THE UNIVERSITY OF MANITOBA

A REACTIVE SULFHYDRYL GROUP IN *ESCHERICHIA COLI* CITRATE SYNTHASE

BY

MICHAEL MAGNUS TALGOY

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ABSTRACT

Citrate synthase from acetate grown E. coli (strain K12 3000) has been subjected to sulfhydryl modification using reagents DTNB, 4,4'PDS and IAA.

DTNB reacts with the enzyme to modify 1 sulfhydryl group per subunit quickly and one or more groups more slowly. The rate of reaction for the first group is saturable with respect to DTNB, and exhibits a pH dependence with a pKa of 8.3 $\pm$ 0.2. NADH, an allosteric inhibitor, and ACoA, a substrate, inhibit the reaction, while OAA, the other substrate, has little or no effect. KCl increases the rate of reaction of the initially modified group by increasing the V_{max} for the DTNB-enzyme reaction, and eliminates reaction of secondary sulfhydryls. The kinetic parameters for citrate synthase are altered upon modification by DTNB, such that the Hill coefficients for KCl and ACoA saturation are lower. As initially reported by Danson and Weitzman (Biochem. J., 135, 513 (1973)), DTNB modification causes the enzyme to lose its sensitivity to NADH and some of its activity. I have found that modification by DTNB wipes out the ability of the enzyme to bind NADH and that treatment with DTT regenerates those functions to a considerable degree.

Modification of citrate synthase by 4,4'PDS leads to rapid inactivation as 2.0 groups per subunit are modified. The reaction is inhibited by NADH, and modification of 1 group per subunit eliminates NADH binding by the enzyme. KCl enhances the reactivity of the first group while diminishing that of the second group. Modification with one reagent, followed by exposure to the other, indicates that DTNB and 4,4'PDS react with the same enzyme sulfhydryl group.

Modification of citrate synthase by IAA leads to incorporation of more than 2 carboxymethyl groups per subunit, with a concomitant loss of NADH binding capacity and 4,4'PDS and DTNB reactivity. The overall rate of incorporation was very similar to the rate of loss of NADH binding sites, indicating that the sulfhydryl associated with NADH binding is not selectively modified. The rate of loss of DTNB and 4,4'PDS reactivities were very similar; this observation supports the finding that they react with the same sulfhydryl group.

A model is presented to interpret all these data, in which it is suggested that the susceptible sulfhydryl group exists in or near the NADH binding site, so that modification prevents NADH binding because of steric hindrance. The partial inactivation seen with DTNB and complete inactivation with 4,4'PDS are then seen as a result of heterotropic interactions with the active site. Differing orientations within the site could explain the differing effects of these two reagents on activity.

Several adenylate analogues of NADH bind to the NADH site of citrate synthase, as indicated by their ability to inhibit NADH binding and to protect the enzyme from DTNB and 4,4'PDS modification. These analogues include ADP-ribose, 2'5' and 3'5' ADP, 5'AMP, 3'AMP and NADPH. At low ionic strength, all except 3'AMP activate the enzyme to different extents. The finding that adenylates bind to the allosteric site of E. coli citrate synthase suggests a closer similarity than has been suspected between this enzyme and the citrate synthases of strictly aerobic Gram-negative bacteria, whose NADH inhibition is readily reversed by 5'AMP (Weitzman and Jones; Nature; 219; 270 (1968)).

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ACoA	Acetyl Co-enzyme A
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
CoA	Co-enzyme A
DEAE-cellulose	Diethylaminoethylcellulose
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylene diamine tetraacetate
g, mg, μg	gram, milligram, microgram
IAA	Iodoacetic acid
IAM	Iodoacetamide
Kd	dissociation constant
M, mM, μM	molar, millimolar, micromolar
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
nm	nanometer
OAA	oxaloacetate
OD	optical density
4,4'PDS	4,4'dithiodipyridine
POPOP	1,4-bis 2-(5-phenyloxazolyl) -Benzene
PPO	2,5-Diphenyloxazole

