined from a linear extrapolation of $1/\varepsilon^*$ v. $1/[E]_t$ to infinite enzyme concentration on the basis that equation 2.32 can be approximated by:

$$\frac{1}{\varepsilon^*} = \frac{1}{[E]_t} \cdot \frac{K_D}{\varepsilon_b} + \frac{1}{\varepsilon_b} \qquad (2.33)$$

As pointed out by Dwek (1972), this approximation can be more suitable for a graphical solution than the approximation that $[E]_f = [E]_t$ in equation 2.32 when ε_b is determined by extrapolation of $1/(\varepsilon^* - 1)$ v. $1/[E]_t$. Both graphical methods were used to obtain an initial estimate of ε_b. Substitution of the conservation equation $n_M.[E]_t = n_M.[E]_f + [EM]$ in equation 2.32 leads to the quadratic:

$$n_M.[E]_t.(\varepsilon_b-1)^2 - ([M]_t + K_D + n_M.[E]_t)(\varepsilon^*-1(\varepsilon_b-1) + [M]_t.(\varepsilon^*-1)^2 = 0. \qquad (2.34)$$

If K_D and n_M are known, then this equation can be solved for ε_b for each value of ε^*, and the average taken for the titration. However, for a constant percentage experimental error in the measurements, there will be increasingly large variations in the value of ε_b as ε^* approaches 1, so that the average will be unfairly weighted. This can be avoided if a value is assumed for ε_b, ε^* calculated, compared with the observed value, and the sum of the squares of the percentage errors in prediction calculated (cf. Reed et al. 1970). The value of ε_b which gave the minimum sum of the errors was found by trial and error for given values of K_D and n_M. The errors were also minimised with respect to K_D and n_M, although the experimental results are relatively less sensitive to changes in the assumed values for these. The calculations were programmed on a Hewlett Packard 9100B calculator.

ε_b was also determined independently of any assumptions about K_D and n_M by measuring the concentration of free Mn^{++}, in solutions of which the enhancements were also measured, from

its electron spin resonance spectrum.

K_D and n_M were determined by a titration of a constant concentration of enzyme with varying concentrations of Mn^{2+} - an "M titration". Equation 2.22 was then used to derive the binding curve. Further analysis of the binding curves was attempted by the methods of Scatchard (1949) and Hughes and Klotz (1956).

The concentrations of solutions of $Mn\ Cl_2$ used in these experiments were calibrated with respect to standard solutions of $Mn\ SO_4$ by atomic absorption spectrophotometry, using a Perkin-Elmer model 303 instrument.

k) Gel Electrophoresis

Polyacrylamide gels were prepared in 15 x 0.5 cm glass running tubes by the polymerisation of "Cyanogum 41" catalysed by N,N,N',N'tetramethylethylenediamine and ammonium or potassium persulphate. The following systems were used:

(i) 0.37M Tris-glycine, pH 9.5, for both gel and tank buffers (Hjerten et al. 1965). Samples were dialysed against half-strength buffer before mixing with glycerol and bromophenol blue and application to the top of the gels. The gels were stained with 0.1% amido-black in 7% acetic acid for 1h, and then de-stained in about half an hour in a transverse electrophoretic de-stainer with 10% acetic acid as electrolyte.

(ii) 6M deionised urea and 0.9M acetic acid in the gels, and 0.9M acetic acid in the electrode vessels (Panyion and Chalkley, 1969). Samples were dialysed against the gel buffer plus 1% 2-mercaptoethanol before application to the gels. The gels were fixed and washed in 7% trichloroacetic acid, and then stained with amido-black as described above. Some of these gels

were also stained by the periodic acid - Sciff's base method of Fairbanks et al. (1971) to test for the presence of carbohydrate.

(iii) 0.1M phosphate-sodium, pH 7.2, 0.1% SDS for both gel and tank buffers. Samples were dissolved in 6M urea - 1% SDS - 1% 2-mercaptoethanol - 0.05M phosphate-sodium (pH 7.2) and dialysed against this buffer before electrophoresis. Bromophenol blue was added to the samples before application to the top of the gels. After removal from the tubes at the end of electrophoresis, the gels were cut to the length of the bromophenol blue front. They were then fixed and washed to remove SDS in 7% trichloroacetic acid, 25% propan-2-ol. Staining was with 0.1% Coomassie blue in 7% acetic acid, 25% propan-2-ol, and de-staining by washing in 7% acetic acid, 25% propan-2-ol. The mobility of the proteins was measured relative to the bromophenol blue marker, and was used to determine molecular weights from a calibration curve of log.(molecular weight) v. mobility obtained with proteins of known molecular weight (Shapiro et al. 1967; Dunker and Ruekert, 1969). The proteins used as standards are given in the Table on the next page (Table 2.1).

TABLE 2.1

Protein	Mol. Wt.	Reference
Human transferrin	76,600	Tanford (1968)
γ-Globulin, L + H chains	73,500	Rutishauser et al. (1968)
Rabbit muscle pyruvate kinase (subunits)	59,000	Steinmetz and Deal (1966)
γ-Globulin, H chains	50,000	Rutishauser et al. (1968)
Ovalbumin	45,000	Castellino and Barker (1968)
Aldolase (subunits)	37,000	Taylor and Lowry (1956)
Pepsin	35,000	Bovey and Yanari (1960)
Carbonic anhydrase	31,000	Lindskog (1960)
Chicken muscle triose phosphate isomerase	26,000	McVittie et al. (1972)
γ-Globulin, L chains	23,500	Rutishauser et al. (1968)
Myoglobin	17,200	Dayhoff and Eck (1968)

1) Cold-Lability and Inactivation by Fructose Diphosphate

Pyruvate kinase was dissolved in 0.1M Tris-HCl (pH 7.5) and chromatographed on a column of Sephadex G-25 equilibrated with the same buffer to remove any other cations. The enzyme solution was then diluted to a concentration of about 2 mg/ml. The effect of various conditions on the activity of the enzyme was studied by mixing equal volumes of the enzyme solution and of 0.1M Tris-HCl (pH 7.5) containing, where necessary, the additive to be studied. When the effect of low temperatures was to be studied, the enzyme solution at room temperature was added to a tube containing the diluent which had been pre-incubated at the lower temperature.

m) Identification of the N-terminal Amino-Acid

The method for the examination of the N-terminal residue was derived from Hartley (1970) and Gray (1972). Pyruvate kinase (about 10^{-5}M) was dialysed overnight against 8M urea - 0.25M $NaHCO_3$. Aliquots (0.15 ml) were reacted for 2 h at 37°C with an equal volume of 1-dimethylamino-napthalene-5-sulphonyl chloride (dansyl chloride) in acetone (20 mg/ml). The labelled protein was precipitated with 10% trichloroacetic acid, and the precipitate washed twice with 1M HCl. The protein was hydrolysed with 0.2 ml of 6M HCl at 110°C overnight in tubes sealed under vacuum, and the hydrolysate then dried down over NaOH.

The hydrolysate was dissolved in about 0.1 ml of pyridine-water (1:1), and applied to one corner of a 5 x 5 cm polyamide layer sheet. After drying the sample spot for 10 minutes, the chromatogram was developed with 1.5% formic acid in water. The sheet was then dried for ten minutes with a hot-air blower and then chromatographed perpendicular to the original direction with the following solvents:-

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

(ii) ethyl acetate-methanol-acetic acid (20:1:1, v/v).

(iii) 0.05M Na_3PO_4-ethanol (3:1, v/v).

The sheet was dried for 5 minutes after each solvent; the fluorescent spots were viewed under a mercury lamp and their positions recorded. Reference samples of dansylated amino-acids were run on the reverse side of the sheet.

n) Reactions of the Thiol Groups of the Protein

Pyruvate kinase was equilibrated with the buffers used for the reactions by chromatography on Sephadex G-25 (coarse). The reaction with 5,5'dithiobis-(2-nitrobenzoate), (DTNB, or

Ellmann's reagent, Ellmann, 1959) was carried out with excess reagent (0.8mM) in 0.1M Tris-HCl (pH 7.5) - 0.1M KCl. The reaction was started by the addition of sufficient enzyme solution to give a final concentration of about 1 μM in 1 ml of solution in a 1 cm spectrophotometer cuvette. The time course of the reaction was followed by the increase in absorbance at 421 nm with a Beckmann D.U.-Gilford spectrophotometer thermostatted at 25°C. After the reaction with native enzyme had reached completion, a 10% (w/w) solution of SDS was added to the cuvette to give a final concentration of 0.5%, and the reaction of the remaining thiol groups of the enzyme was followed. During the reaction with the native enzyme, the reoxidation of reduced DTNB occurred at a significant rate (see Chapter 5, section g). The rate of this reaction could be estimated from the rate of the fall in absorbance at 412 nm after the reaction with the protein was complete. The kinetics were treated as two consecutive first order reactions; the first is pseudo-first order because of the excess of DTNB, and the second may conveniently be represented as such since the fraction of the reaction observed was small:

$$-SH + DTNB \xrightarrow{k_1} -S\text{-}TNB + TNB \xrightarrow{k_2} \tfrac{1}{2}(DTNB)$$

Experimentally, it is [TNB] which is being measured, and k_1 is greater than k_2. In this case, an equation for [TNB] can be derived from the equation for consecutive first order reactions given in Moore (1963). The symbols used are: a_1, the initial concentration of protein thiol groups that react at rate k_1; a_t, the total number of thiol groups reacting in the native state, and so $(a_t - a_1)$ is the concentration of thiol groups which reacted on a time scale very short compared with that of the experiment. The initial equation is:

$$[TNB] = e^{-k_2 t} \cdot \left[\frac{k_1 . a_1 . e^{(k_2 - k_1)t}}{k_2 - k_1} + \text{constant} \right] \qquad (2.35)$$

Since $[TNB] = (a_t - a_1)$ at t=0, the constant must be:

$$(a_t - a_1) - \frac{k_1 . a_1}{k_2 - k_1} \qquad (2.36)$$

Since k_1 is much greater than k_2, $k_1/(k_2 - k_1) \simeq -1$, and the whole expression rearranges to give:

$$a_t . e^{-k_2 . t} - [TNB] = a_1 . e^{-k_1 . t} \qquad (2.37)$$

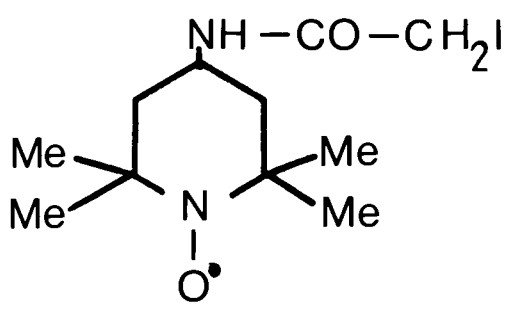
a_t was estimated from the end of the progress curve, where $a_1 . e^{-k_2 . t} \sim 0$, using the observed values of $[TNB]$ and k_2. Ln $\left(a_t . e^{-k_2 . t} - [TNB]\right)$ was plotted against time, to give ln a_1 as the intercept on the ordinate and $-k_1$ as the gradient. This treatment was not necessary for the reaction of the enzyme in the presence of SDS, as the reaction was more rapid and so the amount of air oxidation insignificant.

The reaction of the protein with iodoacetamide was carried out in 0.05M Tris-HCl (pH 7.5) - 0.05M KCl, with 1mM iodo $[1-^{14}C]$-acetamide of specific activity 2.37 mC/m.mole and a protein concentration of about 2 mg/ml. Samples (0.1 ml) were withdrawn at various times and the protein was precipitated with 5 ml of 10% trichloroacetic acid. The precipitates were washed once with 5 ml of 10% trichloroacetic acid, and the protein was dried overnight over silica gel at 37°C. The labelled protein was redissolved in 1M KOH (1.5 mls) and 1 ml samples were added to 'Aquasol' scintillation fluid (10 mls), together with 0.5 mls of glacial acetic acid to quench chemiluminescence. The samples were counted using an LKB Wallac scintillation counter, and the

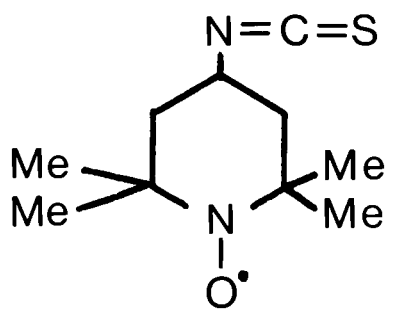
count rate was converted to moles of acetamide by reference to the count rate of an aliquot of the stock iodo$\left[1\text{-}^{14}C\right]$acetamide solution in the presence of unlabelled protein, KOH and acetic acid. The efficiency of counting was calculated as 80%. The concentration of the solution of iodo$\left[1\text{-}^{14}C\right]$acetamide was determined by its absorbance at 268 nm, relative to a calibration curve prepared from a standard solution of iodoacetamide. Count rates for the labelled proteins were in the range 1,500 to 20,000 cpm; the background count was about 60 cpm, and there was a contribution of about 100 cpm contributed by free iodoacetamide which passed through the washing procedure with the protein.

o) Electron Spin Resonance Spectra

The electron has a spin quantum number of $\pm$ ½, and in a magnetic field, an unpaired electron will show transitions between two energy levels, in an analagous fashion to the proton in the phenomenon of NMR. However, lower magnetic fields and higher frequency (microwave) radiation can be used for ESR because of the much higher magnetic moment of an electron compared with a proton. In recent years, a number of stable free radicals have been developed that can be covalently attached to proteins. Two such 'spin labels' were used in this present work; they were:



iodoacetamide spin label



isothiocyanate spin label.

In these compounds, the unpaired electron of the nitroxide radical

is coupled with the nuclear spin of the nitrogen atom to give a three line ESR spectrum. The shape of the spectrum depends on the mobility of the label and the polarity of the medium. The broadening of the spectrum as the mobility decreases is shown in Fig. 2.2; it can be seen that the high field line is broadened first as the mobility decreases, then the low-field line and finally the central line. These spectra are plotted in the derivative form, as is usual with ESR spectra.

When a spin-label is bound to a macromolecule, its mobility may be reduced, with a concommitant broadening of the spectrum; however, the flexibility of the linkage between the free radical and the macromolecule may allow considerable mobility of the former in spite of the constraint by the latter. The binding of ligands to a spin-labelled protein may cause changes in the mobility or local environment of the free-radical moiety that are reflected in measurable changes in the ESR spectrum. As in NMR, the low energy of the transition may result in very high sensitivity of the spectrum to a perturbation of very low energy.

There are three basic parameters of the spectrum that may be monitored: (i) the intensity, and thus any quenching; (ii) the shape of the spectrum, and thus any broadening, and (iii) the separation, or 'splitting' of the lines. Because the intensity of the spectrum is very sensitive to the orientation of the cell in the spectrometer, the measurement of the absolute heights of the peaks would not be adequately accurate for quantitating small changes in the spectrum. However, the three absorption lines differ in their sensitivity to perturbations (e.g. in mobility) and so the ratio of the peak heights may be used as a sensitive parameter of spectral

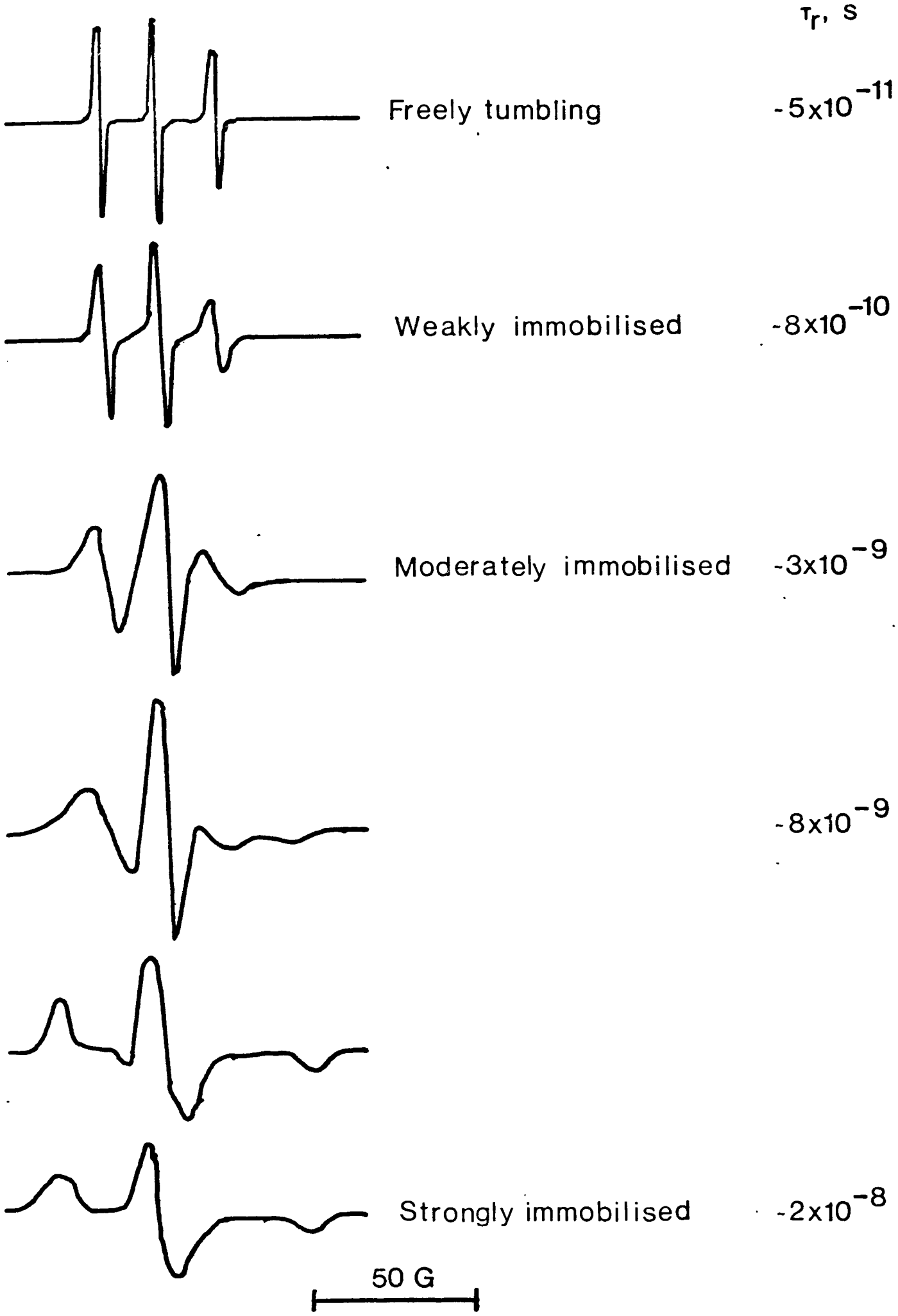


FIG. 2.2 The effect of mobility on the 9.5 GHz spectra of the spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl. The approximate rotational correlation times are given, and the descriptions commonly applied to such spectra indicated. (From Dwek, 1973).

changes (e.g. Jones et al. 1972).

ESR spectra of Mn^{2+} were also measured to determine the concentration of free metal in solutions also containing pyruvate kinase. The spectrum of Mn^{2+} bound to the protein is broadened so as to be undetectable in these conditions, and the free concentration of the ion was obtained from a calibration curve of peak height v. concentration.

The spectra were run on either a Varian E4 spectrometer, or a JEOL spectrometer, operating in X-band.

CHAPTER 3

STUDIES ON PYRUVATE KINASE PREPARED BY THE METHOD OF ASHTON (1971)

a) Preparation of the Enzyme

Yeast pyruvate kinase was prepared by the method that was devised in this laboratory by I.K. Ashton and which is summarised in Chapter 2. The method as originally described was variable in both total yield and specific activity of the enzyme, and the following modifications were made so that a specific activity of about 200 $U.mg^{-1}$ (determined by assay method 1, Chapter 2) could be obtained routinely:

(i) The yeast cytolysate was stored overnight at $4^{o}C$, instead of at room temperature.

(ii) Before chromatography on the CM-cellulose column, the crude ammonium sulphate precipitate was dissolved in a minimal volume of 1M potassium acetate, pH 6.2, to adjust the pH of the protein, and chromatographed on a column of Sephadex G-25 (coarse grade, 100 ml) to remove salts and equilibrate the solution with 5mM potassium acetate, pH 6.2. This resulted in some dilution of the protein solution, but larger ion-exchange columns were used than originally specified by Ashton (1971).

(iii) Similarly, the protein was equilibrated with 0.05M Tris-HCl, pH 7.2, by chromatography on a small column of Sephadex G-25 before chromatography on DEAE-cellulose. Although the preparation was not, even then, completely reliable, it was possible to obtain yeast pyruvate kinase with a specific activity of 200 $U.mg^{-1}$ in a yield which varied up to the equivalent of

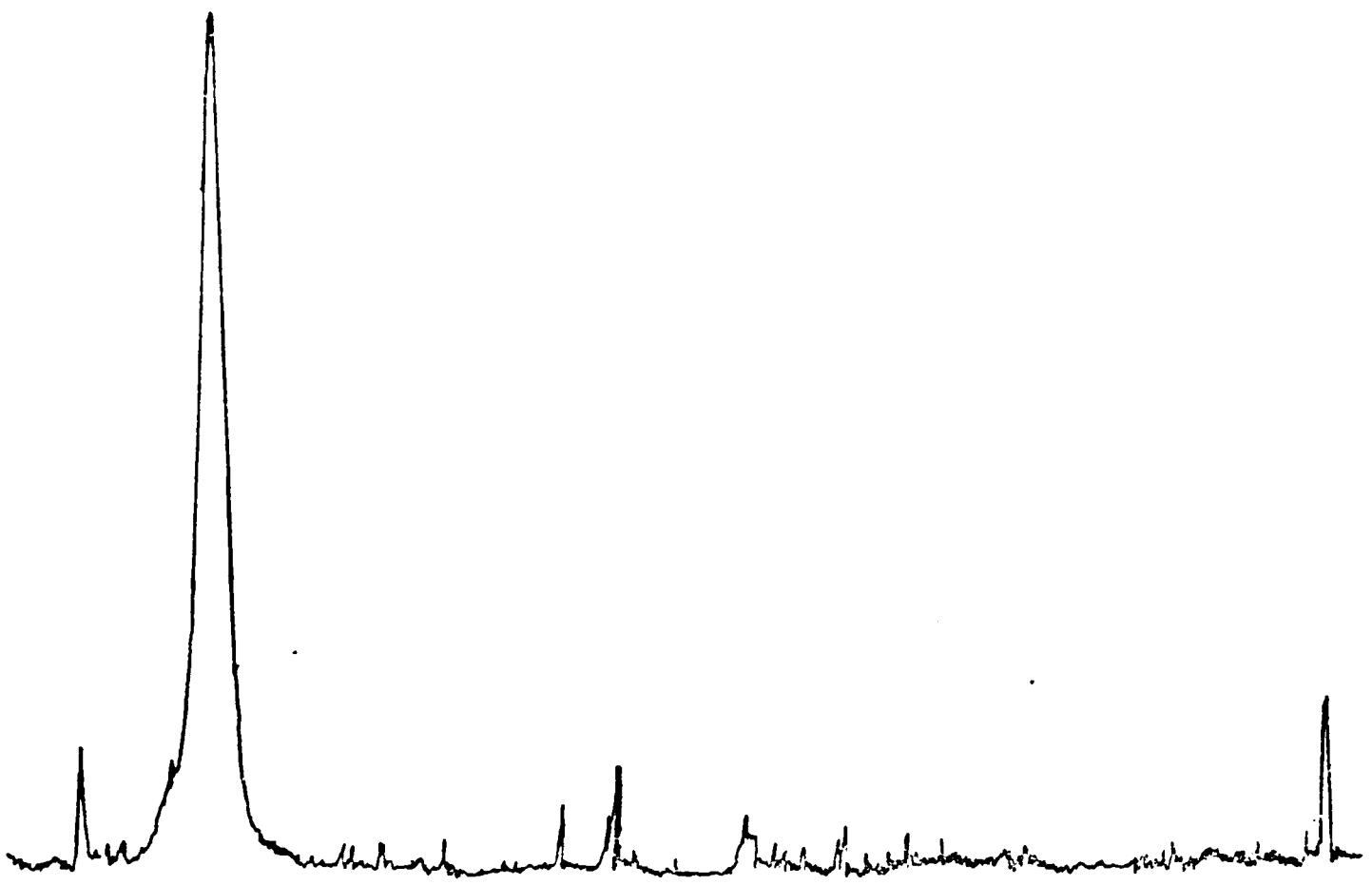


FIG. 3.1 Polyacrylamide-gel electrophoresis of yeast pyruvate kinase prepared by Ashton's method. Micro-densitometer scan of a 6% gel run in 0.2M Tris-glycine, pH 9.5.

600 mg per kg of yeast. This specific activity was comparable with the values obtained by Haeckel et al. (1968) and Hunsley and Suelter (1969a).

The purity of the preparation was checked by electrophoresis in 0.2M Tris-glycine, pH 9.5, on 6% polyacrylamide gels. Usually the protein electrophoresed as a single band of low mobility, but sometimes traces of more mobile impurities were detected. A densitometer trace of a typical gel is shown in Fig. 3.1. The pyruvate kinase was not used for further experiments if the contamination appeared to be greater than 5% from the relative intensity of the staining of the bands.

b) Cold Lability

Experiments were conducted to determine whether the marked loss of enzyme activity in the cold was catalysed by the presence of the allosteric effector, fructose diphosphate, as had been reported by Kuczenski and Suelter (1970a) for pyruvate kinase prepared from S. cerevisiae by the method of Hunsley and Suelter (1969a). It was confirmed that the presence of 1mM fructose diphosphate resulted in an acceleration of the loss of enzyme activity of solutions of the enzyme in 0.1M Tris-HCl, pH 7.5, both at room temperature and at 4°C. (The results were similar to those shown in Fig. 5.8, Chapter 5). This was further confirmation of the similarity of the enzyme prepared in this laboratory to that obtained by Suelter et al., and was another point of difference from the results of Hess and his co-workers who found that the specific activity of the enzyme was not affected by fructose diphosphate (B. Hess, personal communication).

c) Identification of the N-terminal Amino-Acid

Chromatography of the acid hydrolysate of dansylated pyruvate kinase gave a green fluorescent spot with the same chromatographic behaviour as a reference sample of dansyl-alanine. The other spots visible on the polyamide sheet under a mercury lamp were: dansylic acid (fluorescing blue); O-dansyl tyrosine (fluorescing yellow); dansyl amide (fluorescing green); and ε-dansyl lysine (green). The positions of these spots on the chromatograms were similar to those given by Hartley (1970). This confirmed that the identification of the N-terminal amino-acid as arginine by Ashton (1971) was an error resulting from a confusion of this with ε-dansyl lysine, since neither dansyl arginine or any other dansylated amino-acid was detected. Alanine was the sole N-terminal amino-acid detectable in these preparations of yeast pyruvate kinase.

d) Proton Relaxation Enhancement Studies

(i) Binary Complexes

Titrations of 100 μM $MnCl_2$ in 0.08M KCl, 0.02M Tris-HCl, pH 7.2, with pyruvate kinase from three separate preparations are shown in Fig. 3.2. The results almost lay on a straight line when plotted as $1/(\varepsilon^* - 1)$ against $1/[E]_t$, and were extrapolated to an ε_b of about 3.5 at infinite enzyme concentration. The best value of ε_b obtained by a least-squares fit to the results, by the method given in Chapter 2, was within the range 3.4 to 3.6 for values of n_M in the range 4 to 12 and values of K_D chosen to optimise the fit for each value of n_M.

K_D, the dissociation constant, and n_M, the number of the binding sites on the enzyme, for Mn^{2+} were determined by two titrations of a constant concentration of enzyme with $MnCl_2$. The

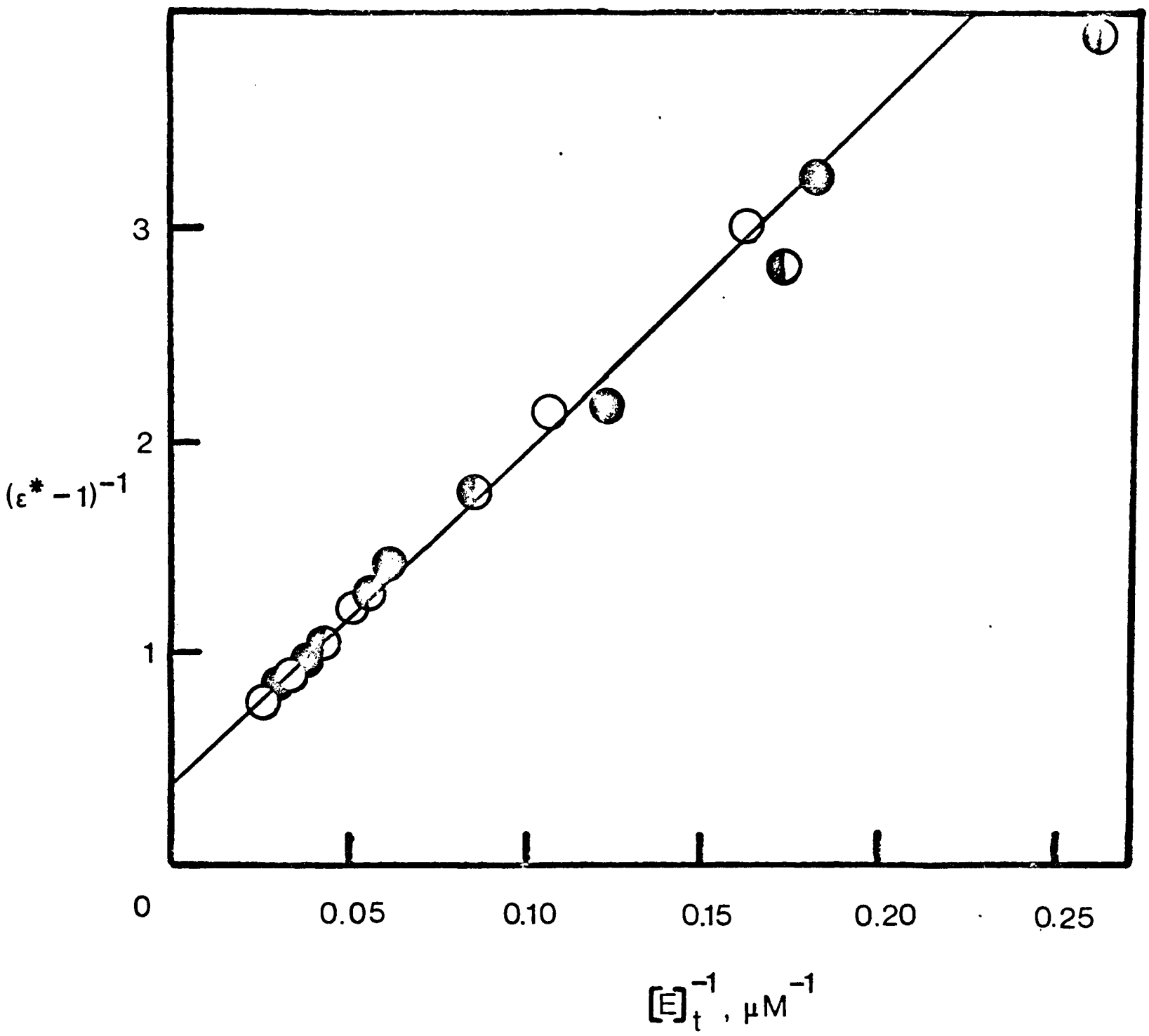


FIG. 3.2 Proton relaxation enhancement titration of $MnCl_2$ with pyruvate kinase. Three separate preparations were used to titrate a constant concentration, 100μM, of $MnCl_2$. The line is calculated for K_D = 275 μM, n_M = 9, and ε_b = 3.57.

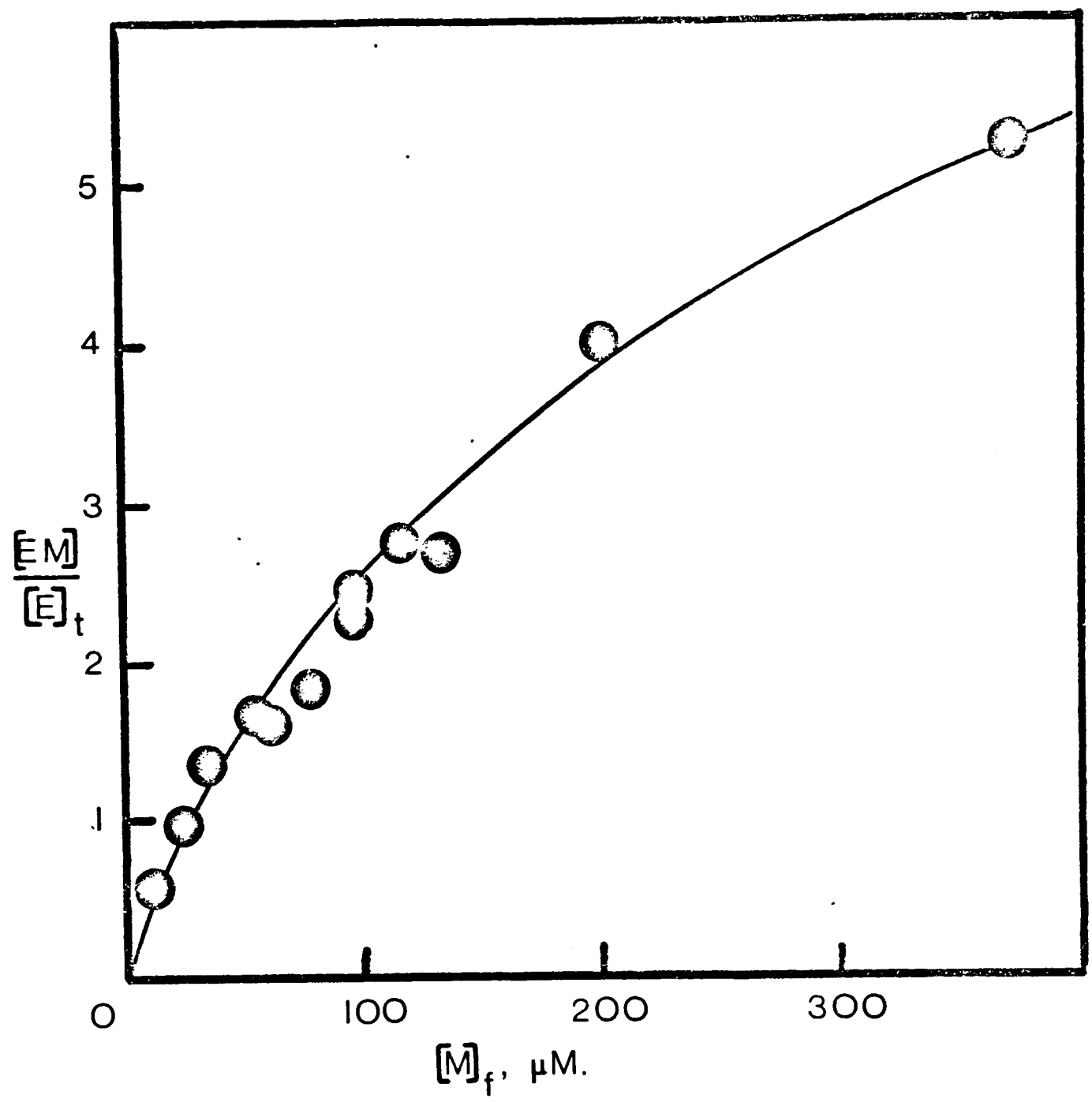


FIG. 3.3a The binding of Mn^{2+} to pyruvate kinase.

The binding curve derived from proton relaxation enhancement measurements. The line is that calculated for $n'_M = 1$, $K'_D = 40$ μM, $n''_M = 9$ and $K''_D = 400$ μM.

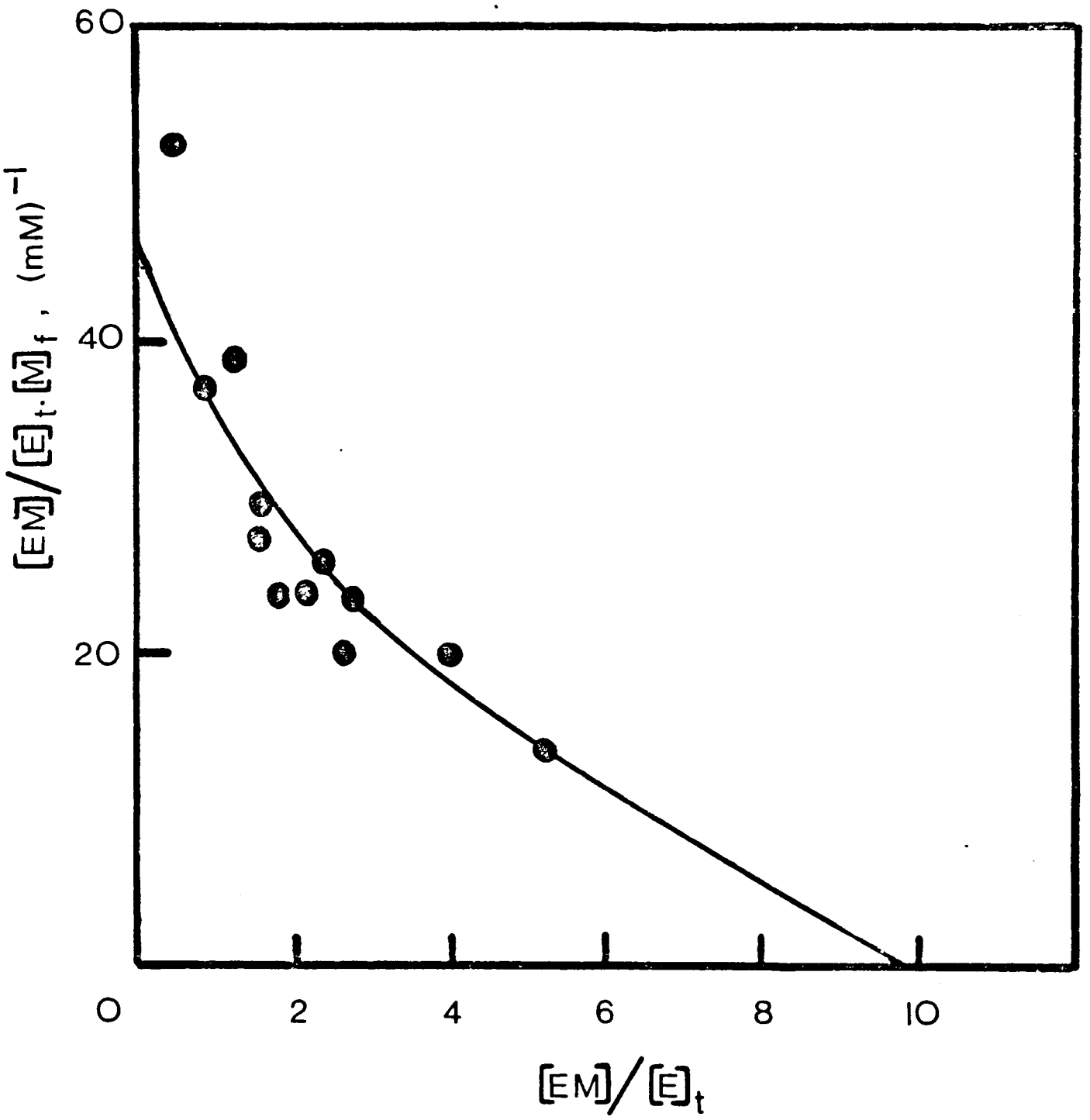


FIG. 3.3b The binding of Mn^{2+} to pyruvate kinase. The results of 3.3a as a Scatchard plot. The line is calculated for the same parameters as that in 3.3a.

concentrations of free Mn^{2+} in the solutions used for one of the titrations were measured from the electron spin resonance signal of the metal ion. The combination of these measurements with the corresponding values of the enhancements, ε^*, gave a value for ε_b of 3.5 (± 0.4, standard deviation) which is independent of any assumption about the nature and the parameters of the binding curve. There was no indication that ε_b varied for different degrees of saturation of the enzyme with Mn^{2+}. The binding curves were then calculated from the enhancement values using $\varepsilon_b = 3.5$. For values of $[M]_b/[E]_t$ greater than 1.5, both a Hughes-Klotz plot and a Scatchard plot (Fig. 3.3) of the results were approximately linear and were fitted by a regression line corresponding to $n_M \approx 9$ and $K_D \approx 260$ μM. However, the Scatchard plot is evidently curved, and a unique analysis of the whole binding curve is not possible. Curves calculated for two types of independent binding sites according to the equation:

$$\frac{[M]_b}{[E]_t} = \frac{n'_M}{K'_D/[M]_f + 1} + \frac{n''_M}{K''_D/[M]_f + 1}$$

will fit the results for $n'_M = 1$ or 2, K'_D less than 50 μM, $n''_M = 6$ to 9, and K''_D greater than 400 μM. Since the composition of the solutions for the E titration was such that $[M]_b/[E]_t$ was equal to, or greater than 1.5, it was assumed that they could be fitted for one type of binding site with $n_M = 9$, and a final refinement of the fitting procedure then gave $\varepsilon_b = 3.5\text{-}3.57$ with $K_d = 265\text{-}275$ μM. The 'error contours' for this fitting procedure are shown in Fig. 3.4.

The parameters ε_{MS} and K_{MS} for phosphoenolpyruvate were determined from a titration of 100 μM $MnCl_2$ with phosphoenol-

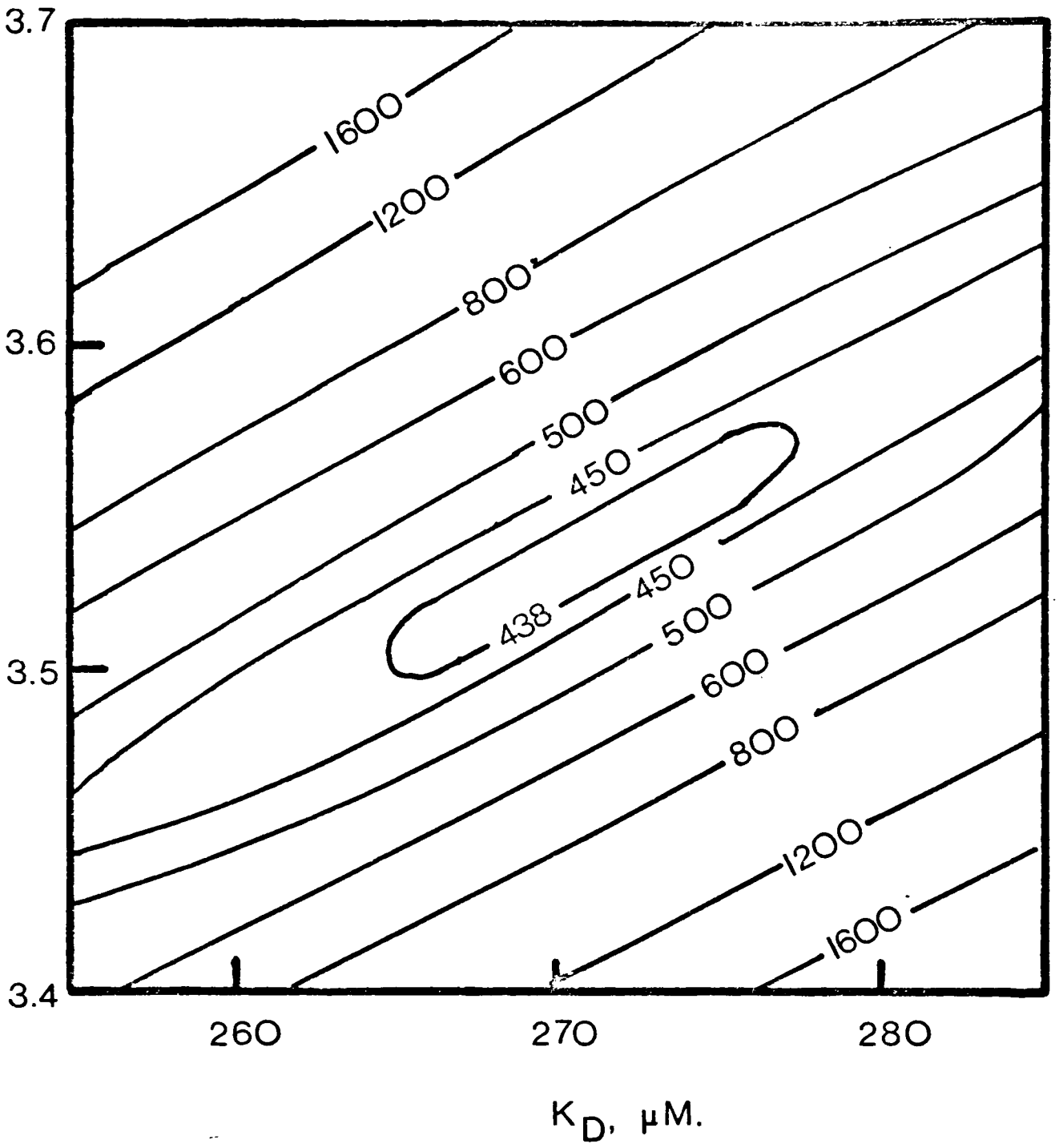


FIG. 3.4 Error contours for the fitting procedure for the determination of ε_b. The contours are labelled with the values of

$$\sum \left[\frac{\varepsilon^*_{calculated} - \varepsilon^*_{observed}}{\varepsilon^*_{calculated}} \right]^2 \times 10^6$$

determined for the results shown in Fig. 3.2, using $n_M = 9$, and 50 combinations of ε_b and K_D within the range of the graph.

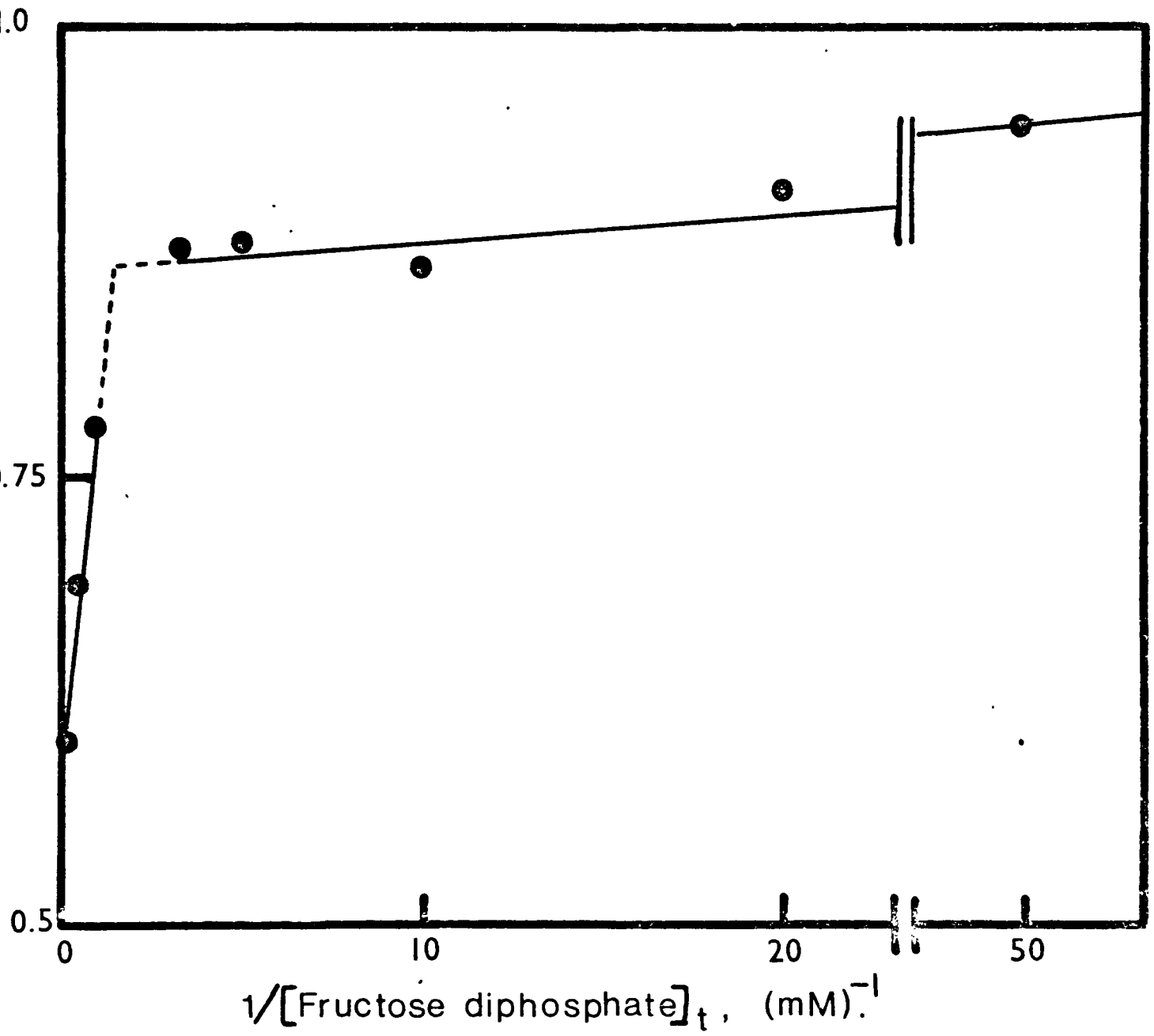


FIG. 3.5 Proton relaxation enhancement titration of 100 μM Mn^{2+} with fructose diphosphate

pyruvate in the concentration range 1-20 mM. The results were fitted in the same way as the E titration, using the assumption that there was only 1 binding site for Mn^{++} per molecule of phosphoenolpyruvate, and K_{MS} was found to be 3.25 mM with $\varepsilon_{MS} = 1.23$. These figures compare with $K_{MS} = 1.15$ mM and $\varepsilon_{MS} = 1.15 \pm 0.4$ at pH 7.5 reported by Mildvan and Cohn (1966), and $K_{MS} = 1.79$ mM found by Wold and Ballou (1957) for the fully ionised form of phosphoenolpyruvate, based on the assumption that only this form was effective in binding the divalent metal ion. (The pK of the last ionisation of phosphoenolpyruvate is 6.4, so a solution of the compound is not fully ionised at pH 7.2).

A similar titration to estimate ε_{MS} and K_{MS} for fructose diphosphate was obviously biphasic when $1/\varepsilon^*$ or $1/(\varepsilon^*-1)$ was plotted against the reciprocal of concentration (Fig. 3.5). Such biphasic titrations are observed in other cases, although generally at higher ligand concentrations (Dwek, 1972). It was only possible to predict the observed enhancements by assuming that both phosphate groups were able to bind Mn^{++}, and that FDP.$(Mn)_2$ was formed at low fructose diphosphate concentrations, whilst $(FDP)_2$.Mn was formed at higher concentrations. The equations for the equilibria were solved by making the approximation that in the range where the second equilibrium becomes important, the only forms of Mn in the solution were FDP.Mn and $(FDP)_2$.Mn (Appendix 1). The observed enhancements were predicted by setting $\varepsilon_{MS} = 1.14$, $K_{MS} = 15$ μM, $\varepsilon_{MS_2} \approx 1.8$ and $K_{MS_2} \approx 6$ mM. Benkovic et al. (1972) observed $\varepsilon_{MS} = 1.7$ and $K_{MS} = 2.7$ mM for fructose diphosphate, but did not report a low-concentration phase in the titration.

These results are summarised in the following Table:

TABLE 3.1

Ligand	n_M	Dissociation constant, K_{EM} or K_{MS}	Enhancement, ε_{EM} or ε_{MS}
Pyruvate kinase	1 or 2	< 50 μM	3.5 - 3.57
	6 - 9	>400 μM	"
phosphoenol-pyruvate	1 (assumed)	3.25 mM	1.23
Fructose diphosphate	2	15 μM	1.14
	2	≈ 6 mM	1.8

(ii) Ternary Complexes

Proton relaxation enhancement titrations of the enzyme with phosphoenolpyruvate were carried out in the presence of 100 μM and 300 μM Mn^{++} (Fig. 3.6). At the enzyme concentrations employed, this corresponds to values of $[M]_b / [E]_t$ of about 2 and about 4 respectively. Since the normalised titrations are superimposable, it would appear that there is no linkage between the substrate and the metal saturation functions; in terms of the equilibrium constants defined in Equations 2.25-2.30 (Chapter 2), K''_{EMS} must equal K_{ES}, since the relative proportions of $[E]_f$ and $[EM]$ are different for the two titrations. It was therefore possible to calculate the value of the enhancement of the ternary complex from the observed enhancement at the plateau of the titration by an analytical solution of the equilibria involved, assuming that the amount of substrate bound to the enzyme was not a significant fraction of the total, and that the affinity of the enzyme for Mn^{++} was unchanged by the binding of the substrate. Thus equations 2.25 and 2.27 from Chapter 2 were combined with the conservation

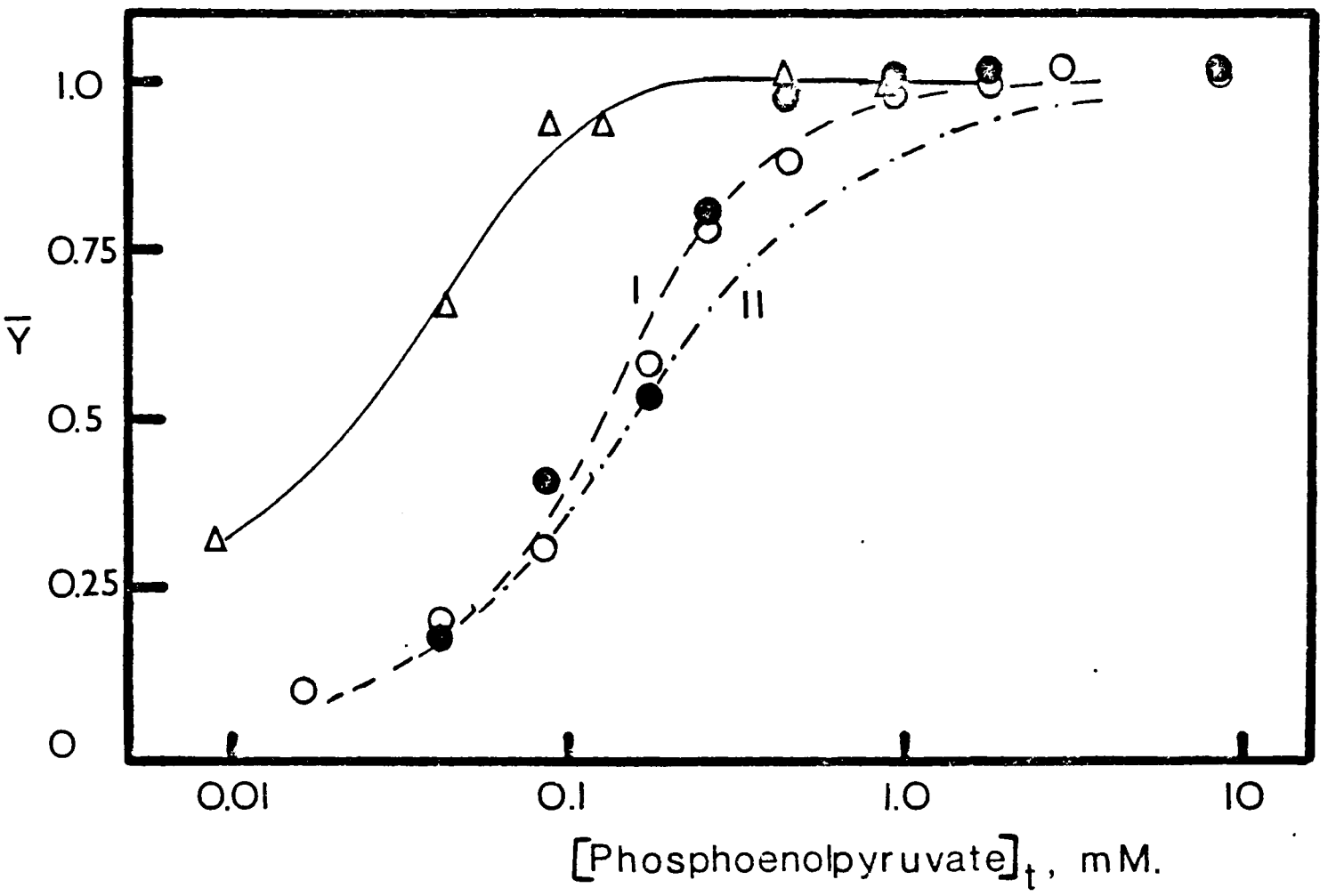


FIG. 3.6 Proton relaxation enhancement titrations of pyruvate kinase with phosphoenolpyruvate in the presence of Mn^{2+} ● , enzyme concentration 23 μM, Mn^{2+} 100 μM; O, enzyme 26 μM, Mn^{2+} 300 μM; Δ, enzyme 18 μM, Mn^{2+} 100 μM, fructose diphosphate 1mM. The ordinate, $\bar{Y}$, is the change in the enhancement expressed as a fraction of the maximal change. For ●, ε* ranged from 2.2 to 1.6; for O, from 2.1 to 1.4, and for Δ, from 1.6 to 2.2. Curve I was calculated for eight equivalent substrate sites with K'''_{EMS} = 100 μM, and Curve II was for one site with K''_{EMS} = 25 μM.

equations:

$$[M]_t = [M]_f + [EM] + [MS]$$

$$n_M \cdot [E]_t = n_M \cdot [E]_f + [EM]$$

$$\text{and } [S]_t \approx [S]_f$$

to give:

$$[EM]^2 - [EM] \cdot \left([M]_t + n_M \cdot [E]_t + K_D \cdot \left(1 + \frac{[S]_t}{K_{MS}}\right)\right) + n_M \cdot [E]_t \cdot [M]_t = 0$$

$$\text{and } [MS] = \frac{([M]_t - [EM])}{\left(\frac{K_{MS}}{[S]_t} + 1\right)}$$

The enhancement of the EM complex (representing, in this case, enzyme saturated with phosphoenolpyruvate, i.e. EMS), was then calculated, using the experimentally derived values of K_{MS} and ε_{MS}, and values of $n_M = 9$ and $K_D = 270$ μM to describe the binding of Mn^{++} to the enzyme-substrate complex. This gave $\varepsilon_{EMS} = 2.4 \pm 10\%$, (mean of 13 values for $[S]_t$ in the range 1 to 10mM and $[M]_t$ 100 μM or 300 μM). Further interpretation of these binding curves is complicated because only the total phosphoenolpyruvate concentration is known, and the fraction of the substrate bound to the enzyme is significant at the lower end of the titration. Also, the number of binding sites for phosphoenolpyruvate which are involved in causing the observed change in enhancement, is unknown. Since $K''_{EMS} = [S]_f$ at the mid-point of the titration, and $[S]_b = n_S \cdot [E]_t/2$ by definition, then:

$$[S]_t = K''_{EMS} + n_S \cdot [E]_t/2$$

and the mid-point of the titration alone does not give a value for K''_{EMS}. Had the change in the observed enhancement been greater, it would have been possible to achieve the accuracy required to determine K''_{EMS} and n_S uniquely from the whole curve

(cf. the theoretical curves in Fig. 3.6). It was qualitatively apparent that the experimental points agree best with theoretical hyperbolic binding curves where n_S is greater than one, but there is the possibility of a slight co-operativity in the binding. If $n_s = 8$, $K''_{EMS} = 25$ μM from the mid-point relationship above, whilst if $n = 4$, $K''_{EMS} = 75$ μM. It should be noted that the mid-point of a titration gives only an apparent dissociation constant for a co-operative binding curve, and not an intrinsic binding constant.

The titration curve of pyruvate kinase with fructose diphosphate is complicated by the titration of free FDP.Mn by excess substrate (Fig. 3.7). From the parameters obtained for the binding of Mn^{++} to fructose diphosphate, it can be calculated that the free concentration of the metal is insignificant at concentrations of the effector greater than 1 mM. Since the enhancements in the titration are all greater than ε_{MS_2} for fructose diphosphate (1.8), it follows that a ternary enzyme-Mn-FDP complex has been formed, which shows no tendency to lose Mn to the excess of free fructose diphosphate and hence a decrease of the enhancement towards 1.8. Also, since the total concentration of the Mn-FDP complex could not have been greater than 100 μM (i.e. $[M]_t$), whilst the concentration of fructose diphosphate rose to a 100 times greater than this without there being a fall in the observed enhancement, the binding of FDP-Mn to the enzyme must be much stronger than that of the effector alone. At concentrations of fructose diphosphate above 1 mM, the following equation will describe the enhancement:

$$\varepsilon^* = \varepsilon_{EMS} \cdot \frac{[EMS]}{[M]_t} + \varepsilon_{MS} \cdot \frac{[MS]}{[M]_t} + \varepsilon_{MS_2} \cdot \frac{[MS_2]}{[M]_t} .$$

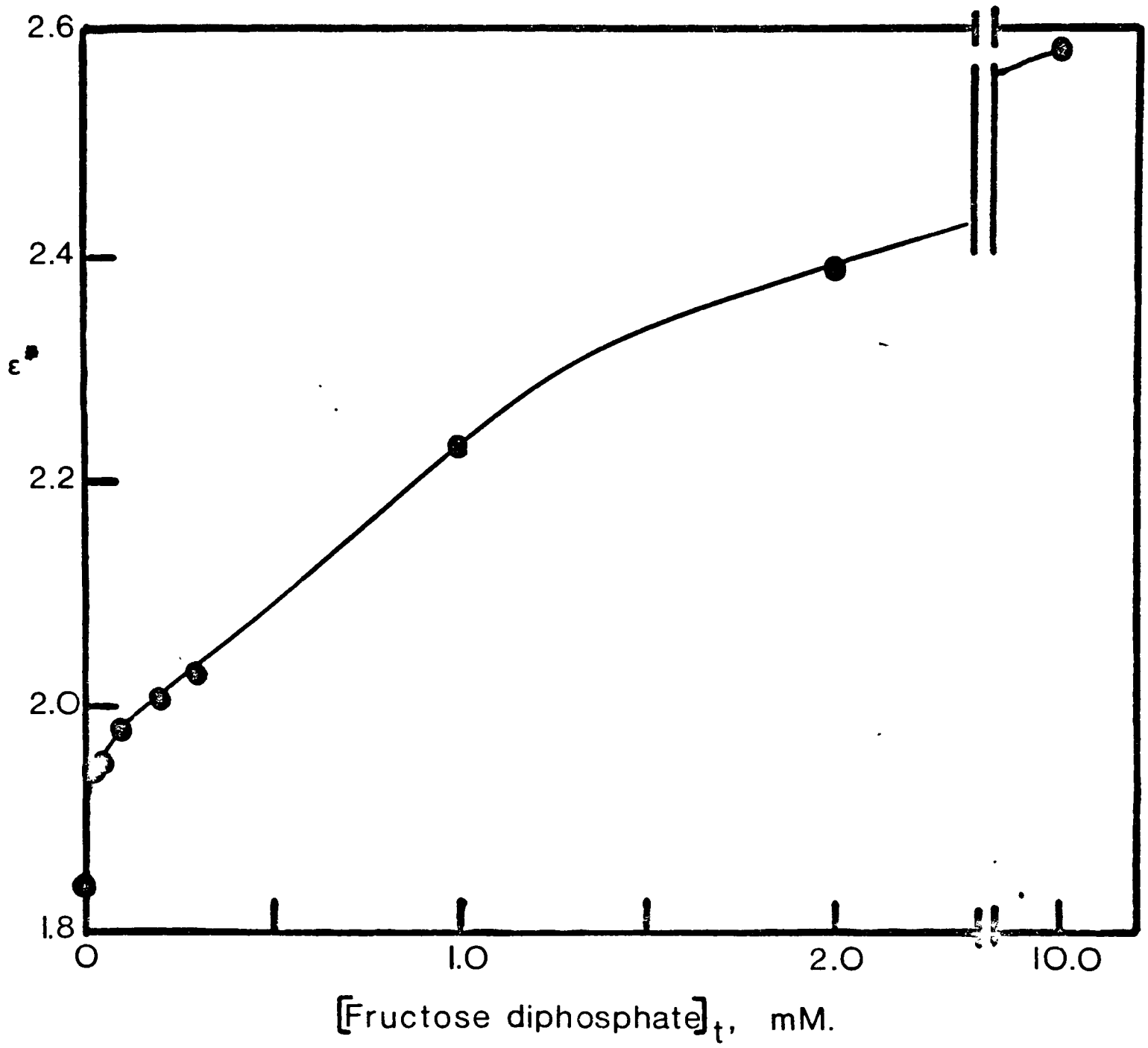


FIG. 3.7 Proton relaxation enhancement titration of pyruvate kinase with fructose diphosphate in the presence of 100 μM Mn^{2+}. The enzyme concentration was 18 μM.

and ε_{EMS} can be calculated for assumed values of [EMS] (e.g. 1 to 4 times the enzyme concentration; see Appendix 2). ε_{EMS} was found to be near constant for a given value of [EMS] at different concentrations of the effector, thus confirming that the rise in enhancement observed at concentrations of fructose diphosphate greater than 1 mM was due to the titration of free FDP.Mn and not to the titration of the enzyme. Thus the titration of the enzyme corresponded to the rise in the enhancement in the first part of the titration curve. The half point of the curve was reached at about 30 μM fructose diphosphate, and since the concentration of the enzyme was 18 μM, the number of sites for the effector could not be as great as 4, since the concentration of the FDP.Mn complex will be less than 30 μM. For $n_S = 2$, the dissociation constant for FDP.Mn from the ternary complex would be of the order of 10 μM, and ε_{EMS} would be 3.9 (± 5%).