SDS	sodium dodecylsulfate
TNB-CN	2-nitro-5-thiocyanatobenzoic acid
tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

INTRODUCTION

Citrate synthase [citrate oxaloacetate - lyase (CoA-acetylating) E. C. 4.1.3.7] catalyzes the reaction through which the acetyl carbons of acetyl coenzyme A enter the Krebs cycle:

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O = \text{Citrate} + \text{CoA} + H^+$$

The Krebs cycle is a major energy producing pathway in eukaryotic cells, and the citrate produced by the reaction catalysed by citrate synthase is broken down stepwise to give back oxaloacetate, yielding 12 molecules of ATP through coupling with oxidative phosphorylation. In cells which contain glyoxysomes, the enzyme functions in the glyoxylate shunt in which two acetyl units are converted to succinate. In bacteria, especially anaerobes, the series of reactions beginning with the one catalyzed by citrate synthase leads to the synthesis of glutamic acid. Thus citrate synthase occupies a key position in the metabolism of most organisms and the control of its activity has been postulated to be important in various regulatory mechanisms.

Molecular Weight Studies

In recent years, a number of investigations have centered around the question of the molecular weight and state of aggregation of the native *E. coli* citrate synthase.

Weitzman and Dunmore (1969) have studied by means of gel filtration the molecular weights of twelve citrate synthases representing three different regulatory types i) NADH-insensitive enzymes from Gram-positive bacteria and eucaryotic organisms ii) NADH-inhibited enzymes

in which the inhibition is relieved by AMP and iii) NADH-inhibited/AMP-insensitive enzymes. These three categories were distinguished by Weitzman and Jones (1968). E. coli citrate synthase is an example of the last group. The citrate synthases in Weitzman and Dunmore's study fell into two groups - "large" molecules that eluted before bovine liver catalase (MW approximately 250,000) on a Sephadex G-200 column and were sensitive to regulation by NADH and "small" molecules that eluted after rabbit muscle lactate dehydrogenase (MW approx 140,000) and were insensitive to NADH.

Faloona and Srere (1969) have carried out molecular weight determinations on the E. coli enzyme using the gel filtration medium Bio-Gel A. They found that at pH 8.1 in the presence of 0.1 M KCl and 0.1 M mercaptoethanol, the molecular weight was approximately 280,000 grams/mole. Removal of potassium did not result in dissociation or aggregation of the enzyme.

Zonal sedimentation and sedimentation velocity experiments by Wright and Sanwal (1971) led to the conclusion that E. coli citrate synthase exists as an equilibrium mixture of monomers of 60,000 $\pm$ 3000 MW, tetramers of 245,000 $\pm$ 10,000 MW and octamers of 486,000 $\pm$ 20,000 MW. Only the tetramer was reported to have enzymic activity and the association-dissociation phenomenon was found to be pH dependent, with predominantly octamer existing at pH 7.0 and the enzyme irreversibly dissociating to form monomers at pH 11.0. A subunit molecular weight estimate of 60,000 to 65,000 grams/mole was obtained through SDS polyacrylamide gel electrophoresis, although it should be noted that it is my feeling that the citrate synthase sample was not adequately "bracketed" in mobility on

gels by known standards, so that there was considerable uncertainty in this estimate.

Srere (1972) has reported that sedimentation equilibrium experiments indicate that E. coli citrate synthase exists as a tetramer of MW 210,000 grams/mole.

Danson and Weitzman (1973) have used SDS polyacrylamide gel electrophoresis to determine a subunit molecular weight for E. coli citrate synthase of 55,000 ± 4,000. Ultracentrifugation studies at pH 8.0, using the same diffusion coefficients as Wright and Sanwal (1971), gave a MW of 57,000 and 230,000 for the two species present. These authors have confirmed the dissociation at pH 11.0 into monomers, as found by Wright and Sanwal, but disagree with the finding that at pH 7.0 in the presence of 2 mM DTT, the enzyme exists only as the octameric species. They have found that under these conditions the tetrameric form predominated, although the enzyme concentration used was much lower than that of Wright and Sanwal (1971). By use of analytical band centrifugation of the active enzyme-substrate complex, these authors confirm that the tetramer is the active species.