(iii) A Quaternary Complex

The titration of the enzyme with phosphoenolpyruvate in the presence of 1mM fructose diphosphate and 100 μM Mn^{++} caused a decrease in the measured enhancement (Fig. 3.6). The half point for this titration was at a substrate concentration of ≈20 μM, and since the concentration of pyruvate kinase was 18 μM, not more than two substrate binding sites were being detected, with a dissociation constant of 10 μM or less. Substitution of the enhancement value at the plateau of the titration in the equations given previously for determination of the enhancement of the ternary complex with fructose diphosphate gave the enhancement of the quaternary complex as 2.0, assuming 2 sites for the effector.

(iv) Discussion

These experiments did not prove to be as rewarding as had been hoped in terms of a fruitful comparison with rabbit muscle pyruvate kinase. Although a number of sites on the enzyme for the metal ion were detected, it is not possible to infer a simple relationship between these and the other ligand sites. The curvature of the Scatchard plots indicates either heterogeneity of binding sites or negative co-operativity between intrinsically equivalent sites. These two possibilities are not readily distinguishable, but even assuming the simplest case of two types of independent binding sites, it is not possible to obtain a unique analysis. However, the curve is explicable without invoking fractional numbers of binding sites per mole of enzyme, as was found necessary by Mildvan et al. (1971) in a similar study of this enzyme by proton relaxation enhancement. Their binding curve gave a curved Scatchard plot which they analysed by the dubious technique of taking four tangents to the curve to give $n_1 = 1/3$, $K_{D1} = 3\ \mu M$, $n_2 = 2/3$, $K_{D2} = 72\ \mu M$, $n_3 = 2$, $K_{D3} = 740\ \mu M$ and $n_4 = 3$, $K_{D4} = 1.8$ mM; i.e. six binding sites in all. Qualitatively, they observed less Mn^{++} bound to the enzyme at any given concentration of Mn^{++} than was obtained in these experiments, and the enhancement of the binary complex was higher ($\varepsilon_b = 15$). Whilst their quantitative interpretation cannot be justified, it is clear that a fractional binding site must be invoked to describe their results, which suggests that either they had heterogeneity of the enzyme, or that manganese caused aggregation of the enzyme. They reported that the enzyme gave many bands (about 10) in gel electrophoresis in dissociating conditions whilst appearing homogeneous in the native state. Unfortunately,

a similar observation was made later with samples of pyruvate kinase prepared by the method of Ashton (see this Chapter, section f(ii)), so that it is probable that there was heterogeneity of the enzyme used in both sets of experiments.

The binding curves obtained by this technique for the substrate and reported here, do presumably reflect the binding of phosphoenolpyruvate to the active site of the enzyme, since the dissociation constants were 25 to 75 μM in the absence of fructose diphosphate, and 10 μM in its presence, and similar values were found for the half-points of the enzyme activity v. substrate concentration curves measured at the same pH (this Chapter, section e). At pH 7.5, Cottam et al. (1972) found 440 μM and 156 μM for the same dissociation constants by proton relaxation enhancement titrations, and 160 μM and 80 μM for the half-saturation concentrations in the enzyme assays. Close agreement between binding data and kinetic constants is not necessary, since the latter only become equivalent to dissociation constants for certain types of enzyme mechanism, and in any case, measurements made on the dependence of activity upon the concentration of one substrate in the presence of saturating concentrations of the others are likely to be different from measurements made in the absence of the other substrates. The important feature of the results was the increase in affinity for phosphoenolpyruvate when fructose diphosphate bound to the enzyme which shows that the allosteric kinetics of this enzyme arise through allosteric equilibrium binding. In the absence of further structural information, it is not possible to decide whether the decrease in enhancement which occurred when phosphoenolpyruvate bound to the enzyme was caused by direct interaction between the substrate and the metal ion, or by a conformational change of the enzyme which resulted in a change of the environment

of the bound metal. (In the latter case, the change in enhancement measures the position of the conformational equilibrium, and not the degree of saturation with ligand; these functions need not be identical).

The activator dissociation constants obtained for fructose diphosphate from measurements of enzyme activity should represent a true dissociation constant; Hunsley and Suelter (1969b) reported a value of about 30 μM in the presence of Mg^{++} and K^{+} at pH 6.2, whilst Haeckel et al. (1968) reported 200 μM at pH 6.0 in the presence of Mg^{++} and non-saturating K^{+} (there is interaction between the binding of K^{+} and fructose diphosphate). Kuczenski and Suelter (1970a) reported a value of 60 μM for the binding of fructose diphosphate in catalysis of cold inactivation of pyruvate kinase, of ~500 μM (1971b) for inactivation at 23°C, and of 0.5-1 mM (1971a) for the quenching of enzyme fluorescence by this ligand. However, none of these results were obtained in conditions exactly comparable to those used here, when a value for $K_{0.5S}$ of about 10 μM was found.

e) Enzyme Kinetics

An attempt was made to measure the dependence of the velocity of the pyruvate kinase reaction on the concentration of phosphoenolpyruvate in conditions closer to those of the proton relaxation enhancement experiments than those used by Haeckel et al. (1968) and Hunsley and Suelter (1969b). The assay system used contained 0.02M Tris-HCl, pH 7.2, 0.08M KCl, 7.5 mM ADP, 0.3 mM NADH and 25 μg/ml of lactate dehydrogenase. However, the $K_{0.5}$ for phosphoenolpyruvate was so low that the initial rates were difficult to determine using absorption spectrophotometry. $K_{0.5}$ was of the order of 20 μM (Fig. 3.8), and was lower than this in the presence of 1 mM fructose diphosphate. Accurate

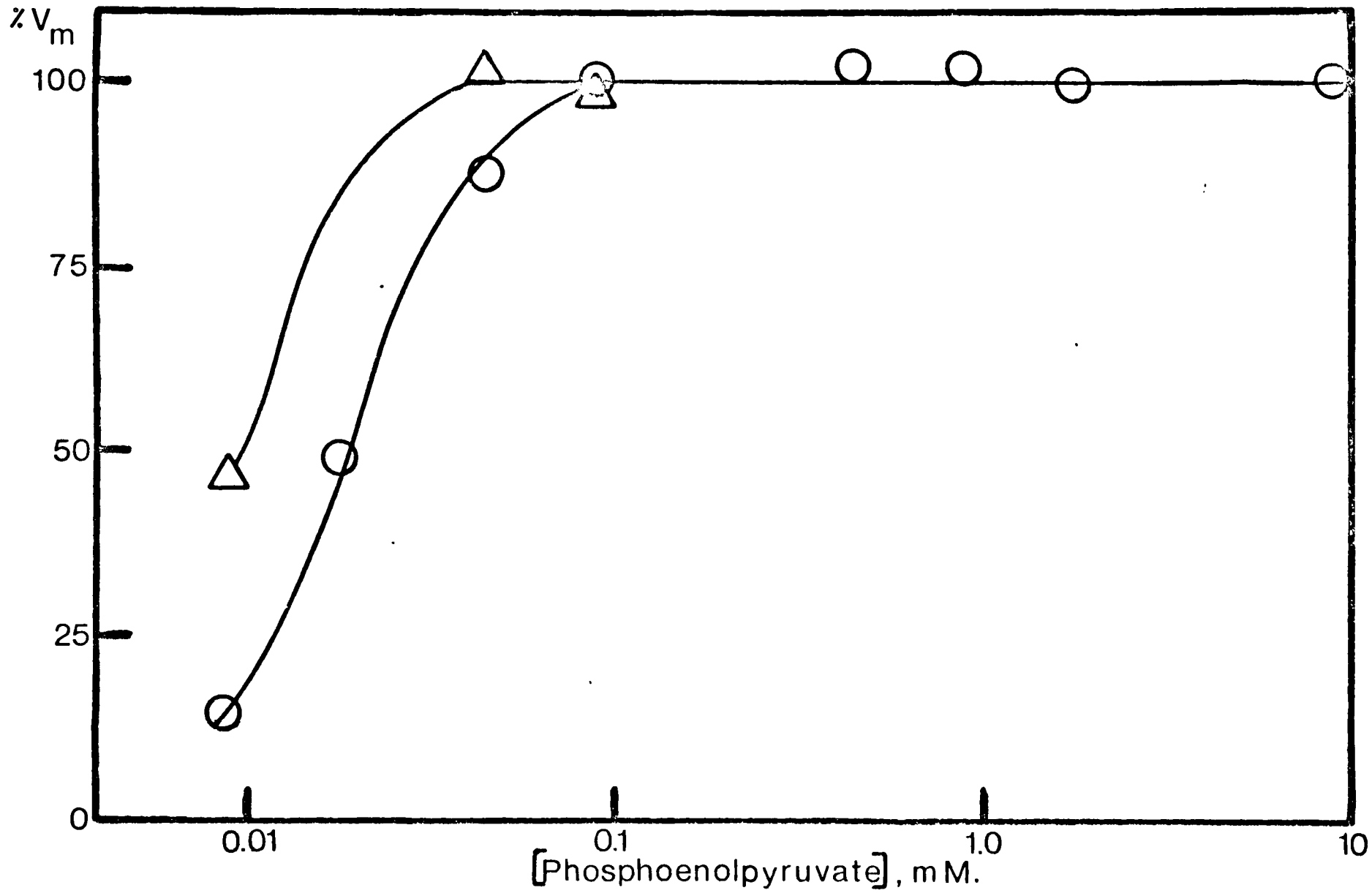


FIG. 3.8 The relationship between the initial velocity of the pyruvate kinase reaction at pH 7.2 and the concentration of phosphoenolpyruvate. The assay conditions are given in the text. O , no fructose diphosphate; Δ, + 1 mM fructose diphosphate.

results could only have been obtained with lower enzyme activities and detection of the utilisation of NADH by its fluorescence emission.

f) Studies on the Subunits of Pyruvate Kinase

(i) Sedimentation Equilibrium

The molecular weight of the subunits of pyruvate kinase was measured by both low-speed equilibrium runs, and by the high-speed Chervenka method. The results are summarised in Table 3.2:

TABLE 3.2

Type of run	Rotor Speed	Solvent	Mean Concn. (mg/ml)	$\bar{M}_w$	$\bar{M}_z$
Chervenka	25,980	6M Guanidinium Chloride + 1% 2-mercaptoethanol	1.25	40,900	40,800
"	35,600	ditto	0.65	48,300	49,500
Low-speed	21,740	Maleylated enzyme in 6M Guanidinium Cl.	1.75	43,800	40,900
"	16,200	ditto	1.90	43,400	44,100
			Mean:	44,100	43,300

Whilst the graphs of $\ln J$ v r^2 and $\ln(\frac{1}{r}.\frac{dc}{dr})$ v r^2 were linear for the enzyme dissolved in 6M guanidinium chloride with 1% 2-mercaptoethanol, the presence of higher molecular weight components was indicated by the upward curvature of these plots at the bottom of the cell for the maleylated enzyme dissolved in 6M guanidinium chloride. These results are not in agreement with those of Ashton (1971), who found a subunit molecular weight of 20,450 for the maleylated enzyme, and a minimum molecular weight

of about 20,000 for the enzyme reduced by 2-mercaptoethanol in 6M guanidinium chloride. Some other centrifuge runs did give non-linear plots suggesting the presence of some material of lower molecular weight, but it was not possible to analyse these results since they were not consistent, and could well have been caused by the technical problems associated with making measurements in 6M guanidinium chloride. There is closer agreement with the values of $\bar{M}_W^o$ = 41,400 and $\bar{M}_Z^o$ = 45,900 obtained by Kuczenski and Suelter (1970b), and these results support their finding that yeast pyruvate kinase has four subunits and a native molecular weight of 160,000 to 170,000.

(ii) SDS Gel Electrophoresis

Ashton (1971) reported that SDS gel electrophoresis of pyruvate kinase dissociated by 1% SDS and 1% 2-mercaptoethanol gave a single band corresponding to a subunit molecular weight of 20,000. Samples of pyruvate kinase were run on 7.5% polyacrylamide gels which were calibrated with proteins of known molecular weight (γ-globulin, pepsin, carbonic anhydrase and myoglobin). The enzyme gave numerous overlapping bands in the range of molecular weights from 19,000 to 80,000 (Fig. 3.9). Thus although the enzyme was pure by electrophoresis at pH 9.5, and has a specific activity comparable with that observed by Hunsley and Suelter (1969a) and Haeckel et al. (1968), it appears that the polypeptide chains have been cleaved to give fragments of various sizes. This suggested that the preparation could not be depended upon to give a unique product, since it evidently allowed proteolytic cleavage to occur to a certain extent at some point in the history of the enzyme. Presumably the 20,000 subunit observed by Ashton (1971) represented some

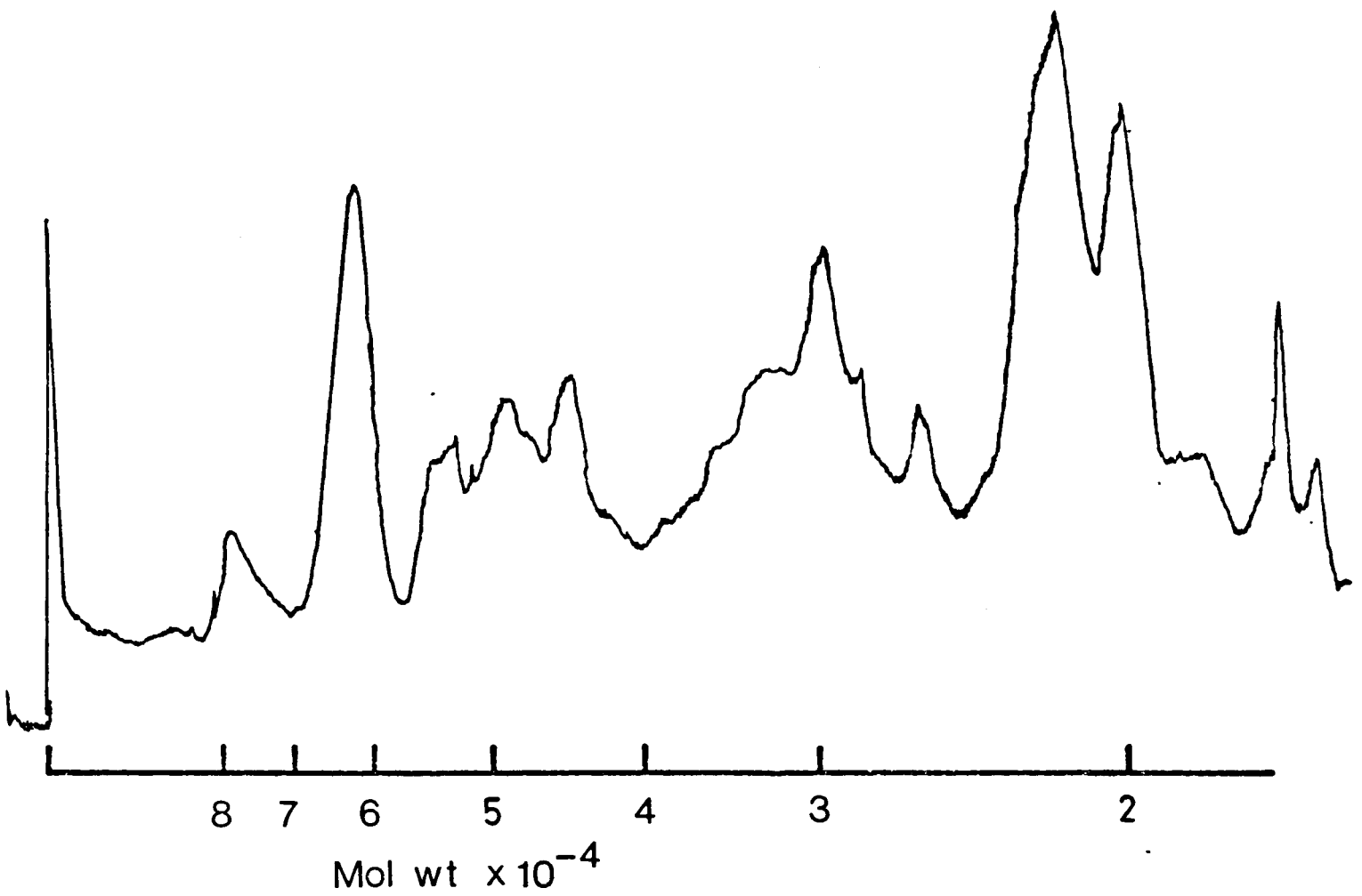


FIG. 3.9 SDS- polyacrylamide gel electrophoresis of yeast pyruvate kinase prepared by Ashton's method. Microdensitometer scan of a 7.5% gel. An approximate molecular weight calibration, obtained with γ-globulin, pepsin, carbonic anhydrase and myoglobin is given.

limiting stage in the proteolysis of the enzyme, and the modifications to the preparation reported in Section (a) of this Chapter were such as to reduce the degree of cleavage.

g) Conclusions

These experiments confirmed some of the similarities between pyruvate kinase prepared from S. cerevisiae by the methods of Ashton (1971) and of Hunsley and Suelter (1969a). Where these authors were in disagreement, about the subunit molecular weight, the results presented here were in support of the value reported by Kuczenski and Suelter (1970b).

The binding studies did not agree well with those of Mildvan et al. (1971) and Cottam et al. (1972), but the discovery of the proteolysis of the enzyme during the preparation raised the possibility that the differences might have arisen through different degrees of proteolysis of the enzyme.

Another possibility was that the differences between the pyruvate kinases from S. cerevisiae and S. carlsbergensis were not due to intrinsic differences of the sources, but due to alterations in the properties of the enzymes from S. cerevisiae by proteolysis. It was therefore necessary to investigate modifications of the preparation of the enzyme from S. cerevisiae so that conditions could be found where proteolysis was prevented, and then to re-investigate the properties of the enzyme.

CHAPTER 4

DEVELOPMENT OF A NEW PREPARATION

a) Introduction

Brewers' or bakers' yeast is a convenient source of many enzymes, particularly the enzymes of glycolysis, since it is readily available in large quantities. However, the polysaccharide cell wall gives the yeast cell a considerable degree of resistance to rupture by mechanical processes of the type commonly used to release enzymes from cells (e.g. ultrasonics, homogenisation, or sudden pressure changes). A method very commonly used to liberate soluble enzymes from yeast is autolysis; this process characteristically involves incubation of a yeast paste, often together with an organic solvent such as toluene, at a temperature between 25^{o}C and 45^{o}C until the yeast liquefies; an aqueous solution containing many soluble enzymes can then be separated from cell debris. Autolysis of yeast occurs by partial digestion of the cell by its own catabolic enzymes, amongst which proteases must be important since the liberation of hexokinase from yeast at 37^{o}C was completely prevented by diisopropylfluorophosphate (DFP), a general inactivator of serine proteases, (Ramel, 1964, quoted by Lazarus et al. 1966). Proteases are released from yeast during autolysis, and three enzymes have been described by Hayashi and Hata (1972), Lenney (1956), Hata et al. (1967) and Doi et al. (1967). Proteinase A has an acid pH optimum (3-4) and is not inhibited by DFP or by reagents specific for the thiol group. Proteinase C has a pH optimum between 4 and 6, is inhibited both by DFP and the thiol-specific reagent p-chloromercuribenzoate (PCMB), and the yield of its activity is increased

by incubation at pH 5. Proteinase B is maximally active near pH 9, is inhibited by DFP and PCMB, and its activity is unstable. The yield of proteolytic activity is said to be lower if toluene is used to promote autolysis than if chloroform is used (Lenney, 1956). The amount of proteolytic activity isolable from yeast has been reported to be higher during growth than in starvation (Sylven, et al. 1959), but on the other hand, Halvarson (1958) has shown that the rate of protein degradation is much greater in resting than in growing cells.

A systematic investigation of proteolytic action during the preparation of yeast hexokinase has been made by Barnhard and his co-workers. Previous to their study, several forms of yeast hexokinase were known, some of which had been shown to arise through the action of proteases during the isolation of the enzyme (Ramel, 1964). The preparative procedure which they adopted (Lazarus et al., 1966) aimed to minimise the exposure of hexokinase to proteases by: (i) the use of a method of cell breakage other than autolysis; (ii) carrying out the preparation at temperatures below $4^{o}C$, and (iii) using DFP to inhibit proteases. Of the several methods of cell disintegration which they tried, they recommended a freeze-thaw method as convenient and effective for the release of hexokinase. DFP was found to inhibit 78% of the proteolytic activity present in their preparation. As a result of this and further studies (e.g. Ramel et al., 1971), it has been established that there are only two types of polypeptide chain associated with hexokinase activity in yeast, and that all the other forms obtained by different preparative methods represent stages in the degradation of these two.

By analogy with these results for hexokinase, it was

anticipated that proteolysis of pyruvate kinase could be prevented by some or all of the following precautions:-

i) performing all steps subsequent to the cell breakage at a temperature not greater than 4°C;

ii) keeping the pH above 6, since the total activity of the yeast proteinases is much greater at lower pH values, and the activation of protease precursors also occurs below pH 6.

iii) using an alternative to autolysis to release the enzyme from the cells.

iv) using specific inhibitors of serine proteases to diminish the activity of those proteases which operate at pH 6 and above.

Whereas the yeast hexokinase preparation required exposure of the protein to pH values in the range 4.5 to 6, none of the preparations of yeast pyruvate kinase used pH values below 6, so no modification was necessary in this respect. The effects of the other conditions on the preparation were investigated, and the results are described in the rest of this Chapter.

b) Results and Discussion

(i) Yeast autolysed; all subsequent purification steps at 4°C

In Ashton's method for the purification of pyruvate kinase from yeast (Ashton 1971), all purification stages after autolysis were performed at room temperature since the purified enzyme was unstable at low temperatures. However, I had found that storing the yeast autolysate overnight at 4°C rather than at room temperature resulted in more successful preparations (Chapter 3, section a), and Hunsley and Suelter (1969a) were able

to prepare cold-labile yeast pyruvate kinase successfully with the fractionation of the crude protein with ammonium sulphate being carried out at 4°C. Probably the enzyme is stabilised during these initial stages by the high concentrations of protein. In Hunsley and Suelter's method, the enzyme was stabilised during the chromatography steps at 4°C by the inclusion of glycerol - to a final concentration of 50% (v/v)- in the column buffers. Purification of pyruvate kinase was therefore attempted by a method essentially similar to that of Hunsley and Suelter, except that their harsh autolysis procedure (at 45°C) and subsequent salt fractionations were replaced by the equivalent steps from Ashton's preparation, which were all performed at 4°C, after the initial autolysis. The two preparations by this technique started with 500 g of yeast, and used a column of DEAE cellulose 30 cm long by 5 cm diameter, and a column of cellulose phosphate (P-11) 10 cm x 5 cm. The final product of these preparations had an enzymic activity of about 300 U/mg (in the conditions of assay method 2, Chapter 2, section g). Although the enzyme gave a single band in electrophoresis on 7.5% polyacrylamide gels at pH 9.5, and sedimented as a single species in the ultracentrifuge (Fig. 4.1), SDS gel electrophoresis in 9% and 10% gels gave a number of bands, corresponding to molecular weights in the range from 60,000 to below 15,000.

The conclusion was, therefore, that further precautions were needed to prevent proteolysis. Replacement of autolysis was the next alteration investigated.

It is of interest to note that the specific activity reported here is higher than those previously reported for this enzyme (see Table 6.1). In the course of these experiments, the

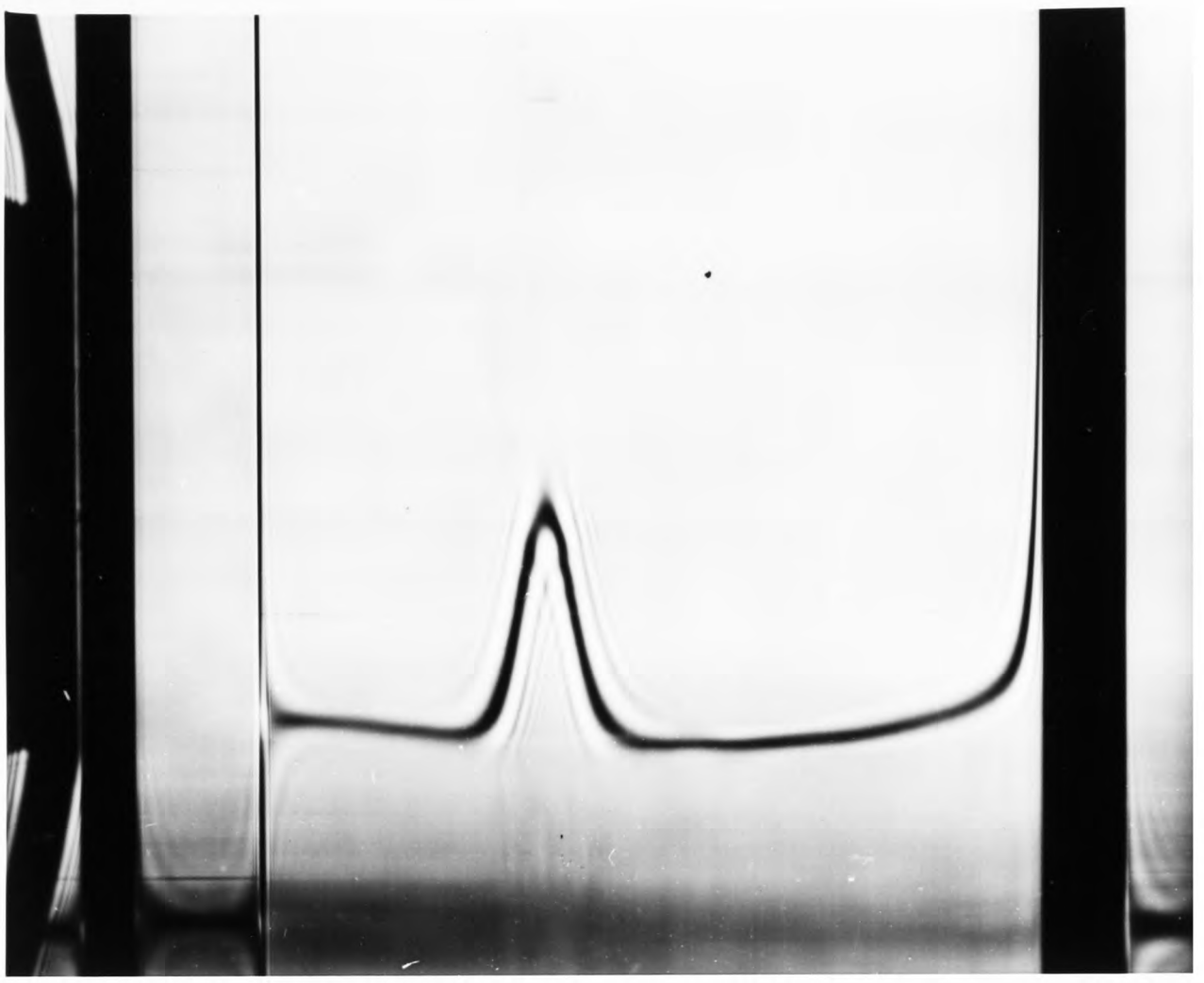


FIG. 4.1 Sedimentation of pyruvate kinase in the ultracentrifuge. The enzyme sample was prepared by the method outlined in Chapter 4, section b(i), and was dissolved in 0.1M KCl, 0.02M Tris-HCl, pH 7.2, to a concentration of 3.7 mg/ml. The photograph was taken 40 min after the rotor reached speed (52640 rev/min). The temperature was 18°C, and the Schlieren analyser angle 60°.

assay method 1 (Chapter 2, section g), had repeatedly failed to give results. Two sources of inhibition were identified: (i) metal ions, of unknown origin, whose inhibition of pyruvate kinase was relieved by the addition of 0.5 mM EDTA to the assay system; (ii) ammonium ions, entering the assay system with the indicator enzyme lactate dehydrogenase, which was stored as a suspension in ammonium sulphate solution. The inhibition by ammonium ions will be discussed again in Chapter 5, section d. Assay method 2 (Chapter 2, section g) was devised, and all activities quoted henceforth refer to those obtained with this system.

(ii) An investigation of different methods of cell breakage

Autolysis is an effective method of cytolysis, which was found to liberate 4 to 12 units of pyruvate kinase per mgm of soluble protein, the concentration of which was determined by the microbiuret method of Itzaki and Gill (1964), calibrated with bovine serum albumin. Hunsley and Suelter (1969a) obtained 6 U/mgm, and Haeckel et al. (1968) reported 12 U/mgm, as the yield of activity with autolysis. The alternatives to autolysis chosen for investigation were the freeze-thawing method of Lazarus et al. (1966) and the passage of a thick suspension of yeast (4 parts by weight of wet yeast to 3 parts of 0.2M Tris-HCl, pH 8, $0^{o}C$) through a "French press". The latter method gave a cytolysate containing about 3 to 7 U of pyruvate kinase per mgm of protein, which was less than the yield obtained from samples of the same yeast by autolysis. There was a marked rise in the temperature of the suspension as it was forced through the needle valve of the press, and it was difficult to keep the temperature of the cytolysate below $4^{o}C$, even when it was collected into a beaker on ice.

100 g of yeast were broken with the French press and used for a pilot preparation by the procedure described in section b(i) of this Chapter, scaled down for a 30 x 2.2 cm column of DEAE cellulose and a 10 x 2.2 cm column of cellulose phosphate. Instead of the typical elution profile from the latter column (e.g. Fig. 4.4), the pyruvate kinase activity was eluted as a leading shoulder on the second of two peaks developed by the gradient, and it was not further resolved from these contaminants after rechromatography on cellulose phosphate. Because cell breakage by this method gave rise to a more difficult purification problem, and because the temperature could not be adequately controlled in the process, freeze-thawing was next investigated.

Lazarus et al. (1966) and Ramel et al. (1971) discuss the efficiency of their freeze-thawing method (which is essentially that described in Chapter 2, section e) at some length. It appears that soluble enzymes are released slowly during the thawing stage at $4^{o}C$, the maximum yield - comparable with that recoverable by autolysis - only being obtained after about 3 days. For the pilot preparations, 100 g of yeast at $4^{o}C$ were suspended in 200 mls of cold toluene, and the yeast was then frozen by placing the suspension in a deep-freeze at $-20^{o}C$ for 6 hrs. The toluene was decanted, and the yeast thawed at $4^{o}C$ overnight by stirring with 75 mls of 0.2M Tris-HCl, pH 8. The preparations were then performed as described in the previous paragraph. Two preparations were carried out on this scale, and both gave elution profiles similar to those shown in Figs 4.3 and 4.4, for the subsequent large-scale preparations. The first preparation gave pyruvate kinase with a maximum specific activity of 200 U/mg. Electrophoresis at pH 9.5 revealed slight

contamination of the leading and trailing edges of the pyruvate kinase peak eluted from cellulose phosphate. Electrophoresis was also carried out in conditions in which the protein would be dissociated - 6M urea, 0.9N acetic acid - to analyse for proteolysis. A scan of such a gel is shown in Fig. 4.2. In addition to the main band, there were two fainter bands of lower mobility, which may have been aggregates of the main component, although the appearance of the bands was not changed by reduction of the protein with 2-mercaptoethanol before electrophoresis. Samples were also run on SDS gels; although there was a major band with a mobility corresponding to a molecular weight of about 60,000, significant amounts of smaller components - down to about 35,000 molecular weight - were present. This was surprising since the urea-acetic acid gels implied greater homogeneity of the subunits. In case the protein had been hydrolysed by a contaminating SDS-activable protease during preparation for the electrophoresis (cf. Pringle (1970) and Diezel et al. 1972), aliquots of the same samples were electrophoresed after a further 24 hrs at room temperature, but the scan profile was unchanged (Fig. 4.2).

The second of these preparations yielded enzyme with a maximal specific activity of 400 U/mg at the centre of the peak eluted from cellulose phosphate, and which gave a single band on electrophoresis in urea-acetic acid gels. The intact molecular weight of the enzyme was measured by two Chervenka equilibrium runs in a buffer containing 0.1M Tris-HCl, pH 7.5, 0.23M KCl, 0.01M $MgCl_2$, 1mM fructose diphosphate and 5mM phosphoenolpyruvate (similar to the solvent used by Kuczenski and Suelter, 1970b). The weight average molecular weights obtained were 222,000 and 227,000, and the corresponding z-

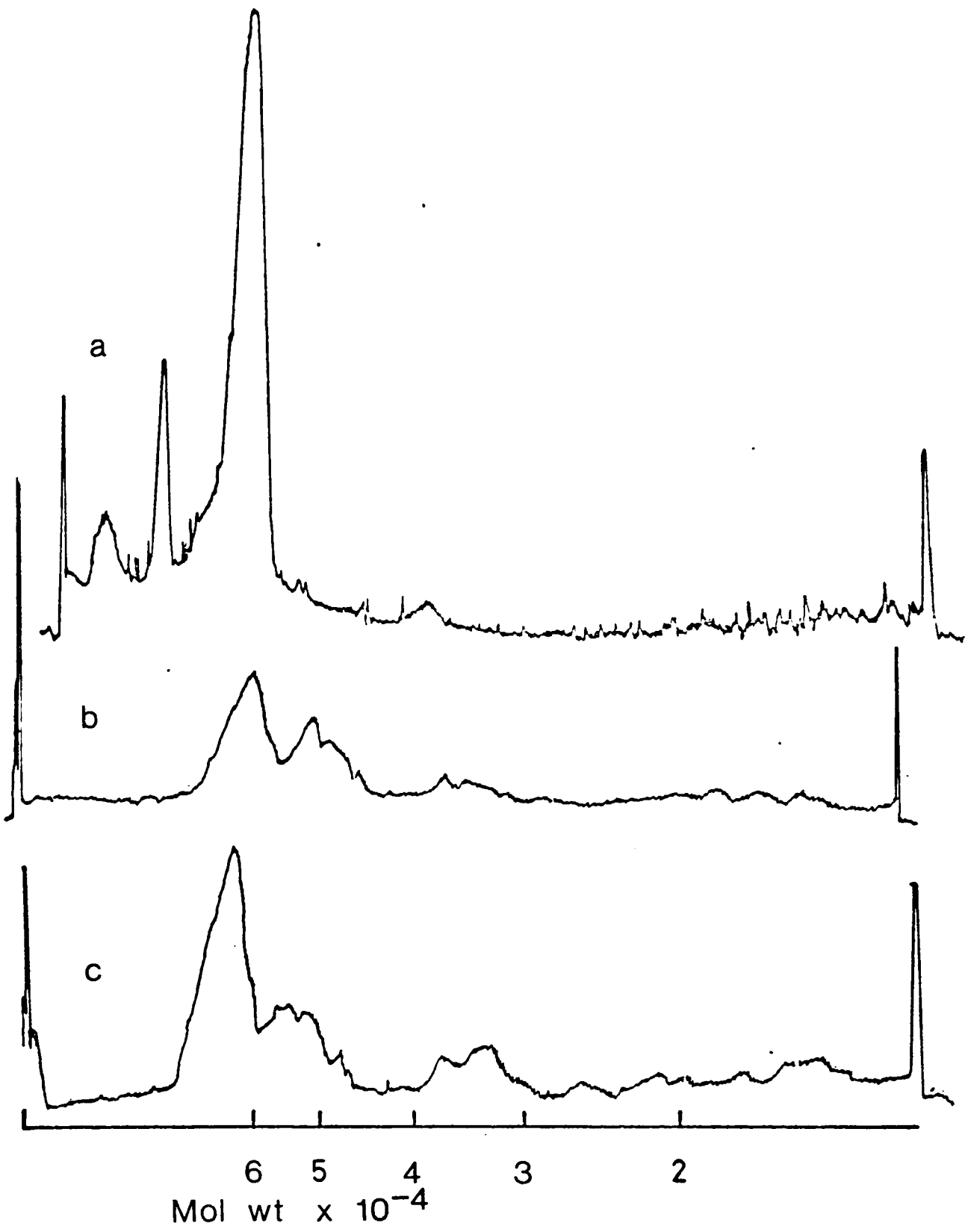


FIG. 4.2 Polyacrylamide gel electrophoresis of yeast pyruvate kinase prepared by the method described in Chapter 4, section b(ii). Microdensitometer scans of (a) 7.5% gel run in 6M urea- 0.9M acetic acid; (b) 7.5% gel run in 0.1% SDS, phosphate buffer pH 7.2. Sample applied to gel after incubation for 1 h with 1% SDS, 1% 2-mercaptoethanol; (c) as (b) after incubation of sample for 24 hrs. For (b) and (c), an approximate molecular weight calibration, obtained with γ-globulin, ovalbumin, carbonic anhydrase and cytochrome c, is given.

average molecular weights were 227,000 and 229,000. These values are significantly higher than previous measurements of the molecular weight of pyruvate kinase from S. cerevisiae, and slightly higher than the value of 190,000 reported for the enzyme from S. carlsbergensis (see Table 6.1). Although the SDS gels showed that proteolysis had not been completely suppressed, the high specific activity and the high molecular weight of the pyruvate kinase prepared by this method justified its further development.

(iii) Large-scale preparation of pyruvate kinase from yeast by freeze-thawing

Three preparations of pyruvate kinase were made using the method outlined in Chapter 2, sections e and f. Examples of the elution profiles from the columns are given in Fig. 4.3 (DEAE-cellulose column) and Fig. 4.4 (cellulose-phosphate column). There were some differences between these preparations, so they will be described individually.

The first preparation was not completely satisfactory. The frozen yeast slurry was transferred to a cold-room at $4^{o}C$, and in spite of the strong air circulation, melted only very slowly. The 2.5 litres of extraction buffer were added after 16 hrs, but the temperature of the slurry only rose above $0^{o}C$ after 48 hrs at $4^{o}C$. At this time, a sample was removed, centrifuged and the supernatant assayed for pyruvate kinase activity. This activity was about 100 U/ml, so the next stage of the preparation was started. In the ammonium sulphate fractionation steps, the solution containing 19 g of ammonium sulphate per 100 ml, which was used for dissolving the crude protein precipitate, was not buffered, as described in Chapter 2, since it was considered that the buffering capacity of the protein itself would maintain the

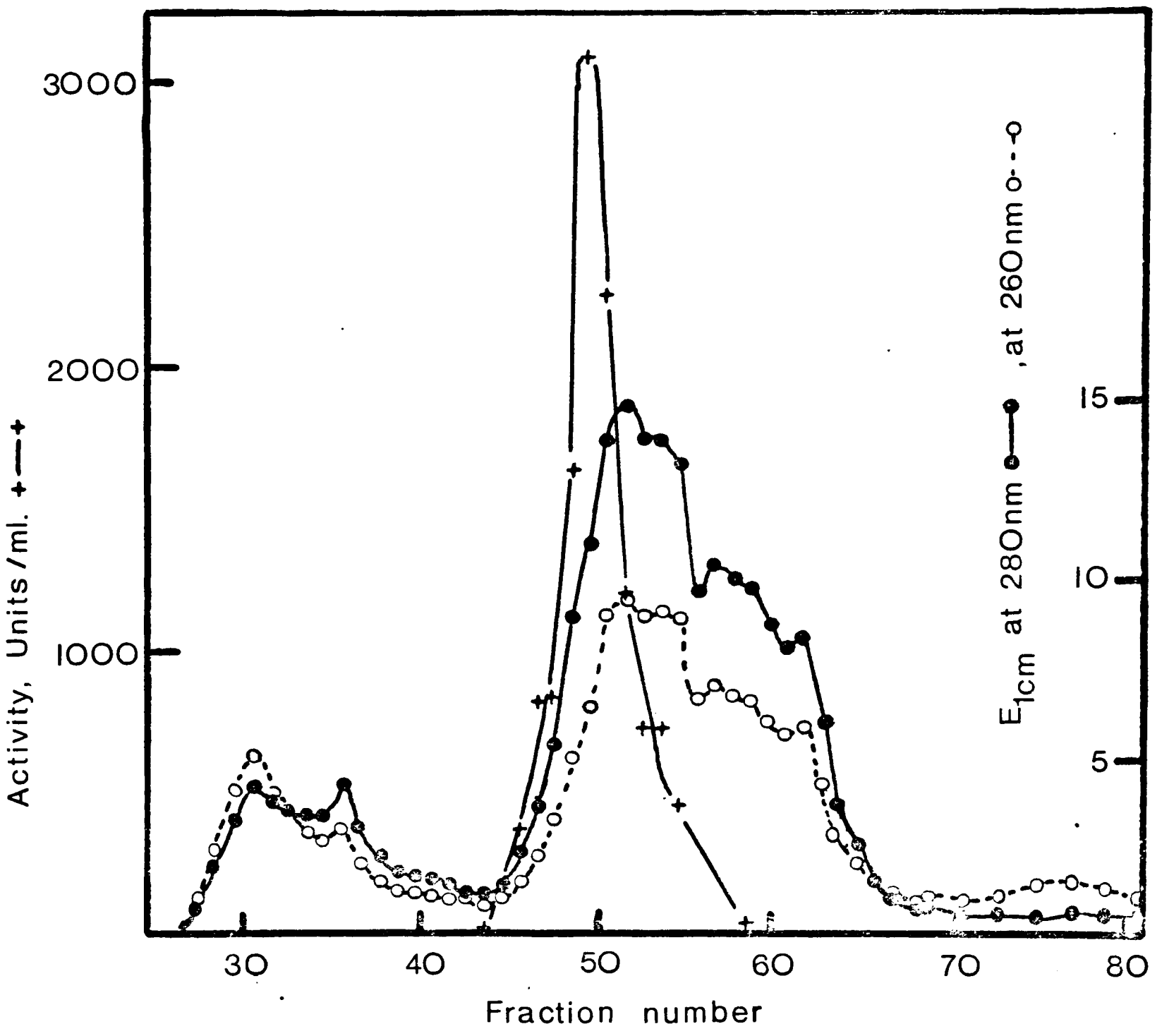


FIG. 4.3 Chromatography of pyruvate kinase on DEAE-cellulose. The elution profile from a 2.3 litre column (70 x 6.5 cm), eluted with 50% (v/v) phosphate-Na^+, pH 7.5, 50% glycerol at a flow rate of 100 ml/hr. Fractions were taken every 20 min.

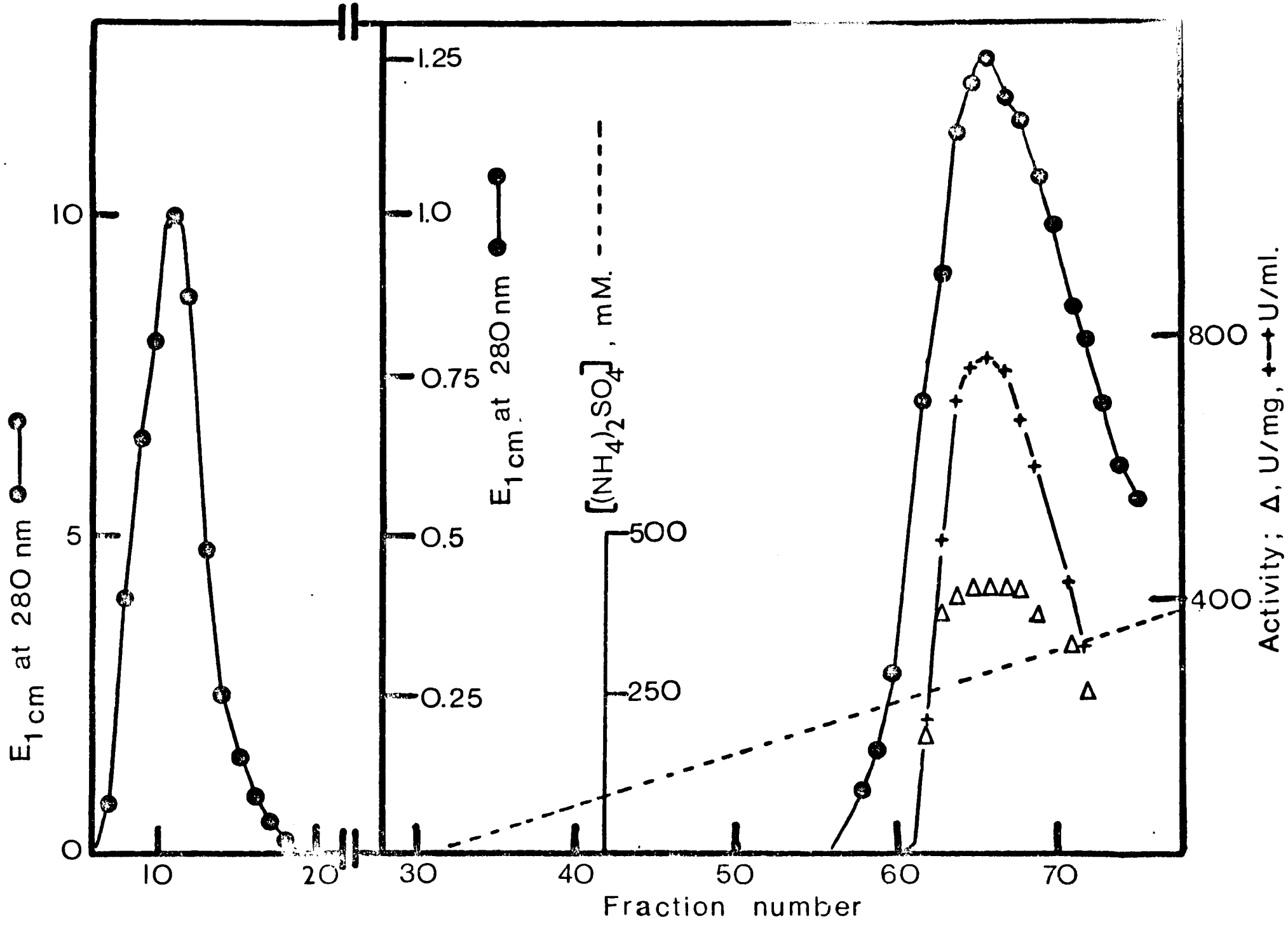


FIG. 4.4 Chromatography of pyruvate kinase on cellulose-phosphate. The elution profile from a 10 x 5 cm column. Elution commenced with 50% (v/v) phosphate-Na^+, pH 6.5, 50% glycerol, followed by a linear gradient of 0-500 mM $(NH_4)_2SO_4$; 20 ml fractions collected every 20 min.

protein near neutrality. However, before loading the protein solution on to the DEAE column, its pH was measured and found to be 5.5. This suggested that the pH 8 extraction buffer had not been sufficient to control the pH of the cytolysate during the melting process. The pH was adjusted to 7.5 with tris before loading the sample on to the DEAE column. The final yield from the cellulose phosphate column was 550 mg of enzyme with a specific activity greater than 360 U/mg, the mean activity being 400 U/mg.

A microdensitometer scan of a 7.5%, urea-acetic acid gel of the protein is shown in Fig. 4.5. A number of contaminating bands were present at low concentrations; their total concentration was probably lower than appears from the scan, since the scanner underestimates the optical density of strong protein bands. The sedimentation velocity profile (Fig. 4.6) showed only a single peak, but there was a significant amount of material with a lower sedimentation velocity behind the boundary. The pyruvate kinase was chromatographed on a column of Sephadex G-200 (160 ml) in 0.05M phosphate-potassium, pH 7.2, in an attempt to remove this material, from which it was partially resolved, since the sedimentation velocity profile (Fig. 4.6) showed a considerable reduction in the amount of contamination. However, a sample from the centre of the pyruvate kinase peak showed no significant improvement in purity when electrophoresed on a urea-acetic acid gel (Fig. 4.5), perhaps because the contaminant was lost in the first instance during the dialysis to prepare the sample for electrophoresis. SDS gel electrophoresis revealed that the enzyme had been degraded, but to a lesser extent than in previous preparations (Fig. 4.5).

The second and third of these preparations were more success-

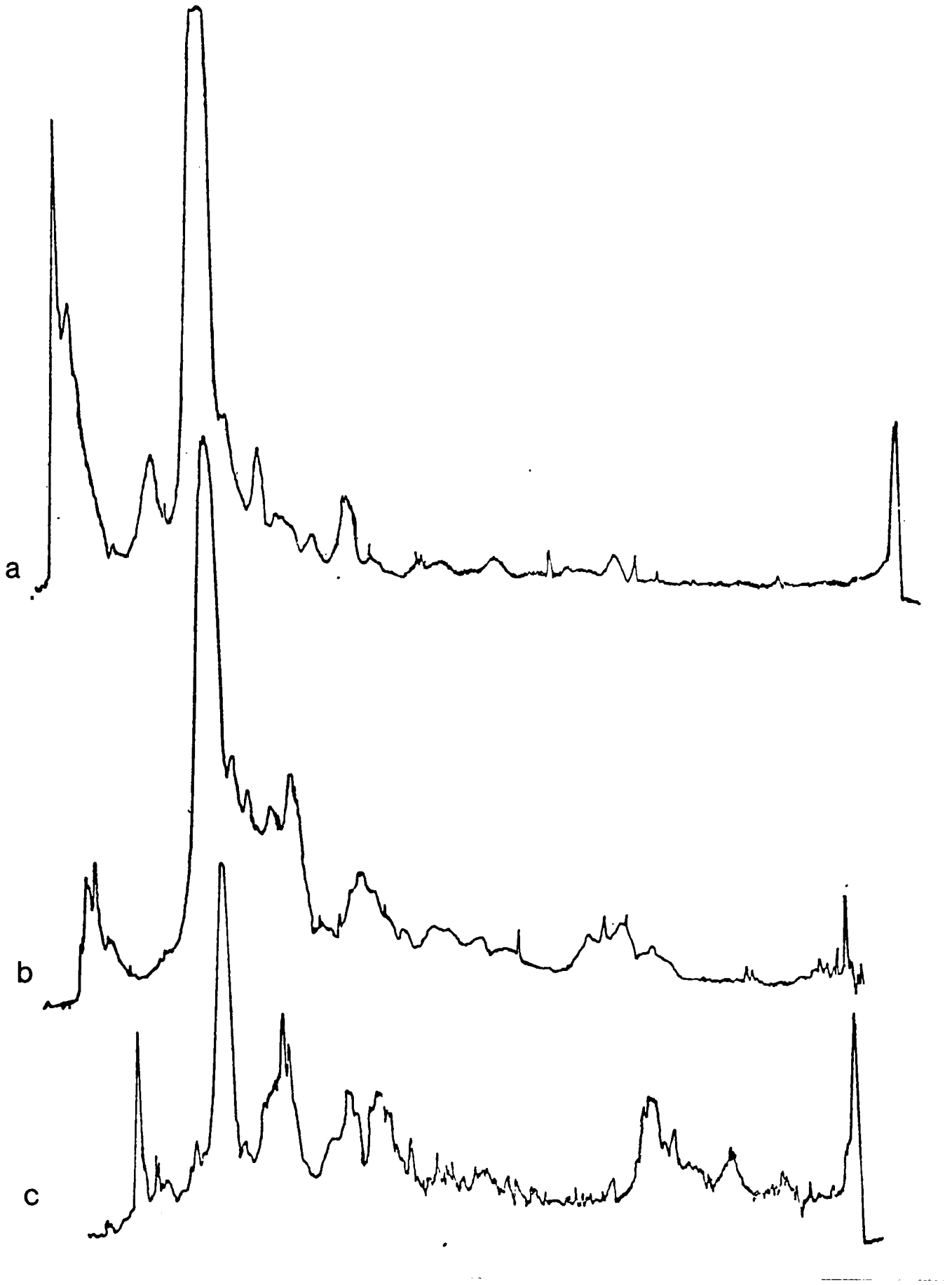
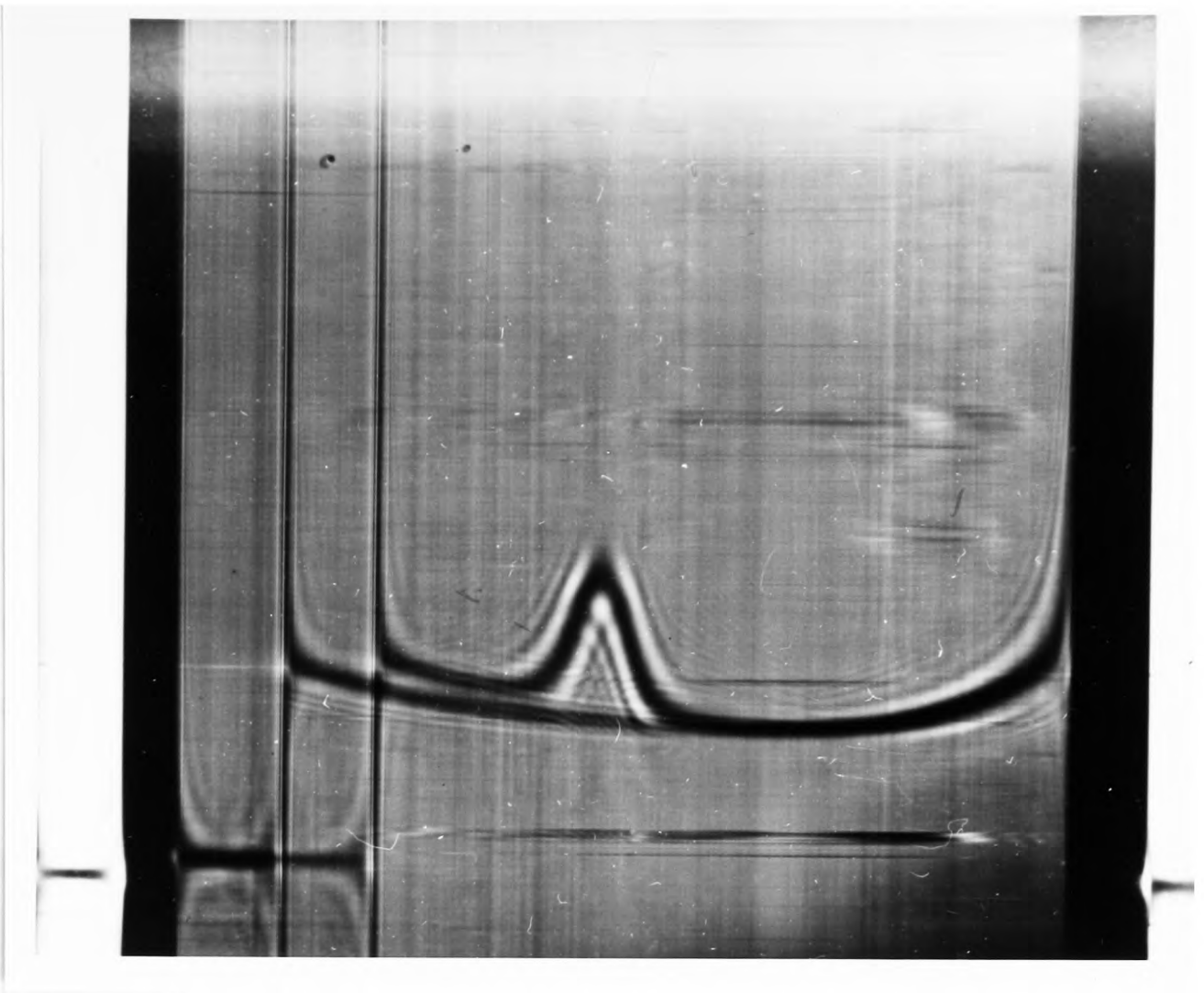


FIG. 4.5 Polyacrylamide-gel electrophoresis of yeast pyruvate kinase; first large-scale preparation from freeze-thawed yeast. Microdensitometer scans of: (a) 7.5% gel containing 6M urea-0.9M acetic acid; (b) as (a), after the preparation had been subjected to chromatography on Sephadex G-200; (c) 7.5% gel, 0.1% SDS.

a



b

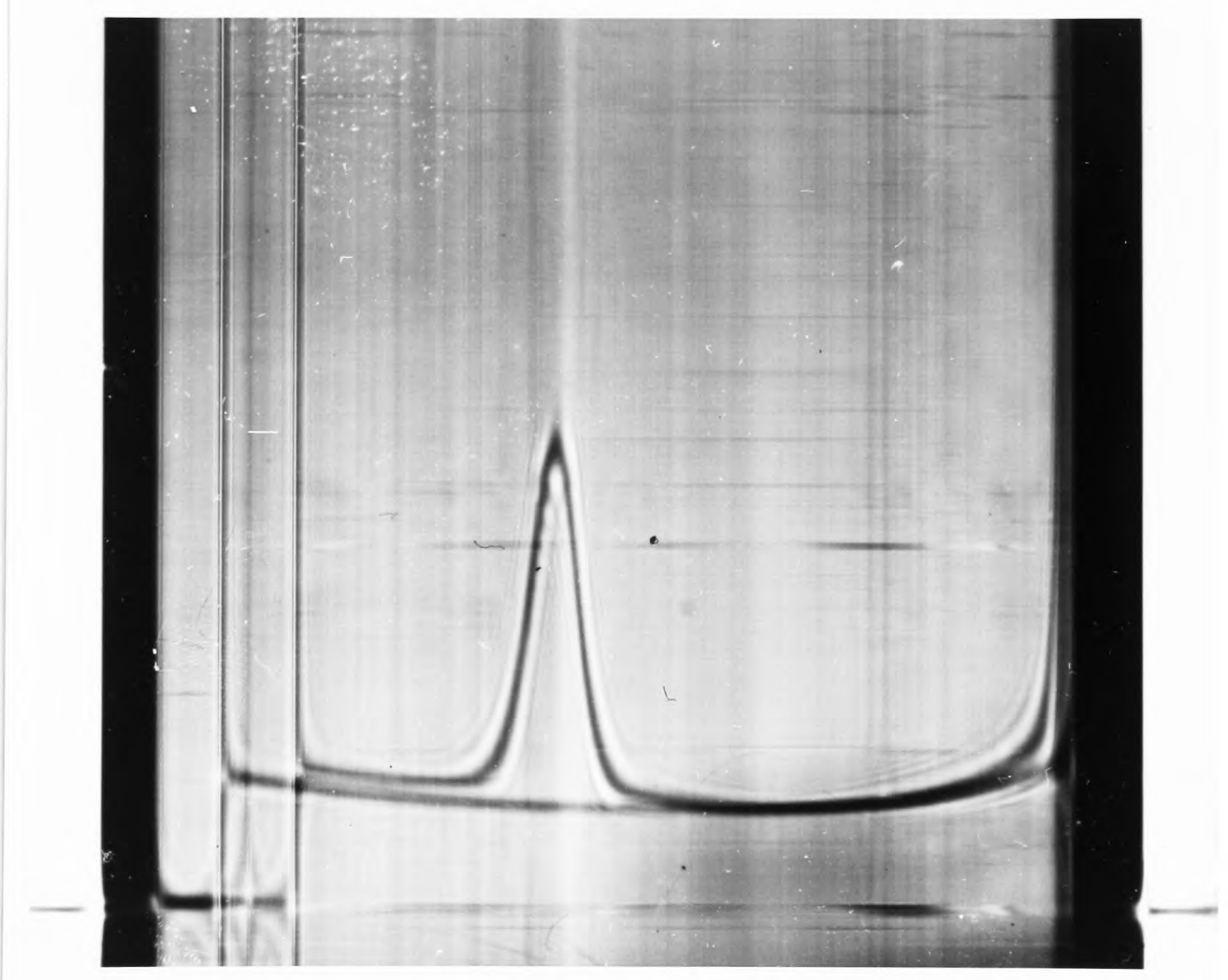


FIG. 4.6 Sedimentation of pyruvate kinase; first large-scale preparation from freeze-thawed yeast.

(a) Before chromatography on Sephadex G-200; 20°C; Schlieren analyser angle 60°. (b) After chromatography on G-200; 22°C; analyser angle 70°. Both photographs were taken 32 min after the rotor reached speed (52640 rev/min). The solvent was 0.1M phosphate-K^+, pH 7.5.

ful. In the second preparation, the frozen yeast slurry was melted in 4°C water bath; this reduced the time taken to melt, and after 36 hrs at 4°C, the pyruvate kinase activity in the cell supernatant was 210 U/ml, so the ammonium sulphate fractionation was then started. It is probable that the release of activity from the cells was not complete (this had been found to take about 72 hrs by Lazarus et al. (1966), who went on to the next stage of their purification of hexokinase after 36 hrs, so that exposure to proteases was limited). Throughout the melting stage, it was found necessary to periodically titrate the cytolysate back to pH 7.5 with dilute ammonia. Phenylmethylsulphonylfluoride, an inactivator of serine proteases (Gold, 1967), in propan-2-ol, was added to the crude protein solution to give a final concentration of 1.5 mM, before dialysis for loading on to the DEAE column. The total recovery of activity from the column chromatography corresponded to 590 mg of pyruvate kinase (about 20% of the total activity present at the start of the preparation), but only the central fractions of the peak eluted from the cellulose phosphate column had a specific activity of 400 U/mg. This protein gave one main band with only traces of contamination on electrophoresis in urea-acetic acid and SDS gels (Fig. 4.7). It was concluded that the shorter time taken for the preparation, the use of phenylmethylsulphonylfluoride to limit proteolysis during the long dialysis stage, and the regulation of the pH were responsible for the improvement in the homogeneity of the product.

The third preparation was similar; the time required for thawing was further reduced by immersing a glass coil, through which water at 4°C was circulated, into the yeast slurry as it stood in the water bath at 4°C. After 10 hrs of thawing, the

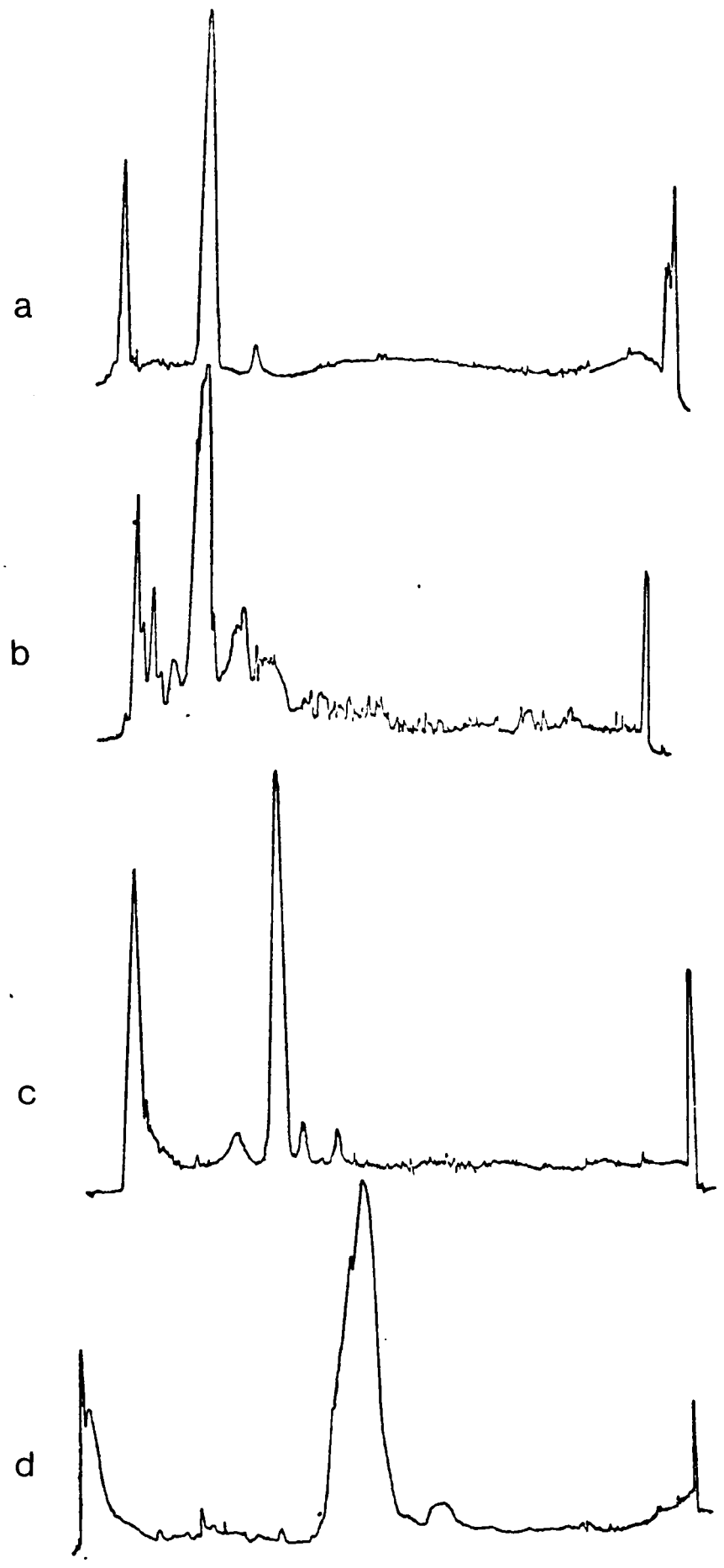


FIG. 4.7 Polyacrylamide-gel electrophoresis of pyruvate kinase; second and third large-scale preparations from freeze-thawed yeast. (a) Second preparation; 7.5% gel run in 6M-urea, 0.9M acetic acid. (b) Second preparation; 7.5% gel run in 0.1% SDS, 0.1M phosphate-Na^+, pH 7.2. (c) Third preparation; 7.5% gel run in 6M urea, 0.9M acetic acid. (d) Third preparation; 6% gel run in 0.1% SDS, 0.1M phosphate-Na^+, pH 7.2

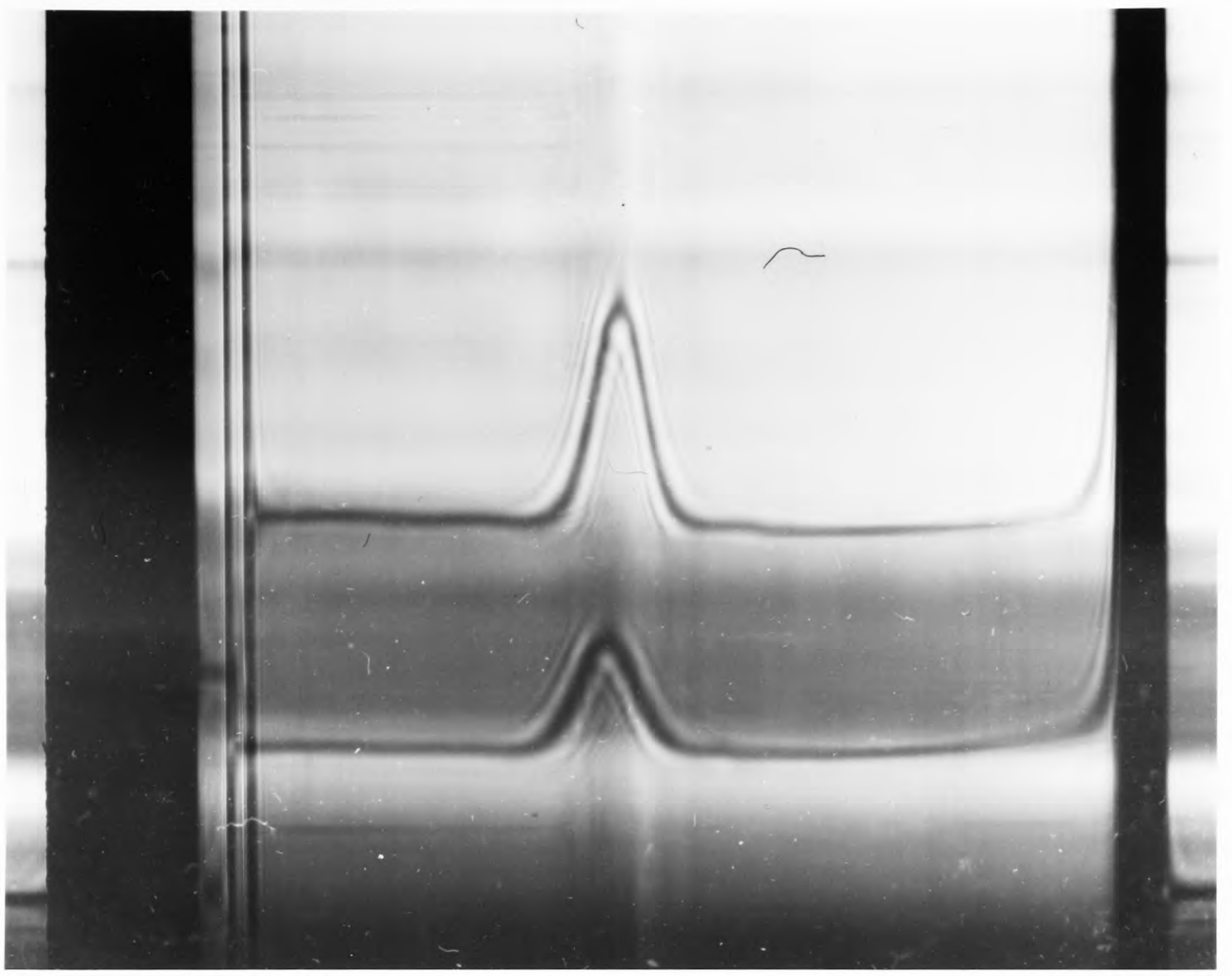


FIG. 4.8 Sedimentation of pyruvate kinase from the third large-scale preparation. The photograph was taken 40 min after reaching speed (59780 rev/min). The temperature was 20.2°C, the Schlieren analyser angle, 70°, and the buffer 0.1M KCl, 0.01M phosphate-K^+, pH 7.5. In the upper trace, the protein concentration was 6.0 mg/ml, and in the lower trace, 3.6 mg/ml.