Tong and Duckworth (1975) have reported a subunit molecular weight for E. coli citrate synthase of 46,000 ± 2000 g/mole, using SDS polyacrylamide gel electrophoresis. Cross-linking with dimethyl suberimidate before electrophoresis led to six bands whose apparent molecular weights were almost integral multiples of 47,000 g/mole. Sedimentation equilibrium experiments in guanidine HCl gave a molecular weight average of 43,400 ± 300 g/mole. Various conditions seemed to

influence the aggregation state of the enzyme. At pH 7.0 and 7.8 monomers through greater than decamers were present; at pH 9.0 only dimers were seen and at pH 10.0, large aggregates (up to 20 subunits) were present. At pH 7.8, in the presence of 0.05 M KCl, dimer and hexamer are the only species present and increasing the KCl concentration to 0.10 M converts all of the enzyme to hexamer.

The native molecular weight of E. coli citrate synthase has been reported to be greater than 250,000 (Weitzman and Dunmore, 1969), 280,000 (Faloona and Srere, 1969), 210,000 (Srere, 1972) or 245,000 (Wright and Sanwal, 1971). The finding of Tong and Duckworth (1975) that the enzyme exists as a heterogenous mixture of various oligomeric forms at the pH and concentration ranges used in these studies renders these values meaningless, since they are based on methods which are incapable of distinguishing the continuous MW variation present. Sedimentation coefficients quoted are reflections of a complex association-dissociation equilibrium, whose values fortuitously could be interpreted in terms of certain oligomeric structures. The subunit molecular weight, on the other hand, is reported to be 47,000 (Tong and Duckworth, 1975); 52,500 (Srere, 1972); 55,000 (Danson and Weitzman, 1973) or 60,000 grams/mole (Wright and Sanwal, 1971). All of these measurements derived in part or whole from SDS gel electrophoresis experiments, and reflect valid disagreement over the true value. The subunit molecular weight of Tong and Duckworth (1975) is the one I have most confidence in, since this value has been obtained using a number of different and sensitive methods to determine a true subunit molecular weight, and it has been duplicated a number of times in the hands of other workers. (data not presented)

Allosteric Properties

Extensive functional studies have been carried out on the enzyme from the time Ochoa's group isolated the enzyme from E. coli in 1950. Originally the enzyme was assayed by coupling it to malate dehydrogenase and following the appearance of NADH at 340 nm. Srere, Brazil and Gonen (1963) developed an assay based on the reaction of Ellman's (1959) reagent, DTNB with the free sulfhydryl of CoA, a product of the enzyme's reaction, to produce a highly colored species.

Weitzman (1966a) was the first to show that NADH inhibits citrate synthase from E. coli, as well as other bacterial sources, but was ineffective towards yeast and mammalian citrate synthase. Inhibition of the enzyme was essentially complete at 0.1 mM NADH and dependent on the amount of ACoA present. No inhibition was observed with NAD^+, $NADP^+$ or NADPH (each at 0.3 mM), but high concentrations of ATP (20 mM) produced 75% inhibition. Thus NADH appeared to be the prime feedback inhibitor in the bacterial system of energy production.

In other studies, Weitzman (1966b) showed that changing the pH from 7.5 to 9.2 abolished the NADH inhibition effect on the enzyme, although the catalytic activity was lowered by only 50%. From this finding, the allosteric nature of the enzyme was first postulated. Weitzman also found that addition of 0.2 M KCl completely removed the sensitivity of the enzyme to NADH, although it did not affect the catalytic activity at pH 8.0. However, in the pH range 8.0 - 9.0, addition of KCl would increase the catalytic activity approximately 40%. KCl also seemed to protect the enzyme from the effects of heat and conferredresistance to thiol reagents that otherwise produced inactivation.