TABLE 4.1

SUMMARY OF THE PREPARATION OF YEAST PYRUVATE KINASE

	Fraction	Volume mls	Protein mgs/ml	Protein Total	Activity U/ml	Activity Total	Activity U/mg.	Yield %
1.	Supernatant from freeze-thawed yeast.	4,080	24	98 g*	146	$6x10^5$	6.1	100
2.	First $(NH_4)_2SO_4$ precipitate resuspended in 0.1M Tris-HCl buffer.	670	24.5	16 g*	672	$45x10^4$	28	75
3.	Supernatant from 2. after centrifugation at 35,000 g for 5 min.	670	19.5	13 g*	608	$41x10^4$	31	68
4.	Second $(NH_4)_2SO_4$ precipitate after solution and dialysis for loading on to DEAE cellulose column.	85	123	10.5 g*	3100	$26x10^4$	25	43
5.	Total eluate from DEAE cellulose column.	-	-	9.3 g+	-	$19x10^4$	-	32
6.	Cut taken from 5. for loading cellulose phosphate column.	510	-	830mg+	300	$15x10^4$	192	25
7.	Total pyruvate kinase eluted from cellulose phosphate column.	-	-	-	-	$14x10^4$	-	23
8.	Pure pyruvate kinase recovered from cellulose phosphate column.	-	-	270mg‡	-	$10x10^4$	340+	16

Protein assays by: *micro-biuret, calibrated with bovine serum albumin (Itzaki & Gill, 1964); +assuming $E^{0.1\%}_{280nm} = 1.0$; ‡ $E^{0.1\%}_{280nm} = 0.65$.

yeast slurry was diluted with 2.5 l of the extraction buffer, and the mixture was then stirred continually. These innovations accelerated the release of pyruvate kinase from the cells; 10 mls of 0.5M phenylmethylsulphonylfluoride in propan-2-ol were added to the yeast after 13 hrs of thawing, and after 23 hrs, the purification was started. Table 4.1 summarises the progress of the preparation. The maximum specific activity of the final product was 380 U/mg, and the purity of the preparation was comparable with the previous one when assessed by electrophoresis in both SDS and urea-acetic acid gels (Fig. 4.7), and by its sedimentation velocity profile (Fig. 4.8).

c) Conclusions

The results presented in this Chapter demonstrate that the careful control of proteolysis is essential in order to obtain pyruvate kinase from yeast in an undegraded form. In addition to the precautions already mentioned, the purifications were conducted with the greatest possible haste. The fastest preparation was the last one, which took a total of seven days, with the DEAE column being loaded at the end of the third day. It might be advantageous to reduce this time still further; it would be difficult to accelerate the chromatography because the viscosity of 50% glycerol at $4^{o}C$ limits the flow rates of the columns, but the time taken for dialysis of the crude protein could be reduced by using a hollow-fibre dialysis unit, which should achieve equilibration of the protein with the buffer within 8 hrs.

The specific activity of the enzyme prepared by the freeze-thaw method was as much as 400 U/mg, which is 60% higher than the previous highest value reported for this protein (Röschlau and Hess, 1972a). The other properties of this pyruvate kinase are presented in the next Chapter.

CHAPTER 5

THE PROPERTIES OF PYRUVATE KINASE PREPARED FROM FREEZE-THAWED YEAST

a) The Molecular Weight and Hydrodynamic Properties of the Native Enzyme

The molecular weight of the native enzyme was determined by high-speed (Chervenka) equilibrium ultracentrifuge runs in the following solvents: 0.1M Tris-HCl (pH 7.5)-0.23M KCl-0.01M $MgCl_2$-1mM fructose diphosphate - 5mM phosphoenolypyruvate; or 0.1M phosphate-potassium (pH 7.5) or 0.1M KCl, 0.01M phosphate-potassium (pH 7.5). The results were more variable than would be expected for this technique; this may have been because the slow aggregation which occurs in solutions of pyruvate kinase was perturbing the equilibrium. A study of the time course of this aggregation (this Chapter, section c) had suggested that there would be a significant loss of enzyme by precipitation in the centrifuge cell in the time taken for a 3mm solution column to reach equilibrium (1½-2 days), and it was this which had dictated the use of the Chervenka method. Some low-speed equilibrium measurements were attempted with 2mm solution columns, which should have reached equilibrium within 24 hrs, but non-linear plots of ln c v. r^2 were obtained. From those Chervenka runs judged as reliable from the linearity of the plots in ln c and ln $(\frac{1}{r}.\frac{dc}{dr})$ v. r^2, over at least two-thirds of the measurable region of the cell (e.g. Fig. 5.1), and from the agreement to within 5% of the corresponding molecular weights, the mean weight-average molecular weight was determined as 214,000 (S.D. $\pm$ 7%), and the mean z-average molecular weight as 213,000 ($\pm$ 7%). The agreement of the two molecular

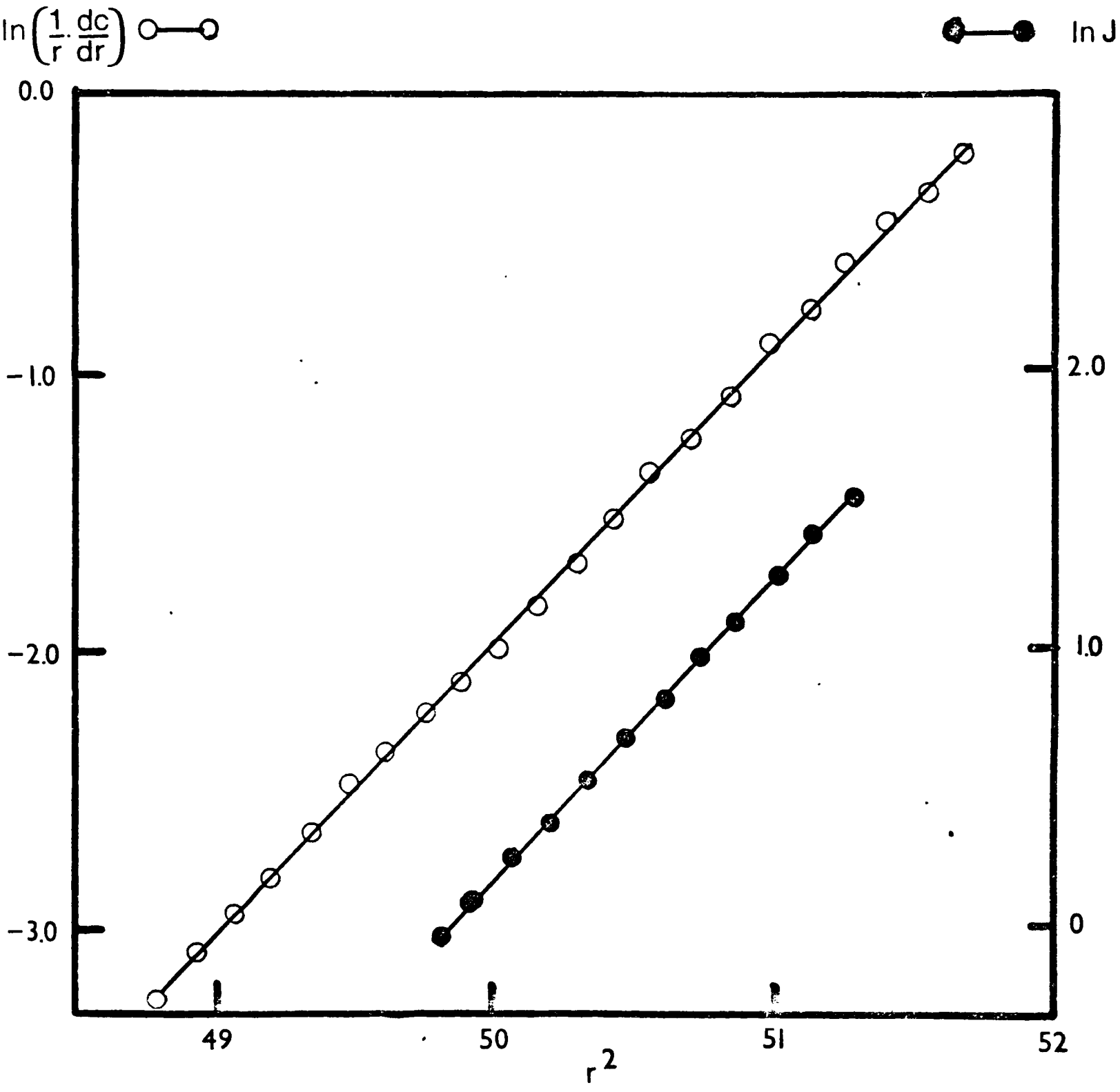


FIG. 5.1 Sedimentation equilibrium of native yeast pyruvate kinase. Plots of $\ln(\frac{1}{r}.\frac{dc}{dr})$ and ln J v. r^2 from a Chervenka run at 9341 rev/min and 20°C. The solvent was 0.1M phosphate-K^+, pH 7.5.

weights indicates that the enzyme was monodisperse. There was no detectable dependence of the molecular weight on protein concentration in the range measured (0.1 - 2 mg/ml, Fig. 5.2).

The sedimentation coefficient of the native enzyme was measured in 0.1M phosphate-potassium buffer (pH 7.5) and in 0.1M KCl with either 0.01M Tris-HCl (pH 7.5) or 0.01M phosphate-potassium (pH 7.5) as the buffer. In 0.1M phosphate-potassium, the $s^{o}_{20,w}$ was 8.65 (S.E.M. $\pm$ 1%) (Fig. 5.3). This is in agreement with the value of 8.7 S obtained in the same buffer at pH 7.0 by Bischofberger et al. (1971), and is significantly higher than the 8.3 S found by Kuczenski and Suelter (1970b), with 0.1M tris chloride as solvent. McFarland and Ainsworth (1972) also reported an $s^{o}_{20,w}$ of 8.2 for pyruvate kinase prepared by the method of Hunsley and Suelter (1969a). However, 0.1M phosphate-potassium was not considered an ideal solvent for sedimentation velocity measurements, both because the curvature of the Schlieren baseline indicated that some redistribution of the low-molecular weight solutes was occurring, and because Svedberg and Pedersen (1940) have indicated the possibility of errors owing to "secondary charge effects" when the masses of the components of the electrolyte are very different. These authors recommended KCl as a suitable electrolyte for use in sedimentation velocity experiments, so measurements were made in two solutions each containing 0.1M KCl and either 0.01M Tris-HCl (pH 7.5), or 0.01M phosphate-potassium (pH 7.5) as buffer. The results obtained in these two solutions were comparable, and were extrapolated together to give a value of 9.50 S (S.E.M. $\pm$ 2%) for $s^{o}_{20,w}$ (Fig. 5.3). That the enzyme had a higher sedimentation velocity in both these buffers than in 0.1M phosphate-potassium indicated that the difference was indeed the result of secondary charge effects in the latter buffer, and was unlikely to have been

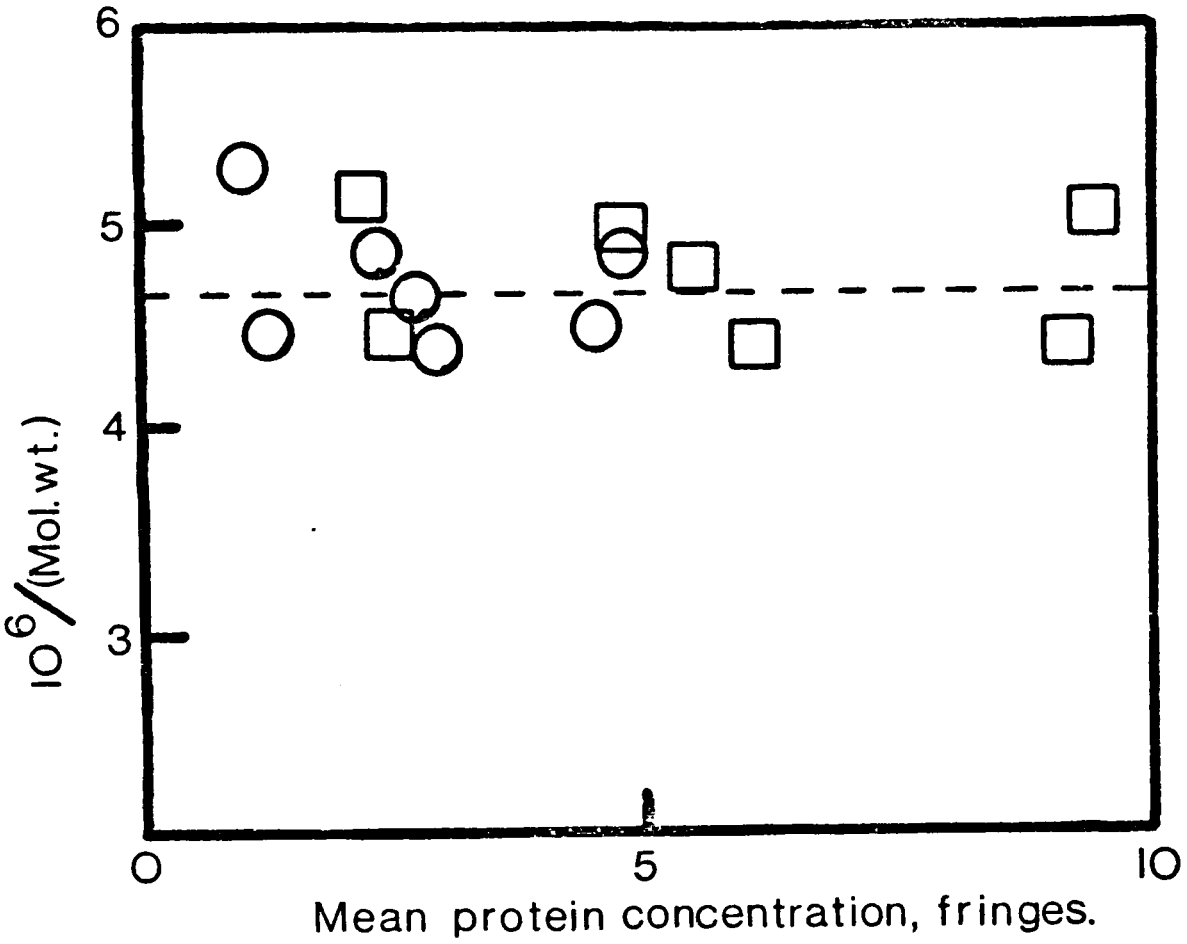


FIG. 5.2 Lack of dependence of molecular weight on protein concentration in the range studied. ○, $10^6/\bar{M}_w$ v. $\bar{c}$; □, $10^6/\bar{M}_z$ v. $2\bar{c}$. The line is the mean value of $10^6/214000$.

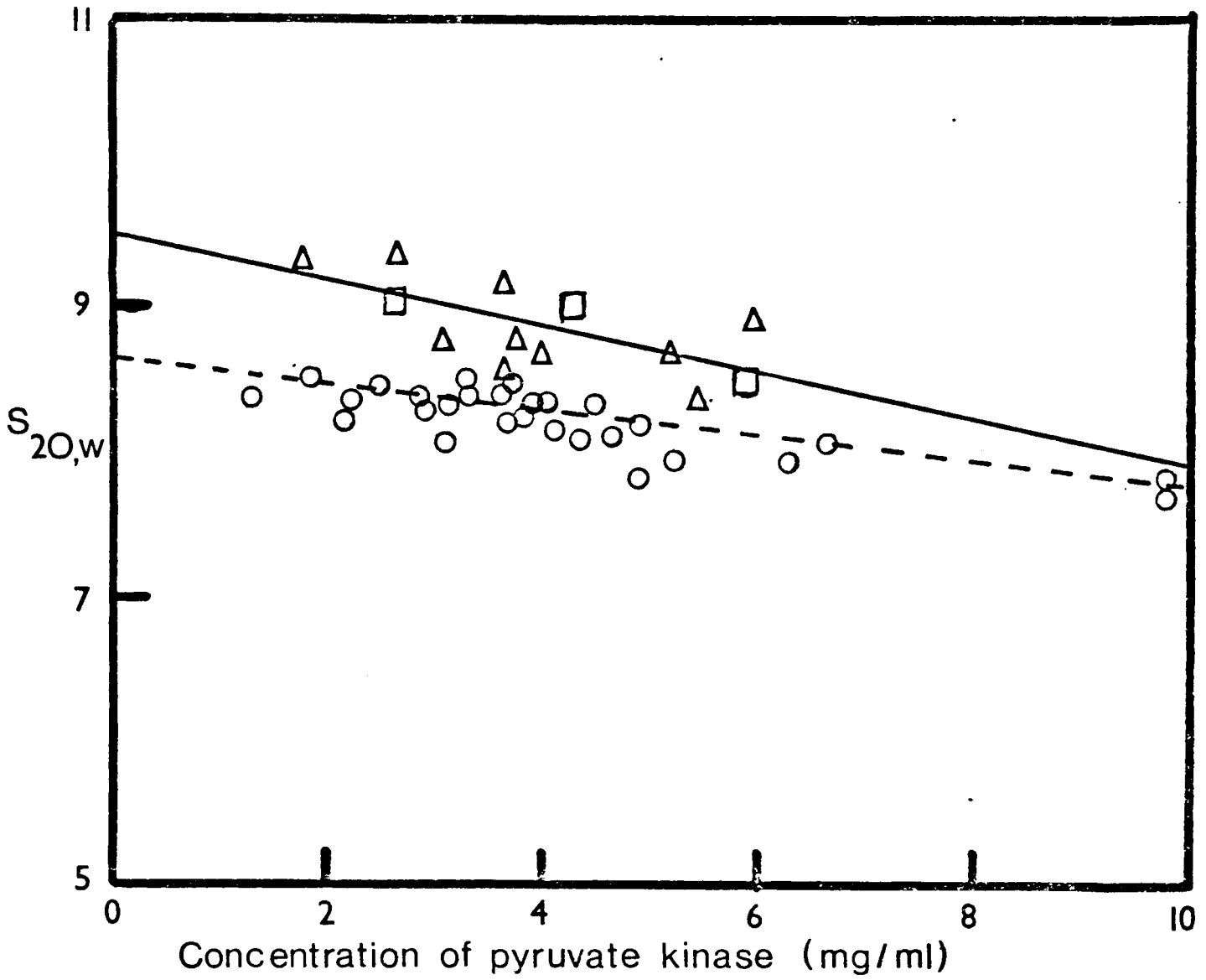


FIG. 5.3 Extrapolation of the sedimentation coefficient to zero concentration of enzyme. Symbols: O, results obtained in 0.1M phosphate-K^+, pH 7.5, and ---- , the least squares line for these points; □ and Δ, results obtained in 0.1M KCl with 0.01M Tris-HCl, pH 7.5, or 0.01M phosphate-K^+, pH 7.5, respectively, with the solid line the regression line for these points.

due to a conformational change induced by phosphate ions. 9.5 S was taken as the correct value for the sedimentation coefficient of yeast pyruvate kinase.

The diffusion coefficient of the enzyme was measured by the laser light-scattering technique in 0.1M phosphate-potassium, pH 7.5, at enzyme concentrations of 1.0, 2.2, and 4.4 mg/ml. There was no detectable dependence on concentration, so $D^{o}_{20,w}$ was taken as the average of the total of nine measurements as 3.99×10^{-7} cm^2 sec^{-1} (S.E.M. $\pm$ 1.3%). There should be no anomalies, arising from secondary charge effects, in this measurement as there is no separation of buffer ions and the solution remains macroscopically homogeneous.

Application of the Svedberg relationship yielded a molecular weight of 217,000 (S.E.M. $\pm$ 3%) for the native enzyme from the combination of the sedimentation and diffusion coefficients. This value is in close agreement with those obtained from sedimentation equilibrium, and the average value of 215,000 was taken as the molecular weight of yeast pyruvate kinase. Comparison of these results with the published results of other investigators (Table 6.1) shows closest agreement with those obtained by Bischofberger et al. (1971) with the pyruvate kinase from S. carlsbergensis, and this suggests that previous preparations from S. cerevisiae have been degraded by proteolytic action. Indeed, Suelter (personal communication) has confirmed that preparations by the method of Hunsley and Suelter (1969a) were subject to proteolysis; he reports a molecular weight of 209,000 for an undegraded preparation with a specific activity of 400-500 U/mg at 30°C.

A theoretical frictional coefficient (f_o) may be calculated from this molecular weight on the assumption that the protein is

spherical and then compared with the observed value, f, which may be calculated from the diffusion coefficient. The ratio f/f_o can give an indication of the asymmetry of a rigid particle, although interpretation is difficult since theoretical values of the ratio are calculable only for simple shapes such as ellipsoids of revolution or rods, and hydration of the particle also increases the value of the ratio. A value of 1.36 was found for the ratio, which agrees with the figure of 1.34 reported by Bischofberger et al. (1971). This corresponds to a large axial ratio for an ellipsoid (6-7 : 1) if asymmetry is the sole contributing factor.

b) The Molecular Weight of the Subunits of Pyruvate Kinase

Measurements of the molecular weight of the subunits were made by both the low-speed and the Chervenka sedimentation equilibrium methods, with the enzyme dissolved in 6M guanidinium chloride plus 1% 2-mercaptoethanol. The plots of ln c v. r^2 and the Lamm plots were either linear, or else showed convex curvature where the concentration was changing rapidly at the bottom of the centrifuge cell (Fig. 5.4); this latter behaviour is typical of a non-ideal solution. The results are summarised in Fig. 5.5, which shows the extrapolation of the molecular weights to zero concentration of protein. The weight-average molecular weight was 55,000 (S.E.M. $\pm$ 4%). The agreement, within experimental error, of the two molecular weight averages indicates that the solution of subunits was monodisperse, and the values obtained, together with the value of the molecular weight of the native enzyme, prove that there are four subunits per molecule of enzyme. This is in agreement with the results of Kuczenski and Suelter (1970b) and of Bischofberger et al. (1970), and disproves the report of Ashton and Peacocke (1971)

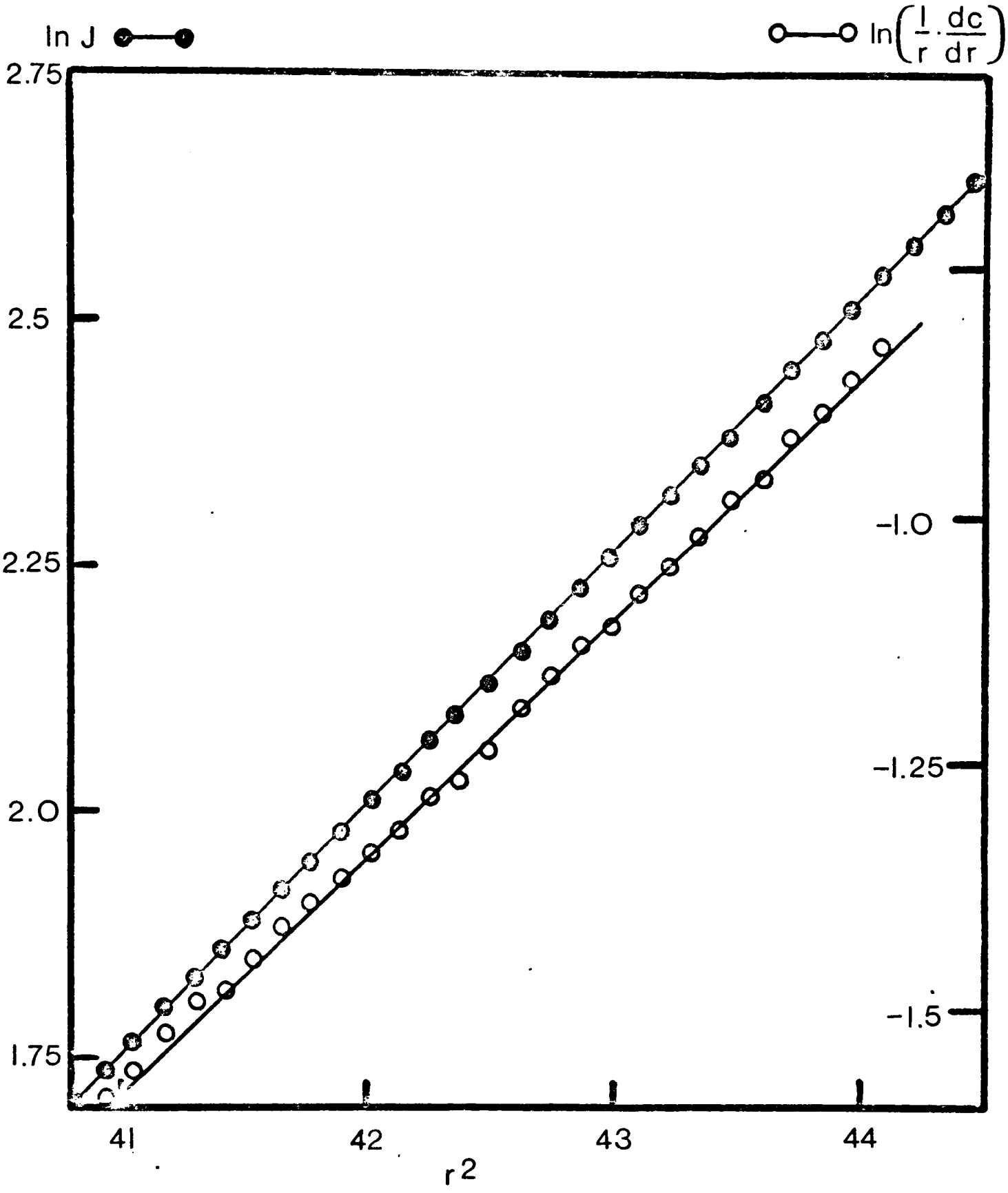


FIG. 5.4 Sedimentation equilibrium of the subunits of yeast pyruvate kinase. Plots of $\ln(\frac{1}{r}.\frac{dc}{dr})$ and ln J v. r^2 from a 3mm column at equilibrium at 14290 rev/min and 20°C. J_o was 9.45 fringes, and the solvent was 6M guanidinium chloride plus 1% 2-mercaptoethanol.

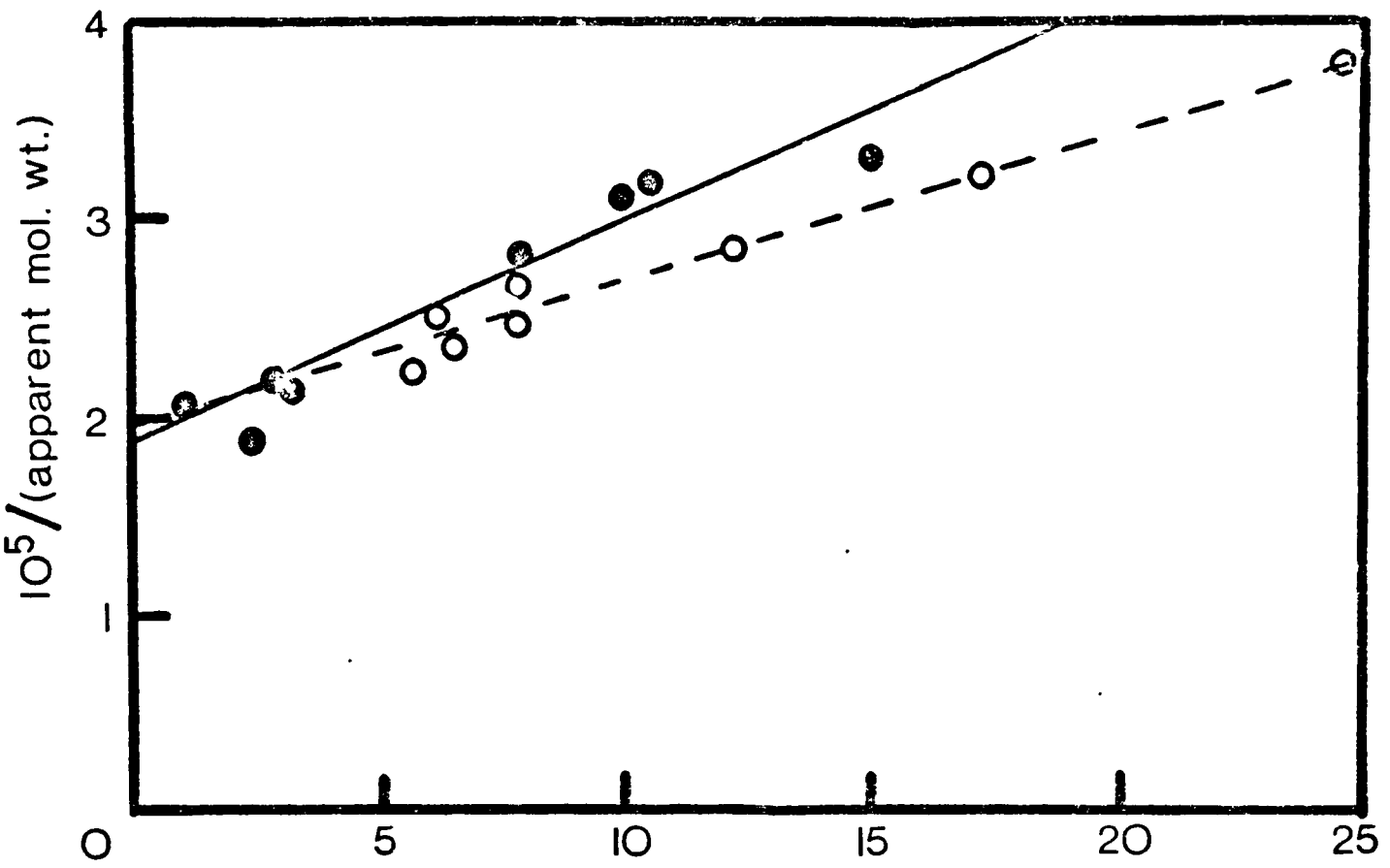


FIG. 5.5 Extrapolation of the subunit molecular weight to zero protein concentration. Symbols: O and ----, points and the regression line for $10^5/\bar{M}_z$ v. $2\bar{c}$; ● and ———, points and the regression line for $10^5/\bar{M}_w$ v. $\bar{c}$.