Weitzman (1967) reported some significant differences from earlier work (Weitzman, 1966a,b) on a purified preparation of citrate synthase, notionally of E. coli origin. These included lack of inactivation by DTNB, NADH inhibition that was noncompetitive rather than competitive with respect to ACoA, sigmoidal dependence of the degree of inhibition on NADH concentration, rather than hyperbolic, and the fact that AMP and ADP could relieve NADH inhibition of the enzyme. Further investigation revealed that the enzyme under study was not from E. coli, but from Acinetobacter lwoffi, a Gram-negative strict aerobe which had contaminated the culture (Weitzman and Danson, 1976).

These findings prompted Weitzman and Jones (1968) to survey a large number of citrate synthases from bacterial sources in order to investigate the seemingly different kinetic details of NADH inhibition of these two Gram-negative bacteria. The organisms could be divided into two major groups according to whether or not their citrate synthases are sensitive to inhibition by NADH. The NADH-sensitive group consisted of the Gram-negative bacteria whereas the NADH-insensitive group comprises the Gram-positive group. The NADH-sensitive group of citrate synthases could be subdivided further into two groups. The strict aerobes, of which A. lwoffi is an example, showed NADH inhibition which could be relieved by AMP, while the facultative anaerobes (e.g. E. coli) showed no AMP reactivation. Various authors have presented exceptions to these patterns (Taylor, 1970; Swissa and Benziman, 1973; Srere, 1972) and these have been discussed by Danson and Weitzman (1976).

Jangaard, Unkeless, and Atkinson (1968) have presented work on the inhibition of E. coli citrate synthase by ATP. They have found that ATP strongly inhibits the enzyme in pH range of 6.0 to 7.3, then the

inhibition decreases with increasing pH until ATP becomes slightly stimulatory above pH 7.6. AMP and ADP inhibit to a lesser degree than ATP at low pH (below 7.0) and stimulate to a greater extent above pH 7.2. OAA in the reaction mixture prior to initiating the reaction seems to protect the enzyme from ATP inhibition. At pH 6.4, ATP decreases the apparent affinity of the enzyme for both ACoA and OAA, in contrast to the mammalian enzymes where only the Km for ACoA is altered. The authors also reported that enzyme activity is influenced by EDTA, Mg^{2+}, K^{+} and changes in the molarity of the buffer.

Faloona and Srere (1969) have investigated the effect of K^{+} on highly purified E. coli citrate synthase. They found that K^{+}, and to a lesser extent other monovalent cations, markedly increased the stability of the enzyme to urea denaturation, and activated the enzyme up to 20 fold, with a concomitant lowering of the apparent Km's for ACoA and OAA. K^{+} also normalized sigmoidal reciprocal plots of velocity versus ACoA concentration. A shift in the ultraviolet absorption spectrum was observed when potassium was added to the salt free enzyme. Since KCl was said to have no effect on the molecular weight, these authors have concluded that the K^{+} effects are due to conformational changes in the enzyme.

Wright and Sanwal (1971) have shown through equilibrium - binding studies that ACoA binds in a cooperative manner, both in the absence and presence of KCl, although cooperative rate - concentration plots in the absence of KCl become Michaelian hyperbolas in its presence. An unexpected finding was the fact that in the absence of ACoA, the maximum amount of binding for NADH did not exceed 5% of the sites available. Both inhibitors,

αKG and NADH, bind to the enzyme in a negatively cooperative manner in the absence of ACoA, but in a positively cooperative manner in its presence. The binding of both inhibitors, especially that of NADH, is considerably enhanced in the presence of ACoA. These results are in conflict with later studies discussed below, and must be viewed with caution, since the enzyme used in these studies contained large amounts of ammonium sulfate.

Danson and Weitzman (1973) have reported that histidine residues appear to be involved in enzyme activity, as well as the αKG effector site, as judged by the pH dependence of the rates of inactivation by photo-oxidation with Methylene Blue, photo-inactivation with Rose Bengal and destruction of activity by diethyl pyrocarbonate. Also, modification with 2-hydroxy-5-nitrobenzyl bromide indicates the participation of tryptophan in the activity of the enzyme. Photo-oxidation studies suggested the involvement of cysteine at the NADH effector site, and this was confirmed by the desensitization to NADH inhibition produced by treatment of the enzyme with DTNB. The authors suggest that the inactivation of the enzyme after modification with this reagent is due to the additional involvement of cysteine in catalytic activity.

Harford and Weitzman (1975) have conducted multiple-inhibition studies on citrate synthase from Pseudomonas aeruginosa, a Gram-negative bacterium in the same class as E. coli, as well as enzyme from eukaryotic and Gram-positive bacterial sources. They have found that ATP acts in all cases as an isosteric inhibitor at the ACoA site but that NADH acts isosterically with the eukaryotic and Gram-positive bacterial citrate synthases and as a specific allosteric inhibitor in the case of the Gram-

negative bacterial enzyme. After desensitization by treatment with DTNB to this allosteric inhibition, only the isosteric nucleotide inhibition found in other citrate synthases is observed.

Duckworth and Tong (1976) have studied the binding of NADH to E. coli citrate synthase using a fluorescence enhancement technique. Contrary to Wright and Sanwal (1971), they found the binding to be strong. The dissociation constant for the NADH-citrate synthase complex increases towards the alkaline pH as if the binding depends on the protonation of a group with a pKa of about 7.05. Over the pH range 6.2 - 8.7, the number of binding sites decreases from about 0.65 to about 0.25 per subunit; this fact is perhaps connected with the partial depolymerization of the enzyme which occurs over the same range. NAD^+ and $NADP^+$ were found to be weak competitive inhibitors of NADH binding, but stronger inhibition was shown by 5'AMP and 3'AMP. ACoA and KCl also inhibit the binding in a weakly cooperative manner. The authors have interpreted their results in terms of two binding sites for nucleotides: i) an active site binding ACoA or 3'AMP and ii) an allosteric site which binds NADH or 5'-AMP and to a lesser extent, other nicotinamide adenine dinucleotides. Occupation of one site prevents binding at the other site.

The above section illustrates some discrepancies concerning the binding of NADH to the enzyme. Weitzman (1966, a, b) was the first to suggest the allosteric nature of NADH inhibitionof E. coli citrate synthase, and this has been confirmed by Harford and Weitzman (1975) and Duckworth and Tong (1975). Wright and Sanwal (1971) found that NADH binding to the enzyme was very weak, but is slightly stronger in the

presence of ACoA, even though NADH inhibition can be overcome by increasing ACoA concentration (Weitzman 1966a, Wright and Sanwal 1971). Even with ACoA present, the binding is too weak to account for the inhibition observed in kinetic experiments. Duckworth and Tong (1975) have reported NADH binding with a K_D of 1.6 μM at essentially the same pH as Wright and Sanwal (1971), and that this binding is weakened by the presence of ACoA. It is my feeling that the findings of Wright and Sanwal must be erroneous and that NADH binds tightly to the enzyme, as would be expected of a potent allosteric effector. Perhaps the discrepancy could result from the differences in temperature between the two studies (11°C for Wright and Sanwal (1971) and 21°C for Duckworth and Tong 1976) or the high contamination of salt in the Wright and Sanwal (1971) enzyme preparation.

The effect of K^+ on the enzyme is dramatically documentated by Weitzman (1966b), Faloona and Srere (1969) and Duckworth and Tong (1976). It is evident that KCl is causing a dramatic conformational change in the enzyme that strongly affects the catalytic and regulatory properties of the enzyme.

It was the finding of Danson and Weitzman (1973) that cysteine was involved in the NADH effector site that prompted my investigation of the sulfhydryl reactivity of E. coli citrate synthase.