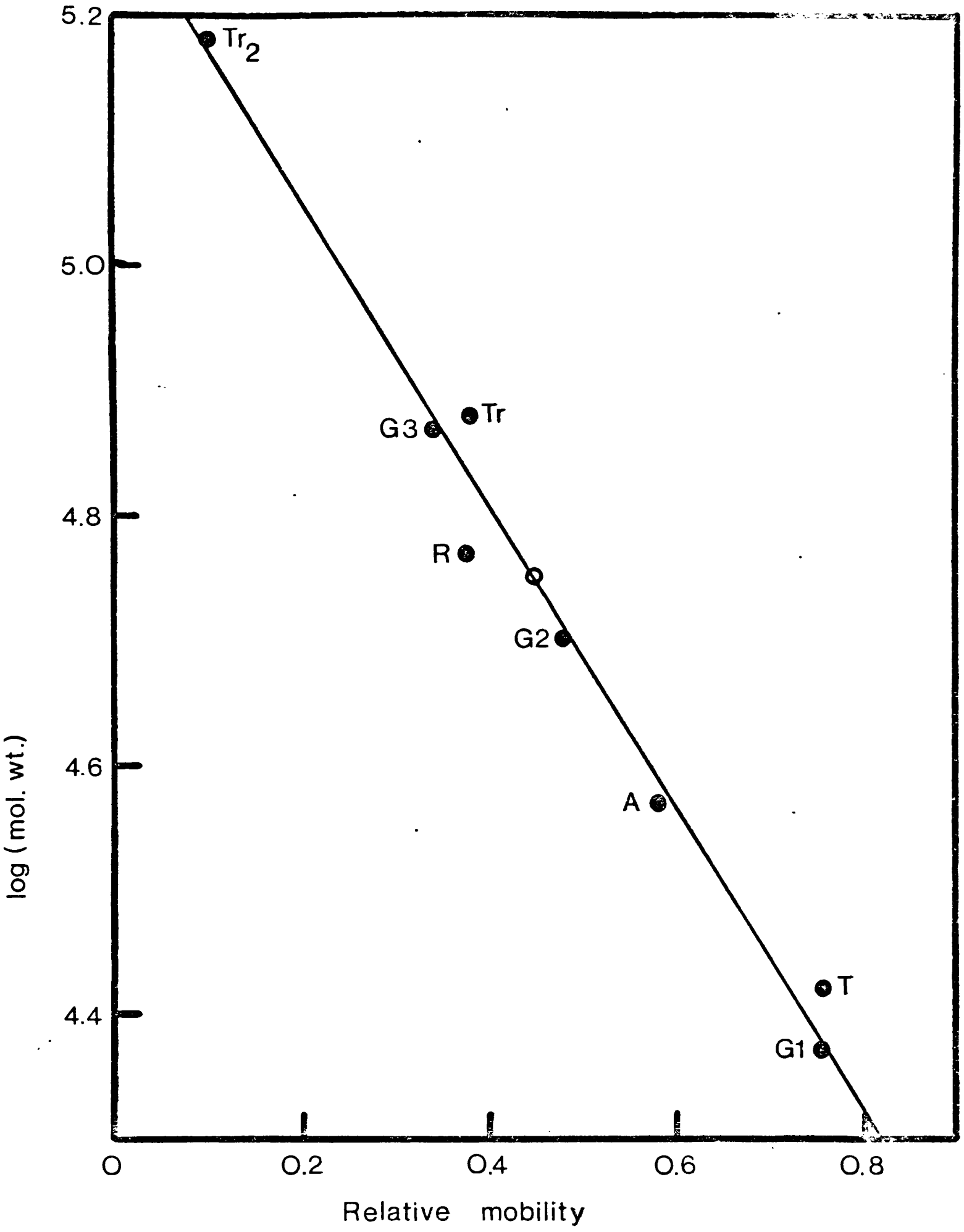


FIG. 5.6 Calibration of 6% SDS-polyacrylamide gels.

Tr = Human transferrin; G1 = γ-globulin, H + L chains; G2 = H chains; G3 = L chains; R = rabbit muscle pyruvate kinase; A = aldolase, and T = chicken muscle triose phosphate isomerase. The open circle is yeast pyruvate kinase.

that there were eight subunits.

The mobility of the reduced, dissociated enzyme in electrophoresis in SDS gels corresponded to a molecular weight for the subunits of 56,000 (Fig. 5.6), which is consistent with the results obtained by sedimentation equilibrium. The mobility was less than that of the subunits of pyruvate kinase from rabbit muscle (molecular weight 57,000-59,000; Steinmetz and Deal, 1966). The presence of a single band in electrophoresis in dissociating conditions (6M urea plus 0.9 acetic acid, or 1% SDS plus 1% 2-mercaptoethanol) suggests that the four subunits are identical. (Bornmann et al. 1972, have shown that the pyruvate kinase of S. carlsbergensis gave the number of peptides predicted, on the basis of the amino-acid composition and the assumption of four identical subunits, when cleaved by cyanogen bromide or trypsin).

c) The Stability of the Enzyme

Pyruvate kinase dissolved in 0.1M KCl with either 0.01M Tris-HCl (pH 7.5) or 0.01M phosphate-potassium (pH 7.5) as buffer, still retained over 50% of its initial activity after two days at room temperature (Fig. 5.7). During this time, the area under the Schlieren peak in successive sedimentation velocity runs showed a decrease of about 15% from its initial value. This loss was attributed to aggregation of the native enzyme, since solutions of pyruvate kinase become visibly turbid as they age. The presence of 1% 2-mercaptoethanol did not prevent the development of visible turbidity.

At low temperatures, the enzyme is markedly less stable; Fig. 5.8 shows the loss of activity in 0.1M Tris-HCl (pH 7.5) at $0^{o}C$ compared with the rate at room temperature; the presence of fructose diphosphate accelerated further the loss of enzymic

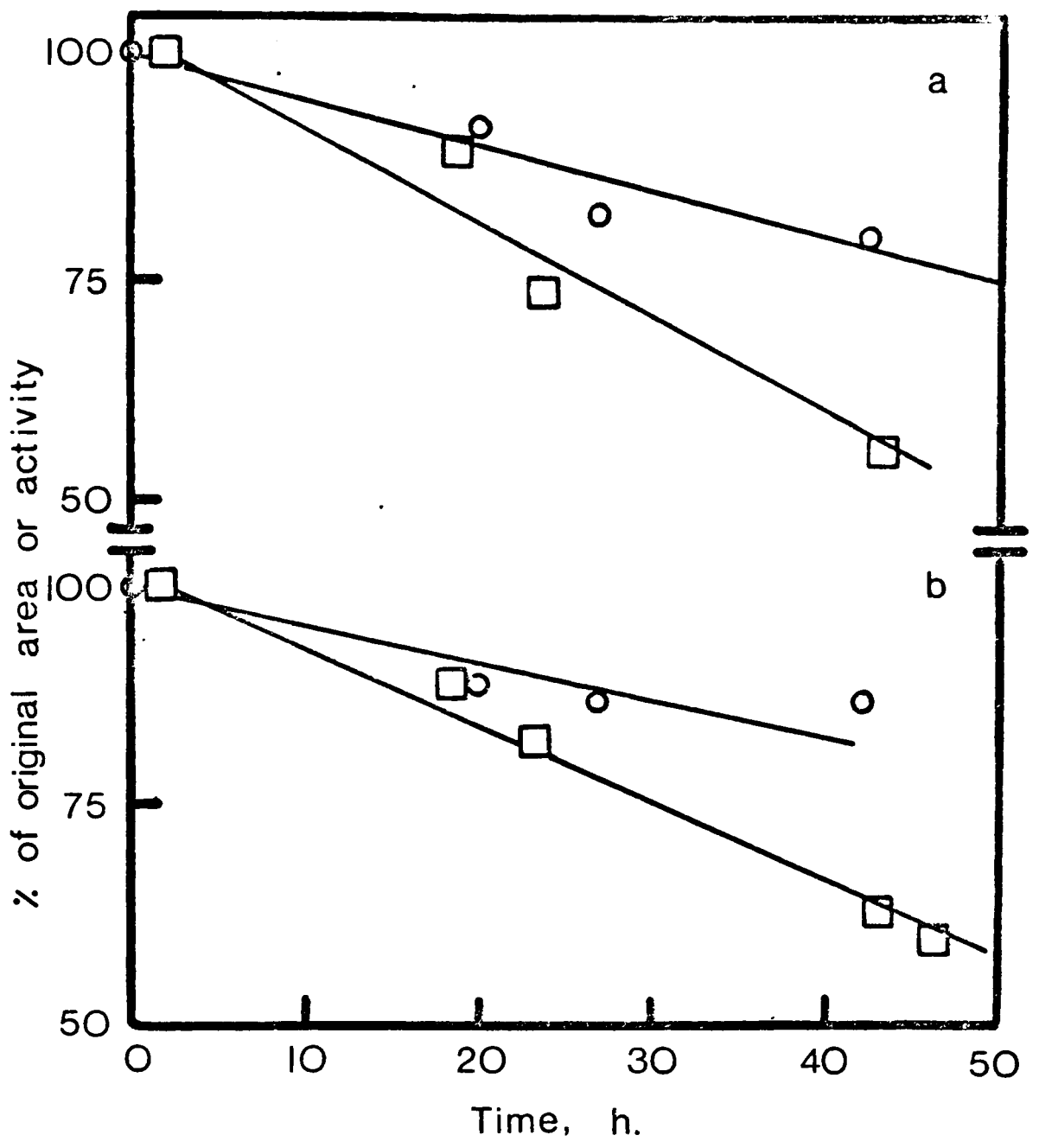


FIG. 5.7 Stability of pyruvate kinase at room temperature. Loss of enzymic activity and the area of the 9.5S Schlieren peak v. time from the first centrifuge run. (a) 5mg/ml of pyruvate kinase in 0.1M phosphate-K^+, pH 7.5. (b) 4.3 mg/ml of pyruvate kinase in 0.1M KCl, 0.01M Tris-HCl, pH 7.5. Circles - % of original peak area; squares, % of original specific activity. (The initial specific activity was 360 U/mg).

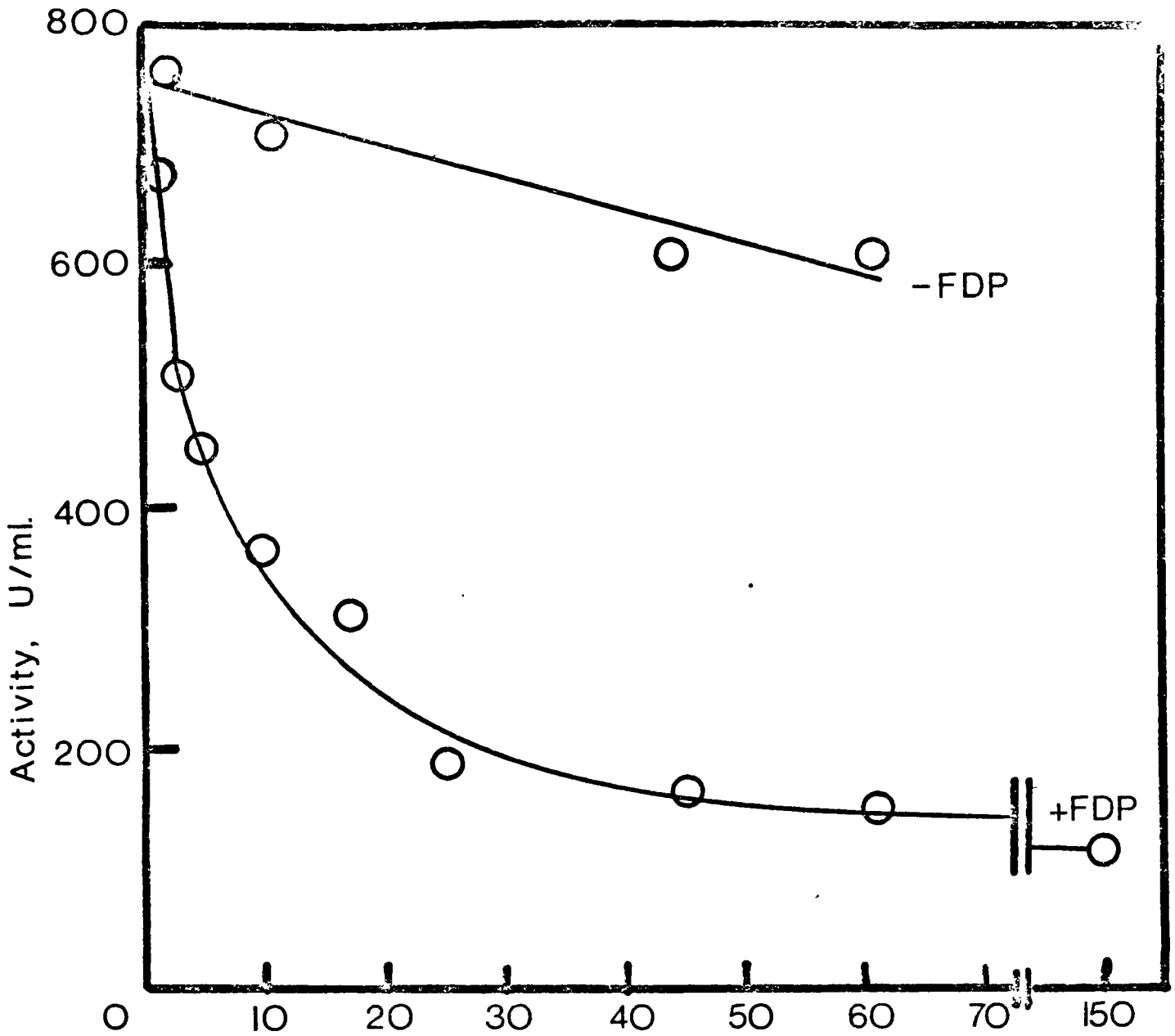


FIG. 5.8 Stability of yeast pyruvate kinase at 0°C and in the presence of fructose diphosphate. The pyruvate kinase concentration was 1.64 mg/ml in 0.1M Tris-HCl, pH 7.5. The initial specific activity was 450 U/mg. At room temperature, less than 5% of the initial specific activity was lost in this period. " + FDP" - experiment in the presence of 1mM fructose diphosphate.

activity. Thus in these respects, the enzyme retained its similarity to that prepared by Ashton's method (Chapter 3, section b) and that prepared by the method of Hunsley and Suelter (1969a), which was used in the original study of these phenomena by Kuczenski and Suelter (1970a). It is therefore apparent that the properties of cold-lability and inactivation catalysed by fructose diphosphate are intrinsic properties of purified pyruvate kinase from S. cerevisiae, and are not created by proteolysis of the enzyme. Since the pyruvate kinase from S. carlsbergensis does not exhibit these properties (B. Hess, personal communication), there must be some genetic divergence between the enzymes from these two strains of yeast.

d) Enzyme Kinetics

The kinetics of yeast pyruvate kinase have been described by both Hunsley and Suelter, 1969b, (for S. cerevisiae) and by Haeckel et al. 1968, (for S. carlsbergensis), and the results revealed few significant differences between the enzymes from the two strains of yeast. One difference, however, was that Hunsley and Suelter reported that the activity of the enzyme when activated by ammonium ions was 50% of that obtained by activation with potassium ions, whilst Hess and Haeckel (1967) found that NH_4^+ was nearly as effective as K^+ for a crude preparation of the enzyme. Further, as reported in Chapter 4, section b(i), preparations of the enzyme from freeze-thawed yeast showed inhibition by NH_4^+, although this effect had never been observed with pyruvate kinase isolated by the method of Ashton.

The dependence of the enzymic activity on the univalent cation was therefore investigated. The assay system contained 0.1M cacodylate-tetramethylammonium hydroxide, pH 6.2, 25 mM

$MgSO_4$, 1mM EDTA, 5mM ADP, 5 mM phosphoenolpyruvate, 0.15 mM NADH, 20 µg of lactate dehydrogenase (added from a solution in 50% glycerol), and varying concentrations of the univalent cation (as the chloride salt). Fructose diphosphate was present at 1 mM when added. The results for K^+ are shown in Fig. 5.9. The half-saturation points are 20 mM and 7 mM in the absence and presence of fructose diphosphate respectively, and the corresponding Hill coefficients are 2.5 and 0.9. The comparable values for $K_{0.5}$ reported by Hunsley and Suelter were 50 mM and 10 mM, and those reported by Hess and Haeckel were 30 mM and 4.5 mM. In the presence of 450 mM NH_4^+, the enzymic activity was found to be only 28% of that obtained with K^+. Also, the extent of the inhibition when both cations were present together showed that the affinity of the enzyme for K^+ was less than for NH_4^+. For comparison, Hunsley and Suelter reported a half-saturation value for NH_4^+ in the presence of fructose diphosphate of 3 mM, and Hess and Haeckel quoted 8 mM.

It seems unlikely that these varying activities with ammonium ions are physiologically significant, and what effect proteolysis may have had in generating these observed differences in cation specificity will probably never be clear, but the observations have been mentioned for comparative purposes.

e) N-terminal Amino-Acid

Samples of pyruvate kinase from both of the pilot-scale preparations from freeze-thawed yeast, and from the third of the large-scale preparations, were reacted with dansyl chloride for identification of the N-terminal amino-acid. An equivalent molar amount of triose phosphate isomerase from chicken muscle (kindly supplied by J.D. McVittie) was also dansylated for comparative

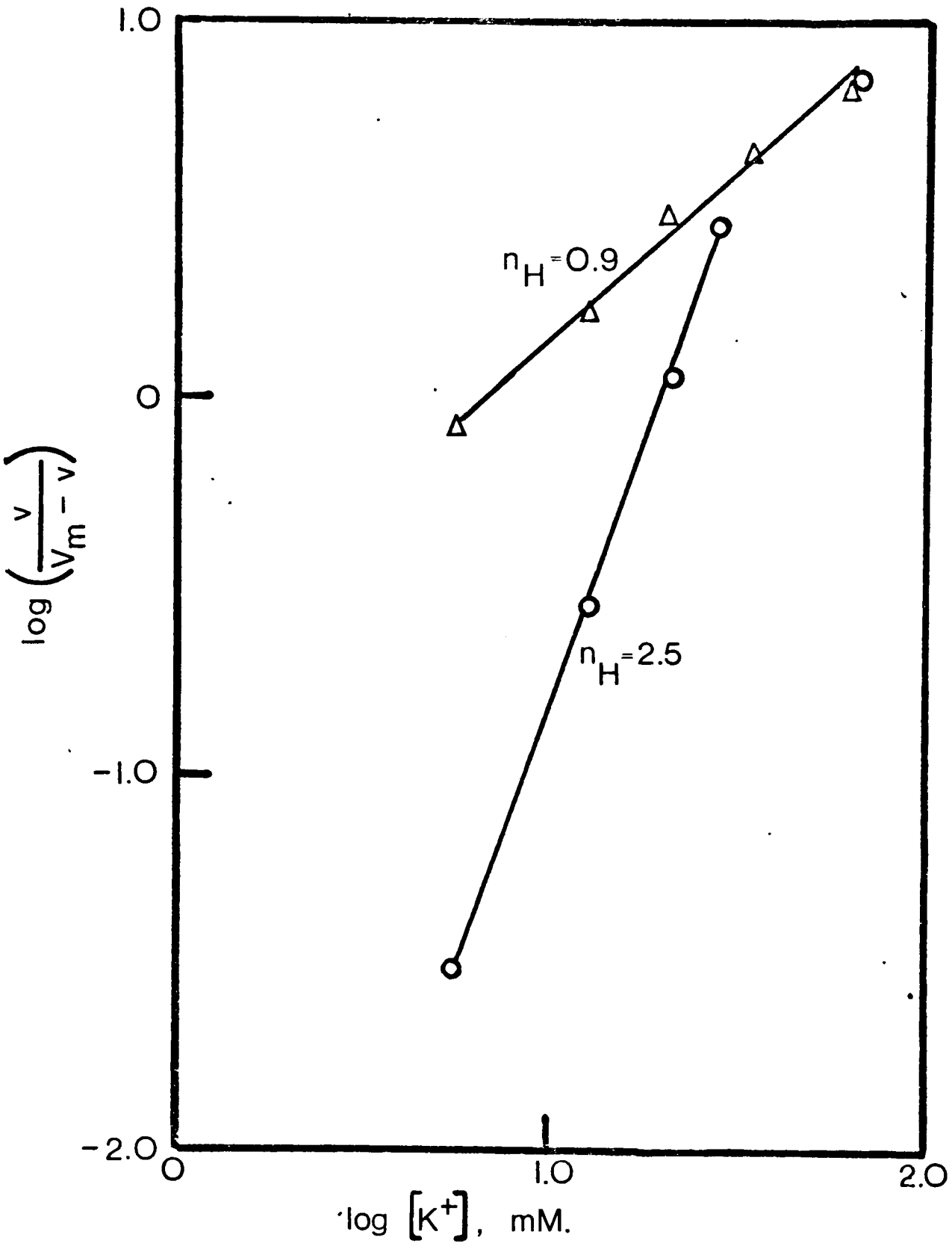


FIG. 5.9 Hill plot for the dependence of the initial rate of enzyme reaction on the concentration of K^+.

O, no fructose diphosphate present; Δ, + 1mM fructose diphosphate. For other assay conditions, see text. V_m, measured in 450 mM K^+, was 380 U/mg both in the presence and absence of fructose diphosphate.

purposes. Although a spot corresponding to dansyl-alanine was just visible on chromatography of the samples from pyruvate kinase, its intensity was not sufficient for it to represent the N-terminal amino-acid of the enzyme sample, and some other spots were also visible at the same very low intensity. Under the same conditions, the N-terminal alanine of chicken muscle triose phosphate isomerase (Furth et al. 1974) was clearly visible. These observations are explicable if the N-terminal amino-acid of pyruvate kinase from S. cerevisiae is acetylated, as has been reported for the enzyme from S. carlsbergensis (Bormann et al. 1972). The faint spots observed on chromatography of the hydrolysed, dansylated protein presumably corresponded to traces of impurities and proteolysis.

The finding of N-terminal alanine in pyruvate kinase prepared by the method of Ashton (Chapter 3, section c) indicates that alanine was consistently liberated as an N-terminal acid during the partial degradation of the enzyme.

f) Extinction Coefficient

The extinction coefficient at 280 nm was estimated from optical density measurements on dialysed solutions of pyruvate kinase, the concentrations of which had been determined by refractive index measurements made in a Brice-Phoenix differential refractometer. The error in converting a difference in refractive index, between a protein solution and its diffusate, into a protein concentration in mg/ml will be small, since the specific refractive increment (dn/dc) for pure proteins is relatively invariant and close to 0.184 ml/g at 546 nm (e.g. Halwer et al. 1951). The presence of carbohydrate may alter the specific refractive increment for glycoproteins, but yeast pyruvate kinase was shown not to contain carbohydrate; the enzyme failed to give

a positive response when a urea/acetic acid gel was stained for carbohydrate by the performic-acid-Sciff method of Fairbanks et al. (1971), and when a solution was estimated for neutral sugar content by the phenol-sulphuric acid method of Dubois et al. (1956). (The latter method was calibrated with glucose, and the sensitivity of the experiment was such that any carbohydrate content must be less than 0.2%).

The value obtained for the extinction coefficient ($E_{1cm}^{0.1\%}$ at 280 nm = 0.66 ml.mg^{-1} cm^{-1} $\pm$ 0.02; mean of 3) is in good agreement with the value of 0.65 quoted for the pyruvate kinase from S. cerevisiae by Hunsley and Suelter (1969a) and by Ashton (1971). Haeckel et al. (1968) reported a value of 0.76 for the enzyme from S. carlsbergensis.

g) Reactivity of the Thiol Groups

This was investigated in order to find suitable conditions for the incorporation of a spin-label reporter group into a specific site on the enzyme; since one of the spin-labels was a derivative of iodoacetamide (Chapter 2, section o), it was expected to be most reactive towards sulphydryl groups.

The reaction of the thiol groups of yeast pyruvate kinase with DTNB was accompanied by a second, slower reaction which led to a decline in the absorbance developed at 412 nm by the generation of the thiobenzoate ion (Fig. 5.10). This was assumed to be a slow air oxidation of the product since it was slower after the solutions had been degassed with a water pump. As a result, the reaction in the presence of excess DTNB did not give a linear plot of $\ln(a_t - [TNB])$ v. t, and so the results were plotted in the form of the equation derived in Chapter 2, section n, (Fig. 5.10).

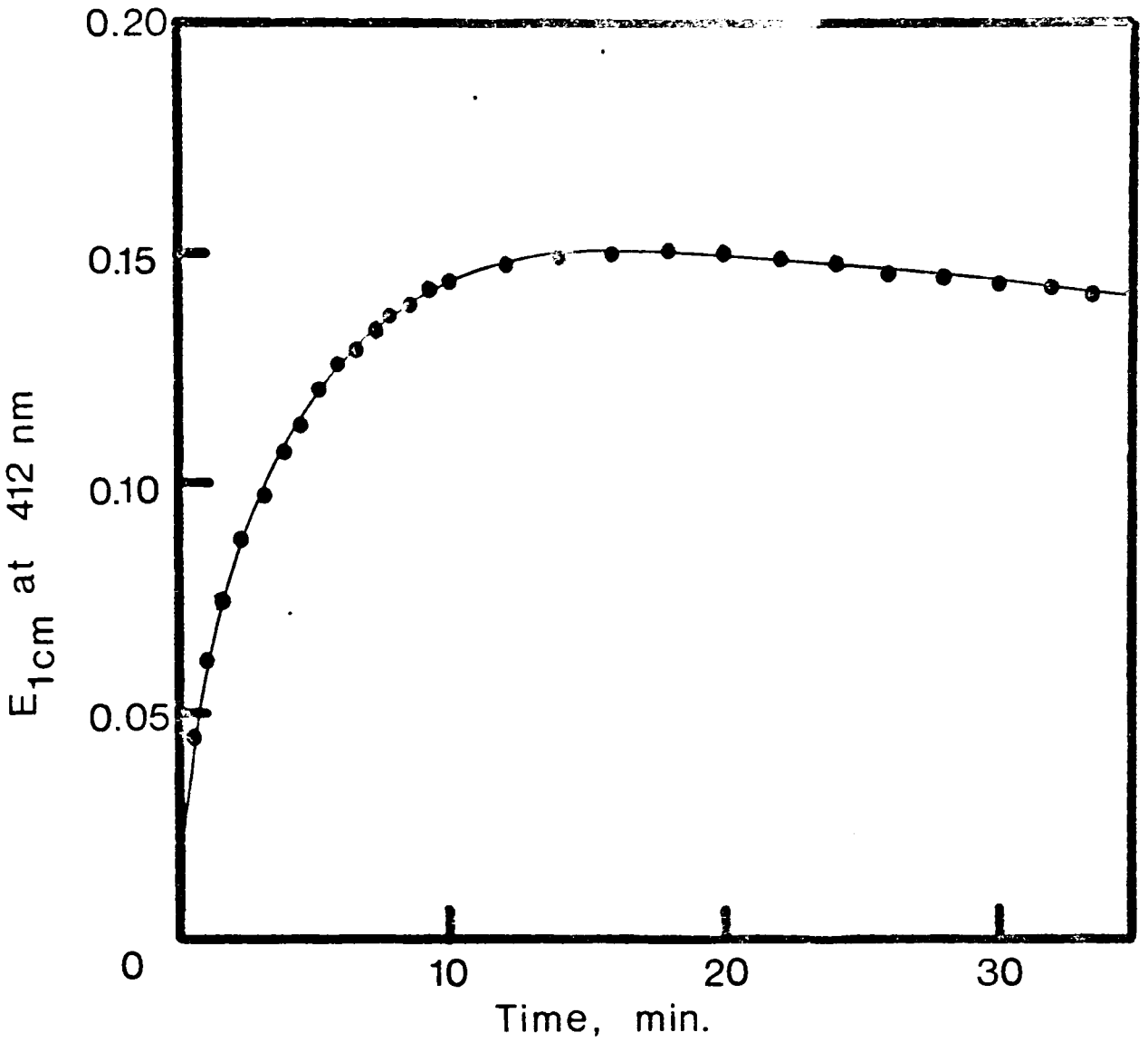


FIG. 5.10a The reaction of pyruvate kinase with DTNB. Absorbance at 412 nm v. time, taken from the smoothed chart record of the reaction of enzyme (0.88 μM) with DTNB (0.81 mM) in 0.1M Tris-HCl, pH 7.5, 0.1M KCl.

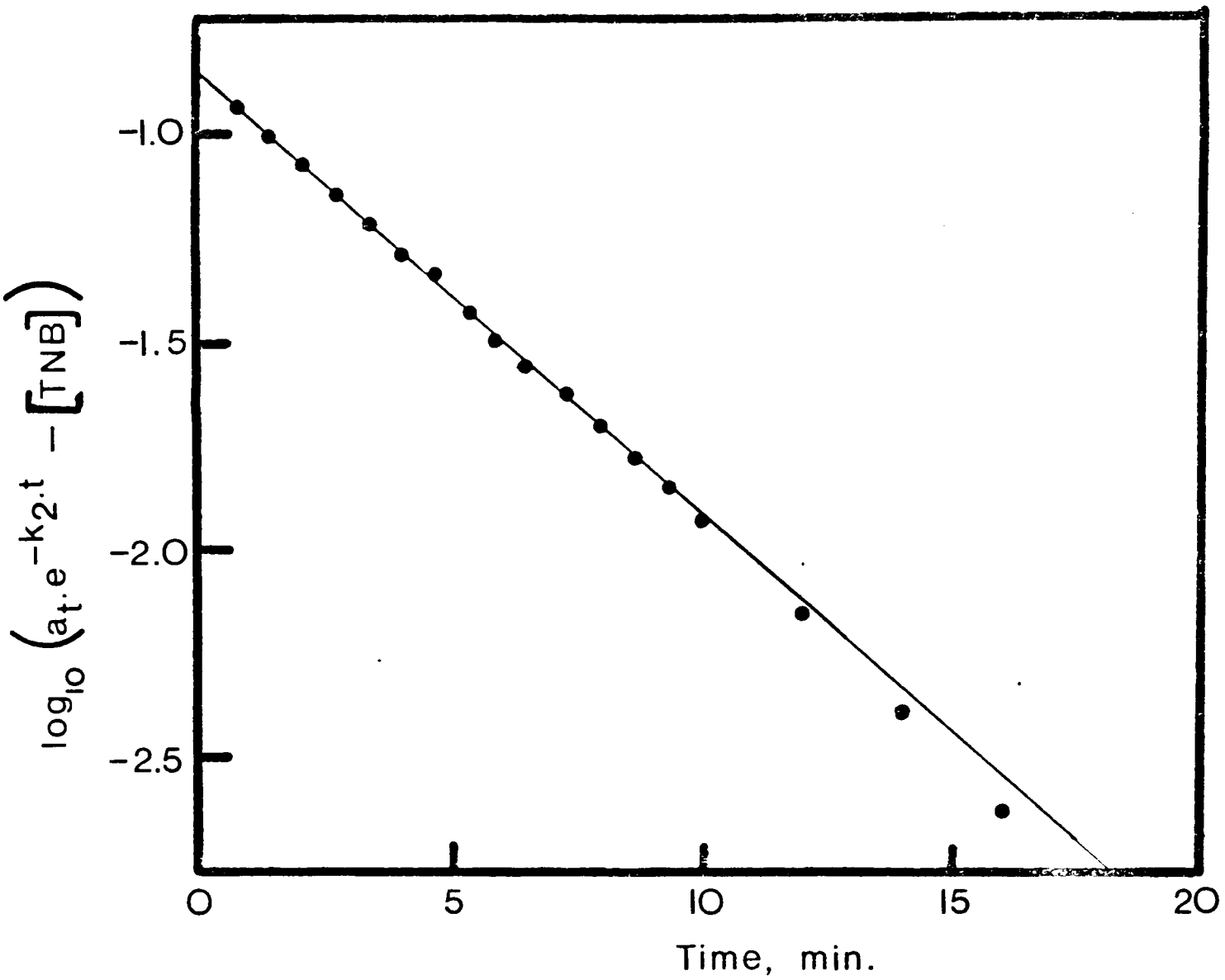


FIG. 5.10b <u>The reaction of pyruvate kinase with DTNB</u>.
The results of (a) plotted according to equation 2.37 with a_t = 0.163 absorbance units. The line is the regression line through the points for the first 8 min.