Sulfhydryl Groups of Citrate Synthase

The sulfhydryl groups of E. coli citrate synthase have been actively investigated with regard to the functional characteristics of the enzyme. Weitzman (1966, b) was the first to investigate the sensitivity of the enzyme to modification by DTNB. He found that in the absence of KCl,

enzyme activity measured as the rate of chromophore formation between CoASH and DTNB (Srere, Brazil and Gonen, 1963) was considerably less than that determined polarographically but that in the presence of 0.2 M KCl, the activities measured by the two methods were identical. Preincubation of the enzyme for 5 minutes with 0.1 mM DTNB at pH 8.0 virtually abolished its activity in the absence of KCl, but if this treatment was carried out in the presence of 0.2 M KCl, no inactivation was observed. Moreover, the addition of KCl (to 0.2 M) to the enzyme after it had been inactivated by treatment with DTNB resulted in considerable reactivation (more than 50%). Very similar results were obtained with the thiol reagents N-ethylmaleimide and $HgCl_2$.

Faloona and Srere (1969) have reported that approximately 5 sulfhydryl groups per MW 280,000 (0.84 per MW 47,000) in E. coli citrate synthase react in the presence of 0.1 M KCl over a period of 100 mins with an approximate overall loss of 20% enzyme activity. In the absence of KCl, 7 groups react (1.18 per MW 47,000) over the same time period with a loss of 32% enzyme activity. The activity continues to decrease slowly for several hours with no further DTNB- enzyme reaction; when K^+ is added to such an incubation it continues to lose activity but at a slower rate. As well, these authors report no inactivation of the enzyme during the DTNB assay procedure (Srere, Brazil and Gonen, 1963). Weitzman and Danson (1976) have suggested that the high ionic strength of the buffers and assay conditions used in these experiments (0.1 M Tris-Cl; contained 0.1 M KCl) prevented these authors from observing DTNB inactivation.

Wright and Sanwal (1971) have investigated the reactivity of the sulfhydryl groups of E. coli citrate synthase with DTNB and 4,4'PDS. These authors report that 2.0 to 2.2 groups per monomer of MW 61,000 g/mole (1.5 to 1.7 groups/47,000 g/mole) react with 0.1 mM DTNB over a period of 6 hours with a gradual loss of approximately 90% enzyme activity. The presence of 0.5 mM ACoA, NADH or αKG did not appreciably alter the number of groups reacting. Modification with 1.0 mM 4,4'PDS at pH 7.9 resulted in loss of approximately 93% enzyme activity over a period of 5 minutes, and complete inactivation in 20 minutes. Approximately 1.0 groups per 61,000 g/mole (0.77 groups/47,000 g/mole) react in the first 5 minutes, and a total of 1.5 groups/61,000 (1.2 groups/47,000) over a period of 6 hours.

Danson and Weitzman (1973) have found that over the first 4 minutes of the reaction of E. coli citrate synthase with 0.1 mM DTNB, 1.0 sulfhydryl group per monomer of MW 57,000 (0.82/47,000) reacts with a loss of approximately 20% of the enzyme activity, but no loss of NADH inhibition. Over a total period of 60 minutes, a total of 2.0 groups will react (1.6/47,000) with a total loss of 65% enzyme activity and a total loss of NADH inhibition. The lag effect in loss of NADH inhibition suggests to these authors that cysteine residues are important both in the catalytic site and the NADH regulatory site. Since rapid inactivation of the enzyme by DTNB was observed even after 10 min of photo-oxidation, the authors conclude these two effects occur at different sites. They also report that when both substrates are added to the enzyme before DTNB, complete protection of enzyme is achieved in the assay mixture.

Danson and Weitzman (1977) have carried out a detailed kinetic analysis of the reaction between DTNB and citrate synthase. In low ionic strength at pH 8.0, 2 thiol groups per tetramer of MW 230,000 (0.40 per MW 47,000) react with a second order rate constant of 2.5×10^4 M^{-1} min^{-1} with a loss of 26% enzyme activity but no change in the NADH inhibition of the enzyme. Six more groups per tetramer (1.22 per 47,000) continue to react in a first order process with a rate constant of 0.076 min^{-1}, resulting in a further 60% loss of activity and complete desensitization to NADH. In high ionic strength (0.1 M KCl), 4 groups/tetramer (0.81 per 47,000 grams/mole), apparently out of the 6 reacting slowly in the absence of KCl, react in a single kinetic set resulting in 70% loss of enzyme activity and complete loss of NADH inhibition. The authors propose that the 4 thiol groups accessible to DTNB in the absence and presence of 0.1 M KCl are those involved in the response to NADH, and that loss of inhibition is only coincident with the appearance of tetrameric protein possessing three substituted thiols, whereas enzyme with one or two modified groups was still fully inhibited by NADH.

The results presented in this paper are a result of extensive curve fitting using a molecular weight which I do not accept. The value for the "tetramer" was determined by sedimentation velocity, a method which must give only an average molecular weight of a number of the different oligomeric species found by Tong and Duckworth (1975). As well, the value for the subunit molecular weight is significantly higher than that of Tong and Duckworth, the most reliable measurement in the literature. In light of this, the authors findings of partial reaction of enzyme with DTNB with no loss of NADH inhibition must be called into question.

It is evident from the above discussion that there exist some disagreements on the effects of DTNB on enzyme activity. Weitzman (1966, b) and Danson and Weitzman (1973, 1977) have reported a rapid loss of enzyme activity as the enzyme is modified by DTNB. Wright and Sanwal (1971) suggest that the loss of activity takes place at a slow rate over a period of 6 hours, and Faloona and Srere (1969) report little loss of enzyme activity as the modification proceeds. Weitzman (1966, b) originally reported that enzyme activity as measured by the DTNB assay method (Srere, Brazil and Gonen (1963)) was less than that determined polarographically, but Danson and Weitzman (1973) and Faloona and Srere (1969) state that complete protection of the enzyme from DTNB inactivation is achieved under assay conditions.

There is little discrepancy over the total number of groups reacting with DTNB. In the absence of KCl, and in terms of groups reacting per subunit of 47,000 g/mole, Faloona and Srere (1969) report 1.18 groups reacting, Wright and Sanwal (1971) find 1.5 to 1.7, and Danson and Weitzman (1973, 1977) report 1.6. In the presence of 0.1 M KCl, Faloona and Srere (1969) find 0.84 groups and Danson and Weitzman (1977) report 0.81 groups reacting.

The most interesting aspect of the modification of citrate synthase by DTNB is the loss of inhibition by NADH reported by Danson and Weitzman (1973, 1977). The authors have suggested that the thiols modified by DTNB are not directly involved in the binding of NADH but that they have an indirect role, such as in subunit interactions which may be essential for the response to the inhibitior. Since my interest

in this enzyme is due to its allosteric nature, the reactivity of citrate synthase to DTNB seemed to be a profitable area on which to focus my attention.

MATERIALS AND METHODS

MATERIALS

Protamine sulfate, trizma (Tris), iodoacetic acid, iodoacetamide, dithiothreitol, 3' and 5' AMP, ADPR, DNAase, PPO and POPOP were obtained from Sigma; Oxaloacetate and NADH were from Boehringer Mannheim; Ellman's reagent (DTNB), 4,4 'PDS (Aldrithiol-4) and 2,2'-PDS (Aldrithiol-2) were from Aldrich; ammonium sulfate, enzyme grade was from Schwarz/Mann ^{14}C-IAA was from Amersham/Searle; ACoA, NADPH, 2'5'ADP and 3'5'ADP were from P-L Biochemicals; N-dansylaziridine was from Pierce; DEAE-cellulose, preswollen microgranule (DE52) was from Whatman. All other chemicals were reagent grade.

Organism

E. coli strain K12 3000 was used throughout this investigation.

Media

Stock LB medium is a solution containing:

Bacto-tryptone	1%
Yeast extract	0.5%
NaCl	1%
1N NaOH	1.0 ml/liter
water to volume	

This solution is autoclaved at 121°C for 20 minutes before use.