It was found that 3.33 (S.D. ± 0.13, 7 results) thiol groups per subunit of pyruvate kinase reacted with DTNB at pH 7.5 in the absence of denaturant. In the presence of 0.5% SDS, 5.67 (± 0.35) thiol groups per subunit reacted. Of the groups reacting in the native state, 0.65 (± 0.2) reacted within the time taken for mixing, whilst the remainder reacted with a half-life of the order of 2 min.

Detailed analysis of the kinetics of labelling by iodoacetamide, as followed by the incorporation of radioactivity from iodo[1-^{14}C]acetamide, was prevented by the eventual denaturation and precipitation of the enzyme before the reaction had reached completion. Qualitatively, it is apparent from the reaction curves (Fig. 5.11) that 0.3-0.5 groups per subunit were reacting rapidly; further groups were reacting more slowly, but after about 1 h, precipitation began to be noticeable. The specific activity was still 400 U/mg after reaction with iodoacetamide for 45 min, by which time 0.5 groups per subunit had reacted, provided that the diluted sample was assayed immediately; otherwise, since the activity of the samples declined with time, (even though the reaction with iodoacetamide would have been stopped by dilution and the change of pH) it was impossible to assign a specific activity to the parent solution. Wieker and Hess (1972) found that pyruvate kinase, from S. carlsbergensis, containing one mole of acetamide per subunit was stable, and similar in its kinetics to the native enzyme.

That iodoacetamide reacted first with the thiols that react rapidly with DTNB was demonstrated by withdrawing samples at various times from a solution containing 1 mM iodoacetamide and 2.3 mg/ml of pyruvate kinase, and reacting these with 40 μM DTNB. Under this condition of a limiting concentration of DTNB,

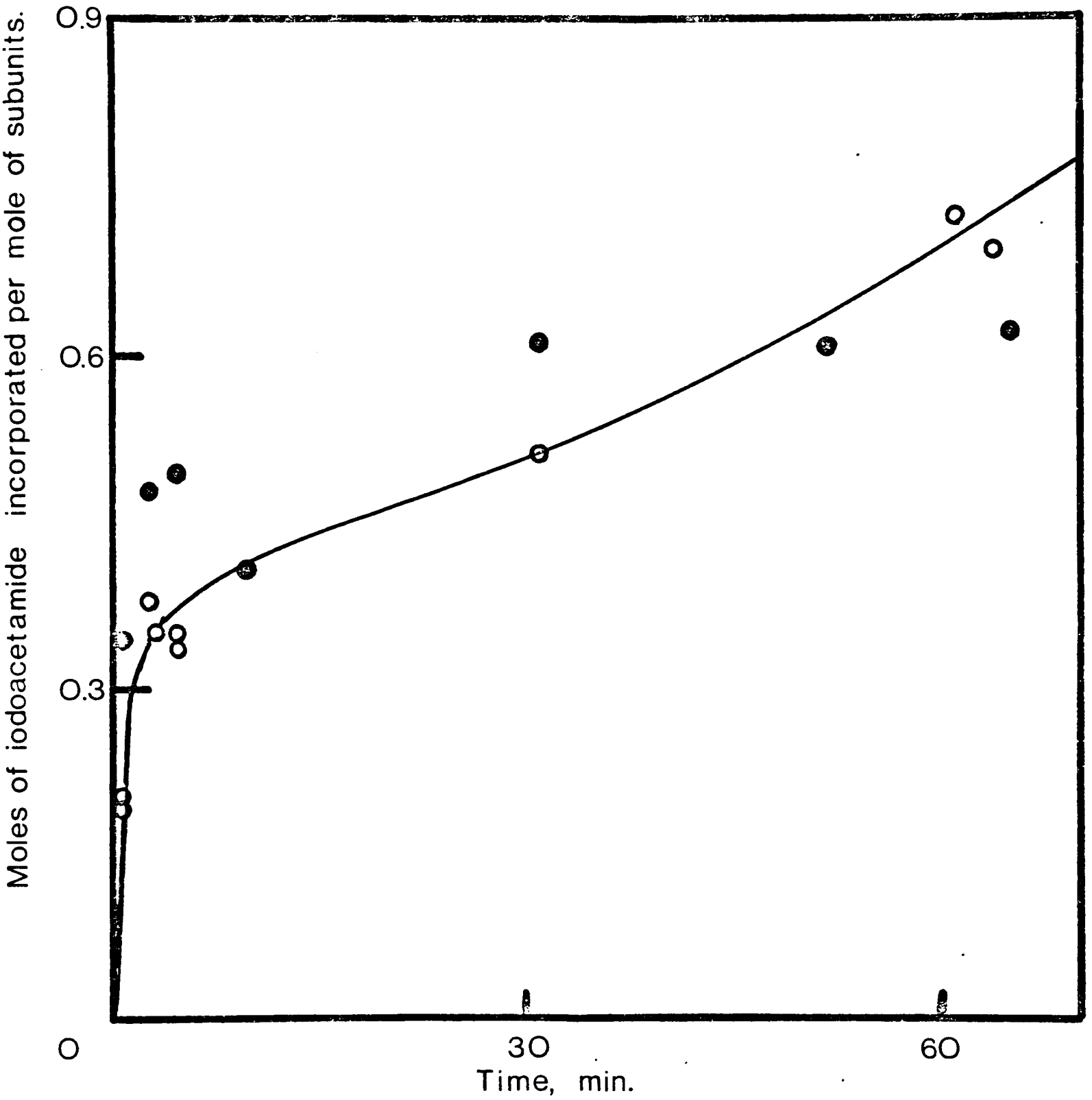


FIG. 5.11a The reaction of the thiol groups of pyruvate kinase with iodoacetamide. O , the reaction followed by the incorporation of ^{14}C; enzyme, 1.6 mg/ml; 1 mM iodoacetamide; 0.1M KCl; 0.1M Tris-HCl, pH 7.5, and temperature 25°C. the reaction followed by the loss of groups reacting rapidly with DTNB; enzyme 2.3 mg/ml, and other conditions as above.

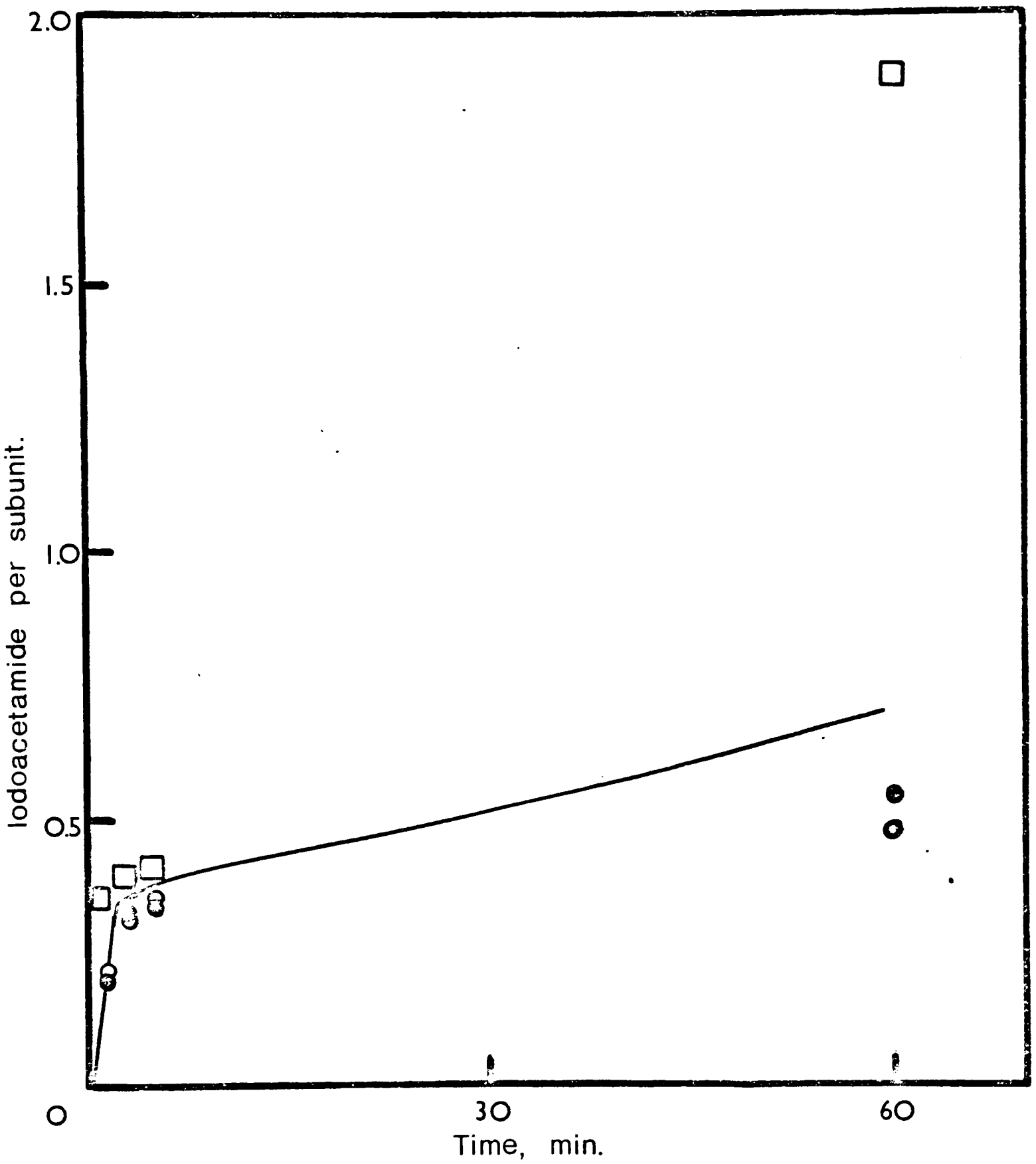


FIG. 5.11b The reaction of the thiol groups of pyruvate kinase with iodoacetamide. The effect of ligands on the incorporation of ^{14}C from iodoacetamide; ○ , as for ○ in (a), plus 1 mM phosphoenolpyruvate and 1 mM $MgCl_2$; ● , plus 1 mM ADP and 1mM $MgCl_2$, and □ , plus 1mM fructose diphosphate and 1mM $MgCl_2$. The line is redrawn from part (a).

the rapidly-reacting thiol groups still reacted within the time taken for mixing, but the remaining groups reacted more slowly so that the reaction curve itself could be extrapolated to zero time to give the number of rapidly-reacting groups remaining available for reaction with DTNB. The reaction with iodoacetamide was shown to be making these groups - estimated by this technique as 0.6 per subunit - unavailable to DTNB (Fig. 5.11).

From these results, the stoichiometry of the reaction of the thiols is inferred to be as follows:

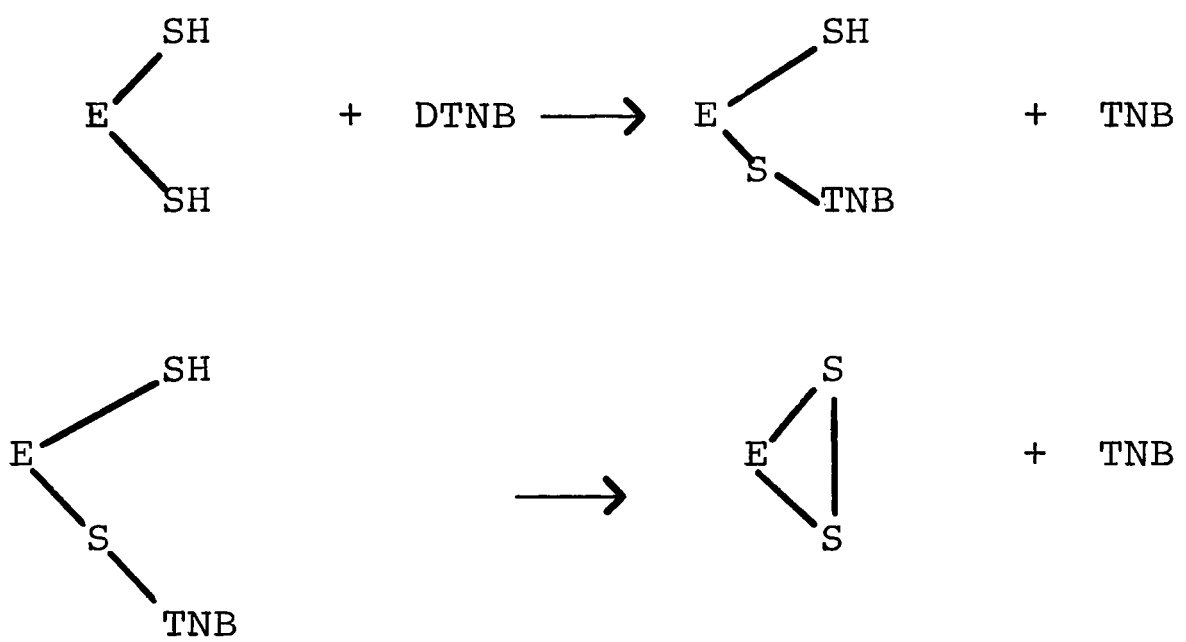
(i) 0.5 groups per subunit reacted rapidly with both DTNB and iodoacetamide;

(ii) 3.5 groups are accessible to DTNB without addition of a denaturant, and

(iii) a further 2.5 groups react with DTNB in the presence of a denaturant, to give a total of 6 groups per subunit.

Bondar and Suelter (1971) reported that 1.5 groups per subunit of pyruvate kinase from S. cerevisiae reacted with DTNB within 10 s and that of a total of 6 thiols, 3.5 reacted in the native state of the protein. Wieker and Hess (1972) found that 1 group per subunit reacted rapidly with DTNB, and 3 groups from a total of 6 reacted without the addition of denaturant. The most significant differences between these results (which are summarised in Table 6.1) are in the number of rapidly-reacting sulphydryl groups. (In fact, the degree of agreement seems better than it ought, since different molecular weights were used to calculate the molar concentrations of enzyme in the three cases). This may reflect genuine differences in the primary structure of the enzymes from the three sources, which either affect the

reactivity of the sulphydryls directly, or which affect the possibility of a rapid disulphide interchange of the type shown by Flashner et al. (1972) to occur in the reaction of DTNB with rabbit muscle pyruvate kinase:-



The latter possibility is mentioned because Wieker and Hess (1972) found that if their yeast enzyme was isolated after reaction of 1 sulphydryl group per subunit, the protein did not contain TNB. The comparison has been further complicated by the recent finding of Yun and Suelter (1973, and Suelter, C.H., personal communication) that a new preparation from S. cerevisiae (already referred to in section a of this Chapter) has 1 fast-reacting thiol group per subunit, a further 2 groups that react in the native state, and a total of 5 thiol groups per subunit. It is difficult to assess the significance of these results in the absence, at present, of information on the preparation and characterisation of this enzyme.

The presence of either of the substrates ADP or phosphoenolpyruvate did not significantly affect the reaction of the enzyme with either DTNB or iodoacetamide. However, the presence of the allosteric effector fructose diphosphate (1 mM) increased the rate of reaction with iodoacetamide (Fig. 5.11b), and the

extent of the reaction with DTNB. In the latter case, more than 3.5 groups reacted per subunit without the addition of any denaturant, although the reaction could not be accurately quantitated as the protein began to precipitate.

h) ESR Spectra of Spin-Label Derivatives of Pyruvate Kinase

It was anticipated, from the results of the previous section, that the "iodoacetamide spin-label", (N-(1-oxyl 2,2,6,6, tetramethyl-4-piperidinyl)-iodoacetamide), would react with the thiol groups of the enzyme, and that it would react first with the two groups per mole of enzyme that react rapidly with iodoacetamide and DTNB.

This was confirmed by monitoring the reaction of pyruvate kinase with 0.2 mM spin-label by withdrawing samples and reacting them with DTNB, when a slow loss of the rapidly-reacting groups was observed.

Several attempts were made to produce a spin-labelled pyruvate kinase which gave a spectrum showing the spin-label present in a single environment. For the first, 30 μM enzyme was reacted with 1 mM iodoacetamide spin label for 20 mins in 0.1M Tris-HCl, pH 7.5, 0.1M KCl. An equal volume of glycerol was then added, and the mixture was set to dialyse against three changes of 50% glycerol, 50% buffer at 4^{o}C for 30 hrs. The labelled protein was then collected by dialysis against saturated ammonium sulphate solution. The ESR spectrum of the protein dissolved in 0.1M KCl, 0.02 M Tris-HCl, pH 7.2 (Fig. 5.12a) showed two components - one weakly immobilised and one moderately or strongly immobilised (cf. Fig. 2.2). For the second attempt, 25 μM pyruvate kinase was reacted with 0.2 mM spin label in the presence of 1 mM phosphoenolpyruvate, 1 mM $MgCl_2$.

After 40 mins, when reaction with an aliquot of DTNB showed that the reaction of the reactive sulphydryl groups was 70% complete, glycerol was added and the mixture dialysed as before. The spectrum (Fig. 5.12b) showed a larger contribution from the immobilised component than previously, so it was concluded that the specificity of the labelling had been improved, and that the more immobile component was given by the spin label attached to the reactive sulphydryls. For the third attempt, the buffer was 0.08M KCl, 0.02M Tris-HCl, pH 7.2, and 1 mM phosphoenol-pyruvate; the enzyme concentration was 40 μM, and the concentration of the spin label was 100 μM, so that the ratio of the reactants was 1 : 2.4. Under these conditions, assay with DTNB showed that the reaction of the sulphydryl groups was much slower than in the previous experiments. After 165 mins, glycerol was added, and the mixture dialysed as before. The spectrum of this sample was contributed mainly by the more immobilised component (Fig. 5.12c) with a smaller contribution from the other component. Unfortunately, none of these preparations showed significant variations in the spectrum (either in the splitting of the peaks, or of the ratios of the peak heights) on addition of any of the ligands phosphoenol-pyruvate, ADP, fructose diphosphate, ATP, or Mg^{++}. Additionally, the first sample was unstable; it precipitated during the course of the experiments, and the specific activity fell to about 10% of its original value. The other samples were more stable, and the specific activity of the enzyme was unchanged.

The reaction of the enzyme with an isothiocyanate spin-label (4-isothiocyanate-2,2,6,6 tetramethyl piperidinoxyl) was also investigated. This reagent would be expected to react principally with the amino-groups on the protein and the pyruvate kinase from

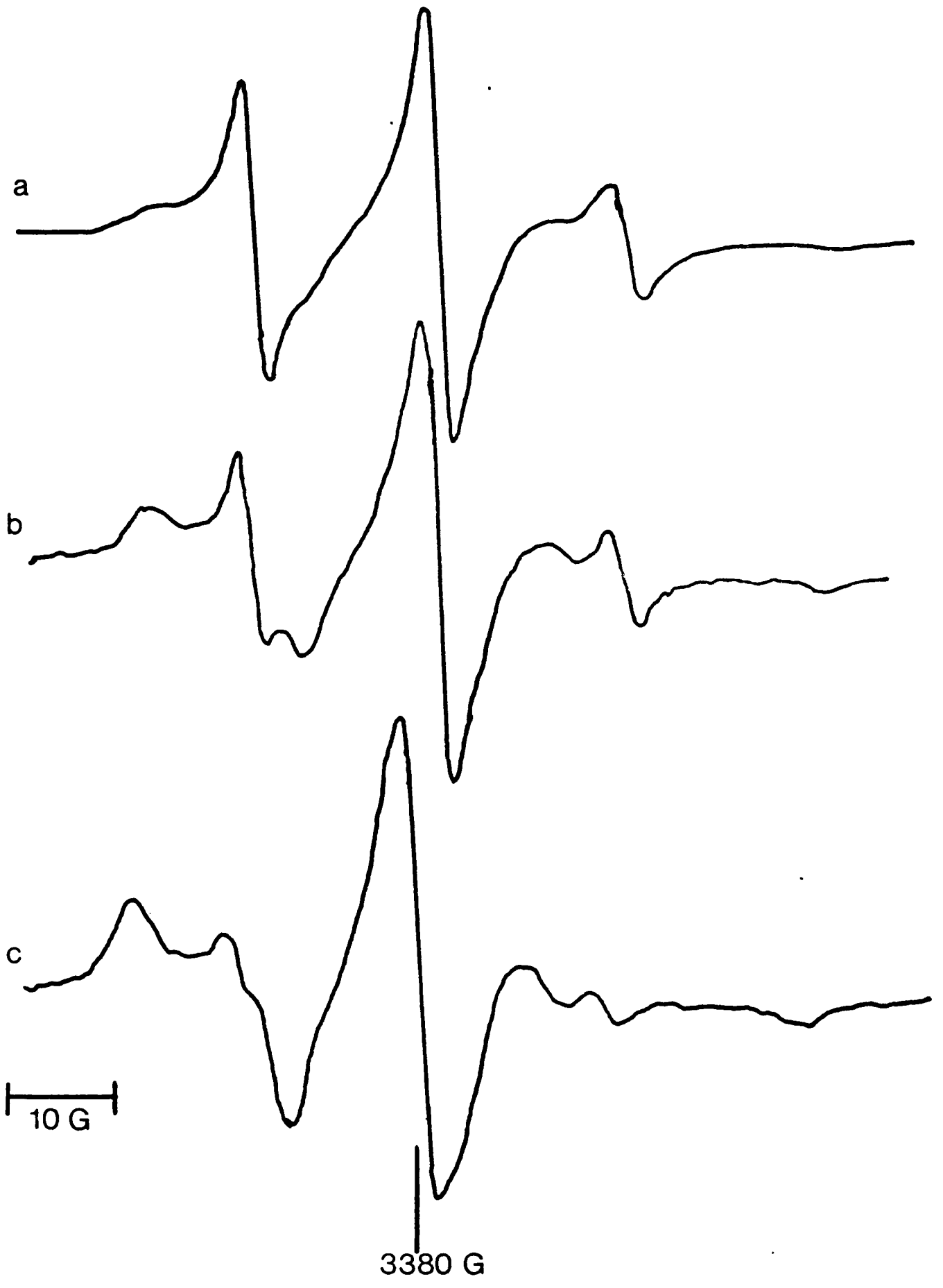


FIG. 5.12 9.35 GHz electron spin resonance spectra of the iodoacetamide spin label bound to pyruvate kinase. The labelling conditions used to prepare the derivatives of which the spectra are shown are given in the text, (Chapter 5, Section h). The spectra were run in 0.1M KCl, 0.02M Tris-HCl, pH 7.2. Approximate concentrations of the labelled proteins were: (a) 4 mg/ml; (b) 2.4 mg/ml, and (c) 4.3 mg/ml.

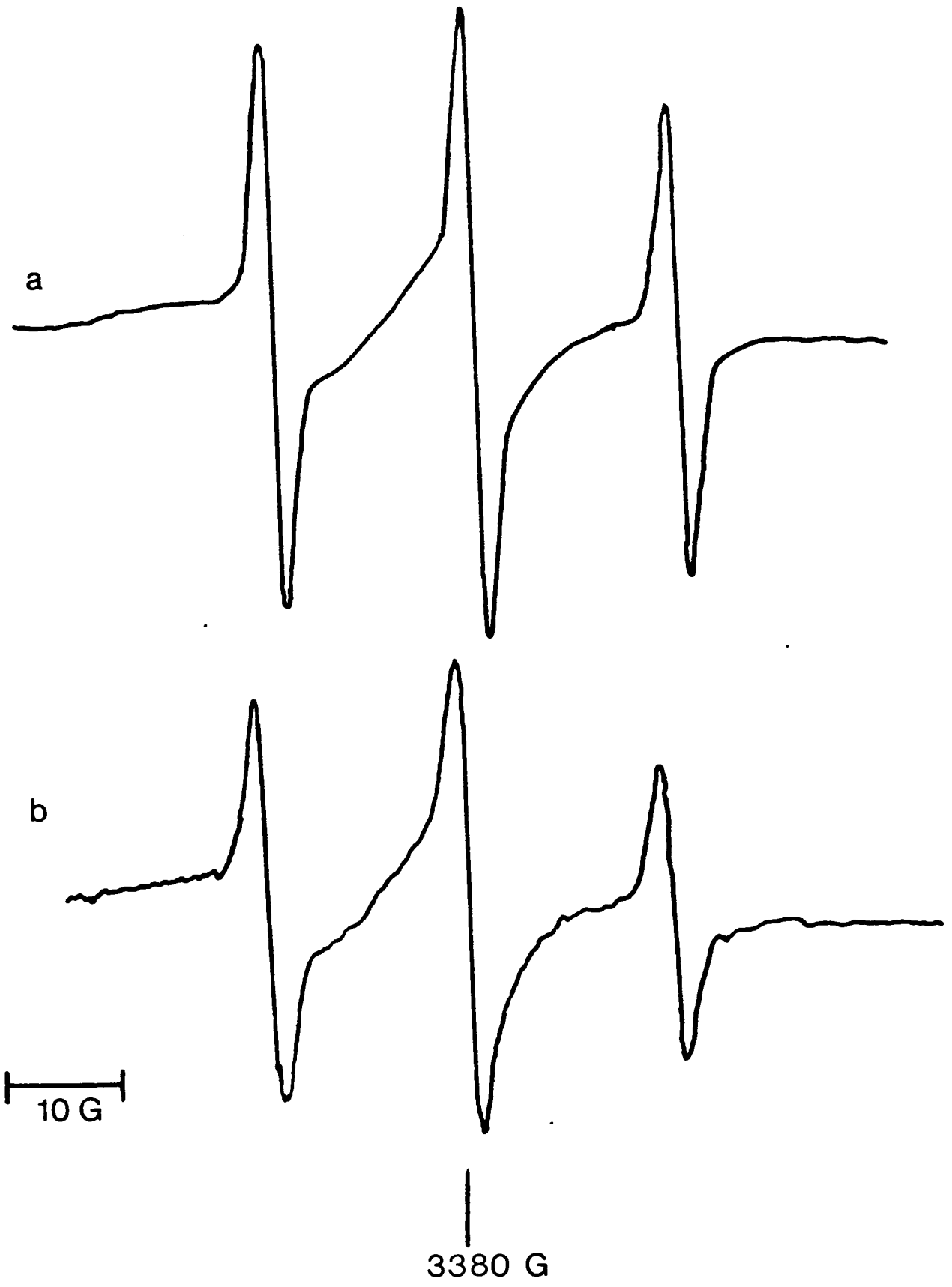


FIG. 5.13 9.35 GHz electron spin resonance spectra of the isothiocyanate spin label bound to pyruvate kinase. The labelling conditions used to prepare the derivatives of which the spectra are shown, are given in Chapter 5, Section h. Approximate concentrations of the labelled proteins were: (a) 4 mg/ml, and (b) 4.3 mg/ml. The solvent was 0.08M KCl, 0.02M Tris-HCl, pH 7.2.

S. carlsbergensis is known to have 4 reactive ε-amino lysine groups per mole (Röschlau and Hess, 1972b). In the first attempt, 0.5mM spin-label was allowed to react with 25 μM pyruvate kinase in 0.1M KCl, 0.1M Tris-HCl, pH 8.0. There was a loss of 25% of the original enzymic activity within the first ten minutes, and after thirty minutes, the activity was 60% of the initial value. The enzyme was then precipitated with $(NH_4)_2SO_4$, and the suspension set to dialyse against $(NH_4)_2SO_4$ overnight at 4°C. The ESR spectrum of the product (Fig. 5.13a) corresponded to very weakly immobilised label. The peak ratios were insensitive to the addition of ligands. A second experiment involved the incubation of 50 μM pyruvate kinase in 0.08 M KCl, 0.02 M Tris-HCl, pH 7.2, with a four-fold molar excess of the spin-label for 1 h. An equal volume of glycerol was then added, and the solution dialysed overnight at 4°C against $(NH_4)_2SO_4$. The spin-label spectrum (Fig. 5.13b) again corresponded to weakly immobilised label, and the peak ratios were insensitive to the addition of ligands. The specific activity of the labelled enzyme was 40% of that of native pyruvate kinase.

Thus, although the spectra show that the isothiocyanate spin-label had reacted at a different site from the iodoacetamide label, neither are sensitive to the addition of ligands.

i) Proton Relaxation Enhancement Measurements

Some of the experiments from Chapter 3 were repeated in order to compare the new preparation with that of Ashton. 100 μM $MnCl_2$ in 0.08M KCl, 0.02M Tris-HCl, pH 7.2, was titrated with pyruvate kinase, and pyruvate kinase (13-27 μM) was titrated with $MnCl_2$ from 50 to 500 μM. It was clear that the measured

enhancements were not consistent with those obtained previously; however, it was not possible to achieve a satisfactory analysis of the results, since a wide range of combinations of the parameters K_D, n and ε_b fitted the results within experimental error. In order to obtain a direct measurement of the binding curve, a 30 μM solution of enzyme was titrated with $MnCl_2$, and the concentration of free Mn^{2+} determined by measurement of its ESR spectrum. It was found that the fraction of the metal ion which was bound by the enzyme was small, so the accuracy of the points obtained for the binding curve (Fig. 5.14) was not high. A measurement of the proton relaxation enhancement factor for one of these solutions revealed that ε_b was much higher than had previously been found; a value of near 40 was calculated, although this figure is uncertain because of the inaccuracy in the ESR measurement. However, calculation of the binding curve from the other proton relaxation enhancement measurements, using this value of 40, gave results which were consistent with the ESR measurements, (Fig. 5.14). It is obvious from the diagram that the binding of Mn^{2+} to this preparation of yeast pyruvate kinase is much weaker than that reported in Chapter 2, or that reported by Mildvan et al. (1971). A possible explanation for the comparatively strong binding of Mn^{2+} obtained in these earlier studies is that it occurred at sites created on the surface of the enzyme by proteolysis. (Mildvan et al. were using pyruvate kinase prepared by the method of Hunsley and Suelter, and in their paper they reported observing many bands in electrophoresis carried out under conditions where the enzyme had dissociated). That both myself and Mildvan et al. claimed that only one enhancement value was needed to describe the binding curve is not necessarily inconsistent with hetero-

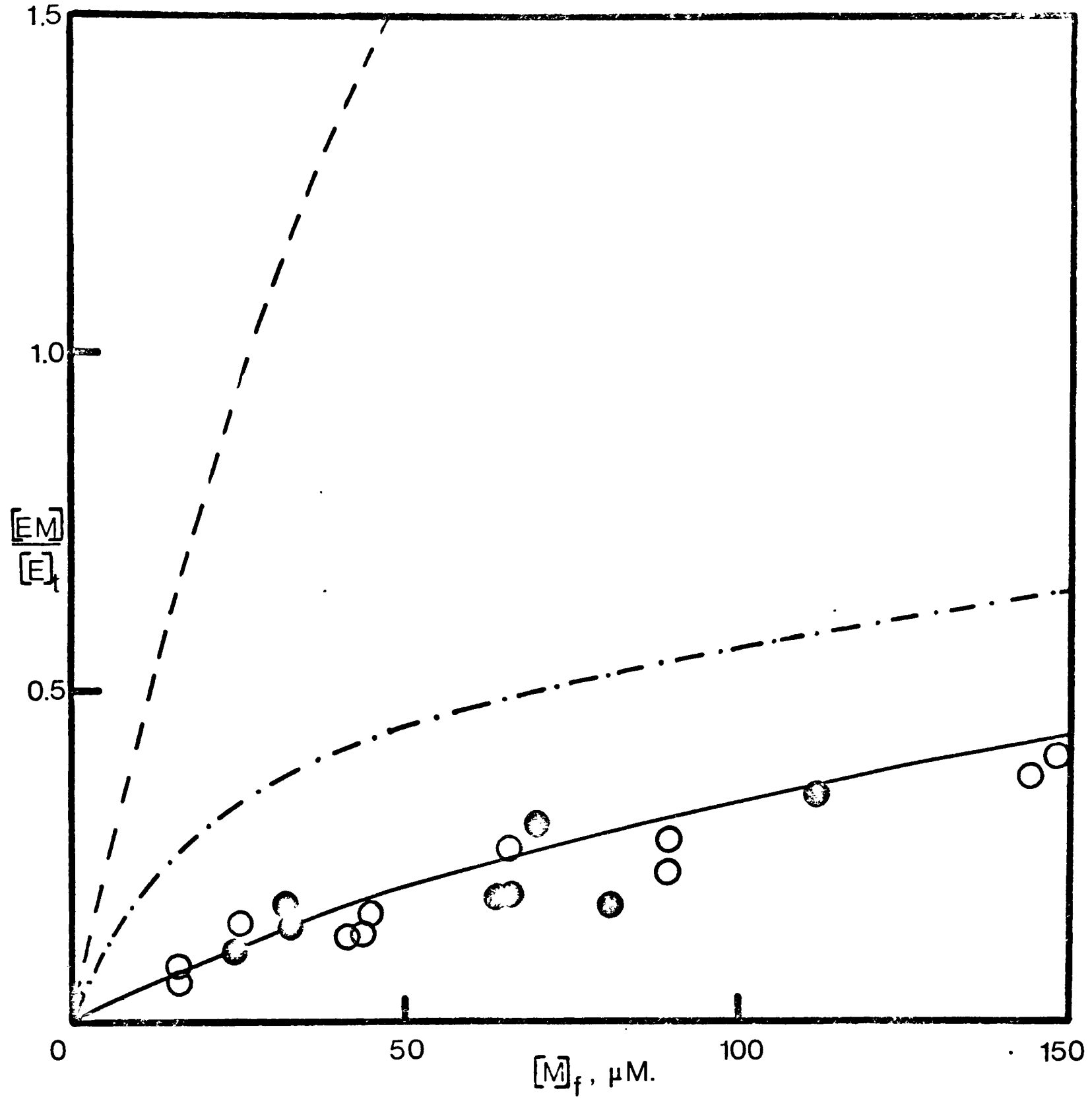


FIG. 5.14 Binding of Mn^{2+} to yeast pyruvate kinase. The conditions were: 25°C; 0.08M KCl, 0.02M Tris-HCl, pH 7.2; enzyme concentrations in the range 10-30 μM. ● , values determined by electron spin resonance spectrometry; O , values obtained by proton relaxation enhancement, calculated with a very approximate value of ε_b obtained from some combined esr and proton relaxation enhancement measurements. ------, results obtained previously (Chapter 3). -·-·-·-·-·, binding curve calculated from results of Mildvan et al. (1971).

geneity of the sites, for if the lifetime of a Mn^{2+} ion was sufficiently short that, in the time scale of the experimental observation, a single Mn^{2+} could "visit" each site, a single average enhancement would be observed. What is not clear is whether the limited binding observed in these latest experiments was weak binding at the active site or binding to non-specific sites. However, the present results would be consistent with the proposal, made by McFarlane and Ainsworth (1972) on the basis of an analysis of the kinetics of yeast pyruvate kinase in the presence of fructose diphosphate, that the essential divalent cation did not bind at the active site until after both phosphoenolpyruvate and ADP had bound (in that order). In this respect, it may be noted that the yeast enzyme differs from the rabbit muscle enzyme, for Ainsworth and McFarlane (1973) showed that the latter exhibited random order binding of phosphoenol-pyruvate, ADP and Mg^{2+}. This could explain why it is possible to demonstrate the binding of one Mn^{2+} ion per subunit to the rabbit muscle enzyme (Reuben and Cohn, 1970), whilst the binding of Mn^{2+} at the active site of the yeast enzyme is insignificant in the absence of both of the substrates.

There is no reason why the uncertainties about the metal binding should affect the results obtained in Chapter 3 for the titrations of the enzyme with phosphoenolpyruvate, although the substrate binding may also have been affected by proteolysis. Similar titrations were made using this new preparation of enzyme, and again the enhancement decreased in the presence of phospho-enolpyruvate, but the total substrate concentration at the half-point was higher than observed previously (600 μM compared with 150 μM). However, since an insufficient region of the Mn^{++}

binding curve had been covered to establish that those enzyme molecules binding the metal ion were a representative sample of a homogenous population, this latest result is of uncertain significance. It was therefore decided that, under the circumstances, no further information could be obtained by this technique.

CHAPTER 6

CONCLUSIONS

a) Preparation of Yeast Enzymes

This study of yeast pyruvate kinase from yeast has underlined the problem of the proteolysis that can occur during the preparation of enzymes from yeast, particularly when autolysis has been used to rupture the cells. In view of the number of glycolytic enzymes from yeast which have now been shown to be vulnerable to proteolytic degradation in the conditions used for autolysis - i.e. hexokinase (Lazarus et al. 1966), phosphofructokinase (Diezel et al. 1973), phosphoglyceromutase (Sasaki et al. 1966), pyruvate kinase and alcohol dehydrogenase (Clark and Jakoby, 1970) - this technique for the liberation of enzymes from yeast must be regarded as unreliable unless proved to be otherwise in any particular case. Proteolysis of hexokinase and phosphoglyceromutase caused a loss of enzymic activity, and so it was thought that since pyruvate kinase prepared by the methods of Ashton (1971) or Hunsley and Suelter (1969a) had a specific activity comparable with that obtained by Haeckel et al. (1968), degradation of the enzyme was unlikely. However, the demonstration of a specific activity of 400 U/mg in Chapter 4 showed that the activity of the enzyme had previously been reduced, and in any case, the activities of phosphofructokinase and alcohol dehydrogenase were not altered by proteolysis.

b) Molecular Weight

The properties of yeast pyruvate kinase reported in Chapters 4 and 5 are summarised in Table 6.1, together with the corresponding results obtained by other workers for the enzyme prepared by the three other published methods. Hess's group have reported several variants (e.g. Röschlau and Hess, 1972a) on their original method of preparation (Haeckel et al. 1968), but their results have been grouped together since the modifications to the preparative method have not been reported to have altered the observed properties of the enzyme. One of the reasons for the alterations in their method of preparation was the presence of traces of a proteolytic enzyme in the pyruvate kinase as originally prepared. The results of Bischofberger et al. (1970) are assumed to have been superseded by those of Bischofberger et al. (1971). Also included in the Table are some recent results obtained in Suelter's laboratory (C.H. Suelter, personal communication) on a new preparation of the enzyme from S. cerevisiae (see below).

As has been noted, the results in Chapter 5 demonstrate that earlier reports (Ashton and Peacocke, 1971; Kuczenski and Suelter, 1970b) which showed the pyruvate kinase of S. cerevisiae to be significantly smaller than that from S. carlsbergensis, were mistaken. The molecular weight now obtained is higher than that reported for the enzyme from S. carlsbergensis, although the difference may not be significant. Recently, C.H. Suelter (personal communication) has obtained evidence for proteolytic degradation in preparations by the method of Hunsley and Suelter (1969a), and has developed an alternative procedure (the details of which are not yet settled) which yields pyruvate kinase with a molecular weight of

TABLE 6.1

A comparison of the properties reported for pyruvate kinase prepared from yeast

Measurement	S.cerevisiae This work	S.cerevisiae a	S.cerevisiae b	S.carlsbergensis c	S.cerevisiae d
Mol. wt. native	215,000	167,000	161,000	190,000	209,000
Mol. wt. subunits	54,000	44,000	20,000	51,000	-
$s^{o}_{20,w}$ native	9.5	8.3	8.0-8.3	8.7	-
$E^{0.1\%}_{1cm}$ at 280 nm	0.66	0.65	0.65	0.76	0.58
Specific activity U/mg, (^{o}C)	340-400 (25)	220 (30)	180-200 (25)	250 (25)	440 (30)
Cold-labile	Yes	Yes	Yes	No	Yes
Thiol group reactivity/mole					
(i) Fast	2	6	-	4	4
(ii) "Exposed"	14	14	-	12	12
(iii) Total	24	24	-	24	20

REFERENCES:

a) Hunsley and Suelter (1969a); Kuczenski and Suelter (1970b); Bondar and Suelter (1971).

b) Ashton (1971)

c) Haeckel et al. (1968); Bischofberger et al. (1971); Wieker and Hess (1972).

d) C.H. Suelter (personal communication), and Yun and Suelter (1973).

209,000. This enzyme seems to be similar to the one described here.

A molecular weight of 215,000 for the enzyme from S. cerevisiae is in the middle of the range of molecular weights reported for pyruvate kinase from other sources, (Table 6.2). Further, assuming that the relationship ' s α(mol. wt.)$^{2/3}$ ' holds (i.e. assuming that f/f_o and $\bar{v}$ are constant for the particles compared; and the values of $\bar{v}$ used are all in the range 0.73-0.74), then the $s^o_{20,w}$ of rabbit muscle pyruvate kinase is predicted as 10.1S, and that of the bovine muscle enzyme as 9.9 S, on the basis of the values of the sedimentation coefficients and molecular weights of the enzymes from S. cerevisiae and bovine liver. The agreement of the predicted with the observed values illustrates that the rather high value of f/f_o reported in Chapter 5 is a property of all the pyruvate kinases subjected to hydrodynamic study. (The value of 8.1 S for the pyruvate kinase of pig liver seems anomalously low; a value of 9 S would be in line with the other results).

A general observation emerging from Table 6.2 is that the molecular weights of the pyruvate kinases which are not activated by fructose diphosphate are higher than those reported for the enzymes sensitive to fructose diphosphate. Presumably the mammalian muscle enzyme evolved from a fructose diphosphate-sensitive form by acquisition of amino-acids in the polypeptide chain, since evolution in the opposite direction would imply the acquisition of an allosteric site for the effector by loss of part of the structure, which seems inherently unlikely.

TABLE 6.2

A comparison of pyruvate kinase from different sources

Source	FDP effect	Native Mol. wt.	Native $s^{o}_{20,w}$	Subunit Mol. wt.	$E^{0.1\%}_{1cm}$ at 280 nm	Reference
S. cerevisiae	+	215,000	9.5	53,000	0.65	This work
Pig liver	+	197,000	8.1	56,000		Kutzbach et al. (1973)
Bovine liver	+	215,000	9.5	54,000	0.57	Cardenas & Dyson (1973)
Rat liver	+	208,000				Tanaka et al. (1967)
Human erythrocyte	+	225,000	9.8			Chern et al. (1972)
" "	+	205,000				Staal et al. (1971)
" "	+	195,000				Blum et al. (1971)
Rabbit muscle	-	237,000	10.0	57,200	0.54	Warner (1958) Steinmetz & Deal (1966)
Rat muscle	-	250,000				Tanaka et al. (1967)
Bovine muscle	-	230,000	9.9	57,000	0.55	Cardenas et al. (1973)
Mucor rouxii	+	182,000				Terenzi et al. (1971)

c) Extinction Coefficient

Although the molecular weights previously reported for pyruvate kinase from S. cerevisiae were too low, the results in Chapter 5 confirmed the other differences that had been noted between the enzyme from this source and that from S. carlsbergensis. Thus, the lower extinction coefficient and the property of cold lability and its enhancement by fructose diphosphate are intrinsic properties of the enzyme from S. cerevisiae and were not a result of proteolytic attack. Since the extinction coefficient was unchanged whilst the molecular weight was decreased by 20%, either the polypeptides lost in proteolysis fortuitously contained the same proportion of aromatic amino acids as the enzyme as a whole, or else the degradation was entirely random. Against the idea of random degradation are the results of Ashton (1971) and of Kuczenski and Suelter (1970b); as both groups of workers claimed to have obtained a monodisperse product and were in reasonable agreement over the native molecular weight, it would seem that there was a definite end product to the degradation. Also, since Bondar and Suelter (1971) found 24 thiol groups per mole for enzyme prepared by the method of Hunsley and Suelter (1969a), none of the cysteine residues was eliminated. In each of the instances of proteolysis of yeast enzymes cited earlier, a small number of species were obtained as reproducible products. C.H. Suelter (personal communication) has written of finding an extinction coefficient, $E_{1cm}^{0.1\%}$ at 280 nm, of 0.58 $ml.mg^{-1}cm^{-1}$ for his new preparation.

The low content of tyrosine and tryptophan implied by the low extinction coefficient is also a characteristic of pyruvate kinase from other sources (Table 6.2).

d) N-terminal Amino Acid

The absence of a free N-terminal amino group in yeast pyruvate kinase (Chapter 5; Bormann et al. 1972) is a property shared with pyruvate kinase from other sources. Acetylation of the N-terminal amino acid has been demonstrated for the enzymes from rabbit muscle (Cottam et al. 1969) and human erythrocytes (Chern et al. 1972).

e) Cold Lability

That the cold lability of pyruvate kinase from S. cerevisiae is not shown by that from S. carlsbergensis demonstrates that there is some degree of genetic divergence between the two strains of yeast. It has been pointed out by Kuczenski and Suelter (1970a) and by Ashton (1971) that inactivation at low temperatures indicates an important contribution from hydrophobic bonding to the maintenance of the native protein structure. It is interesting to speculate whether inactivation at low temperatures observed in vitro has any physiological significance. Against such an idea are: (i) the absence of such an effect in pyruvate kinase from S. carlsbergensis; (ii) the doubt about whether the same phenomenon occurs in the same manner in vivo, for yeast maintains its viability after storage at $4^{o}C$, and the enzyme can successfully be prepared by freeze-thawing the yeast. Furthermore, enhancement of the effect by the allosteric activator, fructose diphosphate, would seem to be anomalous in this context, although it is unlikely that such enhancement would occur in the cell since the metal ions K^{+} and Mg^{2+} prevent it (Kuczenski and Suelter, 1970a). Ashton (1971) reported that high concentrations of fructose diphosphate stabilised the enzyme at low temperatures

in the presence of K^+, whilst Kuczenski and Suelter (1971b) reported a stabilising effect of high concentrations of fructose diphosphate on the dimer intermediate formed in the activation at 23°C, but showed this to result from the high ionic strength. The de-stabilising effect of fructose diphosphate is probably related to its co-operative binding; Weber (1972) has underlined the fundamental relationship between co-operative binding and changes in the association constants for the inter-protomer interactions; such changes are explicitly included in Koshland's general model of allosteric enzymes (Haber and Koshland, 1967), and implicitly included in the model of Monod, Wyman and Changeux (1965). If the effect given by fructose diphosphate is indicative of the conformational change involved in the allosteric mechanism, then it is significant that phosphoenolpyruvate does not also catalyse the inactivation, as would be expected for a simple two-state model. There is the possibility that the *in vitro* lability may reflect an inherent instability which is necessary so that the concentration of the enzyme in the yeast cell may be regulated in response to metabolic requirements, as has been demonstrated by Barwell and Hess (1971). Interestingly, the reason for the lack of information about forms of pyruvate kinase sensitive to fructose diphosphate, compared with that available for the mammalian muscle enzyme, is the instability of these enzymes in preparation; the rabbit liver enzyme is unstable (Irving and Williams, 1973); the rat liver enzyme is unstable, but sucrose improves its stability (Susor and Rutter, 1968); the human erythrocyte enzyme is labile, but glycerol helps to stabilise it (Staal *et al*. 1971), and the bovine liver enzyme loses much of its activity during preparation (Cardenas and Dyson, 1973). The half-life of pyruvate

kinase in rat liver has been estimated as 1 to 2 days (Szepsi and Freedland, 1968), but, on the other hand, the average life of a human erythrocyte is 120 days, during which no renewal of pyruvate kinase by protein synthesis can occur. Inactivation at low temperatures has been observed for a number of other oligomeric enzymes, several of which may be involved in the control of metabolism:- pyruvate carboxylase from chicken liver (Irias et al. 1969); carbamyl phosphate synthetase (Raijman and Grisolia, 1961); arginosuccinase from beef liver (Havir et al. 1965); beef heart mitochondrial ATP-ase (Penefsky and Warner, 1965); acetyl CoA carboxylase (Numa and Ringelman, 1965); threonine deaminase (Feldberg and Datta, 1971); cysteine desulphydrase from S. typhinurium (Collins and Monty, 1973); aspartate β-semialdehyde dehydrogenase from yeast (Holland and Westhead, 1973), and yeast glyceraldehyde-3-phosphate dehydrogenase (Stancel and Deal, 1968). In many of these examples, dissociation of the oligomer accompanies inactivation, which can be prevented in some cases by glycerol or methanol. The inactivations of pyruvate carboxylase, aspartate β-semialdehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are reversible on warming, although transition to an irreversibly inactivated state is possible. In the cases of the latter two enzymes, the inactivation is catalysed by ATP. The inactivation of yeast pyruvate kinase is not known to be reversible (Kuczenski and Suelter, 1970a), although the native structure must be adequately stable since renaturation with regain of activity has been achieved after denaturation with guanidinium chloride (Tobes et al. 1972) and with trichloroacetic acid (Hess, 1974).

f) Conformations and Allosteric Effects

On the basis of their studies of the cold-lability of yeast pyruvate kinase, and of its inactivation by fructose diphosphate at 23°C, Kuczenski and Suelter (1971b) tentatively proposed that the enzyme was not an isologous tetramer, but a structure in which there were two types of non-equivalent subunits, so that dissociation at 0°C gave enzymically inactive dimers, whilst dissociation at 23°C involved rupture of a different set of inter-subunit bonds and produced active dimers. The fractional reactivity of the thiol groups reported in Chapter 5, would be consistent with such asymmetry in the protein structure; for only two of four thiol groups, present at a given point in the four polypeptide chains, to be reactive implies that the conformation of two of the subunits is different from the other two. (There is the possibility that there are four rapidly reacting thiol groups per mole in equivalent positions, but located so that the reaction of two of them with iodoacetamide or DTNB sterically prevents access of reagent to the others. However, this is considered unlikely since iodoacetamide is much smaller than DTNB). The observation is analagous to that of "half-the-sites reactivity" which has been reported for the binding of substrates and the reaction of substrate analogues at the active sites of a number of oligomeric enzymes (see Levitzki et al. 1971). In the case of "half-the-sites reactivity" the two possible explanations are that either the enzyme is in a pre-existing asymmetric configuration, or the non-equivalence may be induced by ligand binding. Bernhard and MacQuarrie (1973) have proposed that the former alternative is true for the alkylation of the active site of yeast glyceraldehyde-3-phosphate dehydrogenase, although Stallcup and Koshland

(1973a) claim that the latter alternative can be demonstrated for this enzyme as well as for CTP synthetase. A pre-existing non-equivalence would seem more likely in the case of yeast pyruvate kinase, since the thiol group labelled is not involved in the active site, and if substitution does involve a conformational change leading to non-equivalence, it does so without affecting the activity of the enzyme. It has been suggested that substrate-induced non-equivalence of subunits in oligomeric enzymes is related to "flip-flop" mechanisms which facilitate product release from an enzyme (Stallcup and Koshland, 1973b), but it is difficult to see what role any pre-existing non-equivalence could fulfil in the case of pyruvate kinase.

X-ray crystallographic studies have established that cat muscle pyruvate kinase (Muirhead and Stammers, 1974) and human muscle pyruvate kinase (Campbell et al. 1971) have two two-fold axes of symmetry, i.e. they are both isologous tetramers.

The experiments on the labelling of thiol groups by DTNB and iodoacetamide (Chapter 5) show that the binding of the effector fructose diphosphate to the enzyme produces a conformational change which results in greater reactivity and which is not elicited by phosphoenolpyruvate or ADP. Wieker and Hess (1972) found that fructose diphosphate protected the thiol groups of the pyruvate kinase of S. carlsbergensis from reaction, as did Bondar and Suelter (1971) for pyruvate kinase from S. cerevisiae. The details of the effects of fructose diphosphate observed by Wieker and Hess suggest that a distinct conformation is induced by this ligand, although steric protection of the enzyme may also be involved.

The attempt to analyse ligand binding and conformational

changes of yeast pyruvate kinase by magnetic resonance techniques did not, unfortunately, yield much useful information. It did not prove possible to introduce a spin label at a site where it was sensitive to ligand-induced changes, and the original experiments by proton relaxation enhancement could not be confirmed with the undegraded enzyme because of its weak binding of Mn^{2+}. This latter observation excludes the possibility that the active site of the enzyme forms a strong binary complex with Mn^{2+}, and therefore the complexes with the metal reported in Chapter 3 probably did not have metal bound at the active site. If this is so, then the changes in enhancement observed in the presence of ligands with the proteolysed enzyme would have been due to changes in the environment of the bound metal caused by conformational changes. Other attempts to examine ligand-induced conformational changes have not produced many results. Difference spectroscopy in the ultra-violet cannot be used as the slight turbidity which usually develops in solutions of pyruvate kinase is accentuated by addition of substrates and effectors, and is sufficient to prevent accurate difference measurements. Kuczenski and Suelter (1971a) observed a significant quenching of the fluorescence emission of yeast pyruvate kinase when the effector fructose diphosphate was bound, which they attributed to a conformational change; other ligands produced only a small effect. Johannes and Hess (1973) have attempted to interpret initial rate kinetics in terms of conformational equilibria. Interestingly, they proposed that, in addition to an 'R' and a 'T' conformation (Monod et al. 1965), a mixed tetramer, R_2T_2, was necessary to fit their results to a concerted-type model of an allosteric enzyme. Unfortunately, they did not attempt to demonstrate that this model was superior to other models in its ability to fit the results of their kinetics

experiments, and the initial assumption of only two conformations is open to doubt, provided that the enzyme from S. carlsbergensis is not fundamentally different from that of S. cerevisiae. Thus, with pyruvate kinase from S. cerevisiae, the effects of fructose diphosphate on cold inactivation, quenching of fluorescence and thiol group reactivity, all of which may be parameters of ligand-induced conformational changes, are not shown by phosphoenol-pyruvate, which should promote the same conformational state in any simple two-state hypothesis. It may be argued that these observations do not disprove that a two-state mechanism is not conceptually valid; as pointed out in Chapter 1, not all observable aspects of a conformational change need be of significance in modulating inter-subunit interactions; however, Kuczenski and Suelter (1970a, 1971b) have shown that fructose diphosphate is modifying the interactions when catalysing cold lability because it promotes dissociation. If then, a two-state hypothesis is inadequate, and Johannes and Hess (1973) did find the model of Monod, Wyman and Changeux wanting, then additional experimental approaches must be devised if parameters derived from model fitting are to be validated. Unfortunately, Whitehead's test (1970) for a two-state system (Chapter 1) is not applicable because it is not clear that there is a true allosteric inhibitor of the yeast enzyme; Mg.ATP does inhibit and exhibit homotropic interactions (Haeckel et al. 1968), but it is known to be a competitive inhibitor at the active site (MacFarlane and Ainsworth, 1972). Following Weber's suggestion (see Chapter 1), the effects of ligands on the dissociation constants for the protomers would need to be established; this might be a difficult task with this enzyme since it would require accurate molecular weight measurements at low concentrations, but frontal analysis in analytical gel filtration might be possible.

In conclusion, some of the main problems in understanding pyruvate kinase are still unresolved. There is as yet no definite agreement on whether ADP.Mg or ADP plus Mg separately, or both, are the true substrates; the catalytic roles of the monovalent and divalent cations are not yet assigned, and there is no definite evidence of what enzymic groups function in the catalysis. Some of these problems may be solved for the mammalian muscle enzyme by future advances in the interpretation of magnetic resonance results, and by the solution of the X-ray structure at a higher resolution than presently available (Muirhead and Stammers, 1974) and location of the active site. For the yeast enzyme, the conditions in which magnetic resonance techniques could be useful have not been found, if they do exist. Work on the X-ray structure of this enzyme is being undertaken at Bristol University, but is not yet very advanced. If progress is to be made with the enzyme in the meantime, then direct binding measurements might be undertaken and the dissociation of the enzyme investigated as suggested previously. (Johannes and Hess, 1973, reported some measurements on the binding of fructose diphosphate, but did not compare the results with the predictions of their model of the conformational states of the enzyme). Unfortunately, the instability of the enzyme will continue to be a handicap, but since most of the sources of pyruvate kinase sensitive to fructose diphosphate also yield an unstable enzyme, this cannot easily be avoided. It is hoped that the work presented here has helped to resolve the main anomalies which existed in the reports on the properties of yeast pyruvate kinase, and will provide a sounder basis for future work with this enzyme.

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APPENDIX 1

An approximate solution for the binding of Mn^{2+} by fructose diphosphate

The linear portions of the biphasic reciprocal plot of the titration (Fig. 3.5) were extrapolated to give initial estimates of the enhancement factors, $\varepsilon_{MS} = 1.18$ and $\varepsilon_{MS'} = 1.8$. Even with very strong binding ($K_{MS}<1$ μM), it was not possible to predict an enhancement as large as the observed value at low molarities of fructose diphosphate, and also to fit the titration at higher molarities, unless it was assumed that both phosphate groups of the ligand were able to bind metal.

$$\text{i.e. FDP} + \text{Mn} \rightleftharpoons \text{FDP.Mn} + \text{Mn} \rightleftharpoons \text{FDP.(Mn)}_2$$

It was assumed that the dissociation constants for the two steps were the same. At the concentrations of fructose diphosphate required to form $(FDP)_2Mn$, the proportion of molecules present as $FDP.(Mn)_2$ would be negligible on a statistical basis, so the second phase of the titration is described as:

$$\text{FDP} + \text{FDP.Mn} \rightleftharpoons (\text{FDP})_2\text{.Mn}$$

Thus:

$$K_{MS} = \frac{[M]_{f.}\ [S]_f}{[MS]_t} = \frac{\left([M]_t - [MS]_t\right)\left(2.[S]_t - [MS]_t\right)}{[MS]_t}$$

where $[MS]_t$ is used to represent the concentration of bound metal i.e. $[MS]_t = [MS] + 2.[M_2S]$. It follows that $[MS]_t$ is the physically significant root of the equation:

$$[M\ S]_t^2 - [MS]_t.([M]_t + 2.[S]_t + K_{MS}) + [M]_t.2.[S]_t = 0.$$

The second step is described by:

$$K_{MS_2} = \frac{[MS]_t . [S]_f}{[MS_2]}$$

At concentrations of fructose diphosphate high enough for MS_2 to be formed, calculation shows that the concentration of free Mn^{2+} is virtually negligible, hence $[MS] + [MS_2] = [M]_t$.

Therefore $$K_{MS_2} = \frac{\left([M]_t - [MS_2]\right).\left(2.[S]_t - [M]_t\right)}{[MS_2]}$$

$$[MS_2] = \frac{[M]_t}{1 + \frac{K_{MS_2}}{2.[S]_t - [M]_t}}$$

The enhancement of the solution is then calculated from:

$$\varepsilon^* = \frac{[M]_f}{[M]_t} + \varepsilon_{MS} \cdot \frac{[MS]}{[M]_t} + \varepsilon_{MS_2} \cdot \frac{[MS_2]}{[M]_t}$$

The model is crude, but there is little point in refining the calculations since there are already more parameters than can be accurately derived from the experimental results. Values for ε_{MS}, K_{MS}, ε_{MS_2} and K_{MS_2} were obtained by calculation of the enhancement with trial values and comparison with the observed results, e.g.

Concentration of FDP	ε* observed	ε* calculation 1	ε* calculation 2
0.02 mM	1.06	1.05	1.05
0.05 mM	1.10	1.09	1.10
0.1 mM	1.15	1.13	1.14
0.2 mM	1.14	1.16	1.17
0.3 mM	1.14	1.18	1.19
1 mM	1.29	1.29	1.31
2 mM	1.45	1.39	1.42
10 mM	1.66	1.66	1.65

Calculation 1 was with $\varepsilon_{MS} = 1.14$, $K_{MS} = 15$ μM, $\varepsilon_{MS_2} = 1.84$ and $K_{MS_2} = 7$ mM, and calculation 2 used the same values for the first two parameters and 1.78 and 5 mM respectively for the other two. The best fit was obtained with $\varepsilon_{MS} = 1.14$ and $K_{MS} = 15$ μM, but as can be seen from the example, the answer is not so sensitive to ε_{MS_2} and K_{MS_2} ; values of 1.8 and 6 mM are an approximate solution.

APPENDIX 2

Calculation of enhancement parameters for the ternary complex of enzyme with Mn^{2+} and fructose diphosphate, when the concentration of the latter is 1 mM.

Since $[M]_f$ will be negligible in these conditions, (cf. Appendix 1, plus the assumption that there is no EM complex in the presence of such a concentration of fructose diphosphate),

$$[M]_t = [EMS] + [MS] + [MS_2]$$

Also from Appendix 1, if $[S]_t$ is used in place of $[S]_f$,

$$[MS_2] = \frac{[M]_t - [EMS]}{1 + \frac{K_{MS_2}}{2.[S]_t}} = \frac{([M]_t - [EMS]) \cdot 2.[S]_t}{2.[S]_t + K_{MS_2}}$$

Combination of these two equations gives:

$$[MS] = ([M]_t - [EMS]) \cdot \frac{K_{MS_2}}{2.[S]_t + K_{MS_2}}$$

Then:

$$\varepsilon^* = \varepsilon_{EMS} \cdot \frac{[EMS]}{[M]_t} + \varepsilon_{MS} \cdot \frac{[MS]}{[M]_t} + \varepsilon_{MS_2} \frac{[MS_2]}{[M]_t}$$

$[EMS]$ is unknown, but if saturation of the enzyme has been achieved, then it equals $n_S.[E]_t$, and it is instructive to solve this for various values of n_S:

Concentration of FDP	ε^* observed	ε_{EMS} for $n_s = 1$	$n_s = 2$	$n_s = 4.$
10 mM	2.6	6.6	4.0	2.9
2 mM	2.4	6.7	3.9	2.7
1 mM	2.2	6.3	3.7	2.6
1 mM + saturating phosphoenolpyruvate	1.6	2.9	2.0	1.7

ABSTRACT

Pyruvate kinase from brewers' yeast (Saccharomyces cerevisiae or Saccharomyces carlsbergensis) is an allosteric enzyme that is activated by fructose diphosphate. The reaction velocity is sigmoidally dependent on the concentration of the substrate phosphoenolpyruvate in the absence of the activator. It was considered to be a particularly suitable choice for study by physical methods in an attempt to understand the mechanism of allosteric effects because there were already available, for comparative purposes, the results of several lines of investigation of rabbit muscle pyruvate kinase, which is not activated by fructose diphosphate. The work described in this thesis follows on from that of Ashton (1971), who devised a method for the preparation of pyruvate kinase from S. cerevisiae, and who found that the molecular weight of the native enzyme was 161,000, apparently contributed by eight monodisperse subunits of 20,000 each. This value for the native molecular weight is in reasonable agreement with that of 168,000 reported by Kuczenski and Suelter (1970) for pyruvate kinase prepared from S. cerevisiae by the method of Hunsley and Suelter (1969). The enzyme prepared by both these methods was cold-labile. A higher molecular weight, 190,000, was reported by Bischofberger et al. (1971) for the enzyme prepared from S. carlsbergensis by the method of Haeckel et al. (1968), and this preparation was not cold-labile. However, both Kuczenski and Suelter (1970) and Bischofberger et al. (1971) reported that the enzyme had four subunits.

In this present study, pyruvate kinase was first prepared by the method of Ashton (1971). The binding of Mn^{2+} to the

enzyme was measured by the magnetic resonance technique of proton relaxation rate enhancement. Both strong and weak binding sites for the metal were detected, although exact analysis of the binding curve was not possible. These measurements were also sensitive to the binding of the substrate phosphoenolpyruvate and of the effector fructose diphosphate, and an increase in the affinity of the enzyme for the substrate was demonstrated in the presence of the latter. In the absence of metal ions, the effector fructose diphosphate was found to catalyse the inactivation of the enzyme. The molecular weight of the subunits was re-investigated, but Ashton's results (1971) were not confirmed; sedimentation equilibrium measurements were consistent with four subunits per mole, but electrophoresis in polyacrylamide gels containing sodium dodecylsulphate revealed heterogeneity of the subunits, presumably caused by proteolytic attack during the preparation of the enzyme.

Various alterations were made to Ashton's method of preparation in an attempt to prevent proteolysis, but eventually a new method was devised in which: (i) the initial autolytic degradation of the yeast cells, which had been used in all three published methods of preparation, was replaced by the use of freeze-thawing to rupture the cells; (ii) the temperature was kept below $5^{o}C$ at all stages of the preparation, and (iii) the protease inhibitor phenylmethylsulphonylfluoride was added in the early stages of the preparation. The specific activity of the enzyme thus obtained, up to 400 U/mg, was significantly higher than had previously been observed in any laboratory.

The molecular weight of yeast pyruvate kinase prepared by this method was determined as 213,000 by sedimentation equilibrium,

and as 217,000 from a combination of the sedimentation and diffusion coefficients. The diffusion coefficient was determined by intensity fluctuation spectroscopy of scattered laser light. Sedimentation equilibrium measurements in 6M guanidinium chloride - 1% 2-mercaptoethanol and electrophoresis in sodium dodecyl-sulphate gels showed that there were 4 subunits, of molecular weight about 54,000, per mole of native enzyme. Like pyruvate kinase prepared from S. cerevisiae by the methods of Hunsley and Suelter (1969) and Ashton (1971), the enzyme was cold-labile, and the inactivation was catalysed by the allosteric effector fructose diphosphate. A free N-terminus was not detected by dansylation, although N-terminal alanine had been observed with the enzyme prepared by the method of Ashton. When the proton relaxation enhancement experiments were repeated, it was found that there were no longer any strong binding sites for Mn^{2+}, and the metal binding was too weak to obtain meaningful results by this technique. The reactivity of the thiol groups of the protein was investigated: two groups per mole were particularly reactive with both 5,5'-dithiobis-(2-nitrobenzoate) and iodoacetamide; 14 groups per mole reacted with the former reagent in the absence of denaturant, and a total of 24 reacted when the protein was denatured. Two 'spin-labels' were covalently attached to the enzyme, but the spectrum of neither was sensitive to the binding of ligands.

The results are compared with those reported by other investigators for pyruvate kinase from yeast, and also with those for pyruvate kinase from other sources.

Some of the work presented in this thesis has also been published (Fell et al. 1972 and 1974).

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