Minimal Medium A is a solution containing

K_2HPO_4	10.5 gm/liter
KH_2PO_4	4.5 gm/liter
$(NH_4)_2SO_4$	1.5 gm/liter
$MgSO_4.7H_2O$	0.1 gm/liter

The minimal acetate medium A contains in addition 1% of sodium acetate, and 0.001% thiamine.

Growth of Bacteria

E. coli cultures were routinely stored at -20°C in a solution of 40% glycerol: 60% stock LB medium. Growth was initiated by inoculating 1 x 10 mls LB medium, which was used to inoculate 3 x 10 mls LB medium. Each of the 3 flasks inoculated 1 x 300 mls minimal acetate medium A. These were combined and used to inoculate 1 x 15 liters minimal acetate medium A in a carboy. This was used to finally inoculate 4 x 15 liters

minimal acetate medium A in carboys, from which the cells were harvested.

At each stage the culture was allowed to grow 24 hours at 37°C with sufficient agitation to ensure good aeration. A typical yield is about 175 grams of wet E. coli cells from the final 60 liters.

At the time of harvesting, a check on the purity of our culture was carried out as follows: Samples from each of the 4 final carboys were taken using sterile techniques and dilutions were plated on LB plates. As well, samples were streaked on λ, T_6 and T_7 phage plates, which are specific for E. coli. Visual inspection of the LB and phage plates indicated that our preparations were essentially pure.

Enzyme Purification

The method used was similar to that of Tong and Duckworth (1975).

The harvested cells were suspended in 2.5 times their weight of standard buffer and stirred overnight at 4°C. The cell suspension was passed through an Aminco French pressure cell at a cell pressure of 18000 lbs/in^2 while being kept on ice. A small amount of DNAse was added to the broken cell suspension and this was stirred for 25 minutes at 4°C.

The broken cells were then spun in 2 runs at 20,000 rpm for 1 hour on a Sorvall RC2B ultracentrifuge. The supernatant was collected and to this was added dropwise over a period of 15 minutes 8.4 mg protamine sulfate per gram of harvested wet cells as a 2% solution. The solution was stirred for an additional 15 minutes and the precipitate was discarded by spinning the solution at 20,000 rpm for 45 minutes and collecting the supernatant.

Solid ammonium sulfate was slowly added over a period of 15 minutes to the supernatant to achieve 55% saturation; the mixture was allowed to stir for an additional 15 minutes at 4°C. The solution was then spun for 30 minutes at 6,000 rpm and the supernatant collected. To this was slowly added over a period of 15 minutes sufficient solid ammonium sulfate to achieve 70% saturation. This solution was stirred at 4°C for an additional 15 minutes and then spun at 6,000 rpm for 30 minutes. The pellets were dissolved in a minimal amount of standard buffer containing 0.05 M KCl and stored overnight at 4°C. Typical recoveries at this point were 57-69% of the enzyme activity initially extracted.

Redissolved precipitate was layered on a Sephadex G-25 coarse column (6 x 32 cm) previously washed with the above buffer and was eluted with the same buffer. Fractions of approximately 15 mls volume were collected at a flow rate of ~ 3.7 mls/minute. Conductance of fractions was determined by a Radiometer Conductance meter and OD_{280} read on a Gilford 2400-2 spectrophotometer. Protein fractions with low conductance were pooled and bound to a pre-equilibrated DEAE cellulose column (2.5 x 23 cm). The column was washed overnight at 4°C with standard buffer containing 0.05 M KCl; then a linear gradient of KCl from 0.05 M to 0.3 M in standard buffer, total volume 2 liters, was applied to elute the column at a flow rate of 1 ml/min. Fractions with a specific activity above 15 units/mg were pooled and concentrated by either ultrafiltration or a combined Millipore/Amicon concentration scheme. This concentrated solution was applied to a Sephadex G-200 column (2.8 x 80 cm) and eluted with standard buffer containing 0.05 M KCl. Fractions with a specific activity

above 60 units/mg were pooled and again concentrated using ultrafiltration or the Amicon system. The resulting solution was designated the stock solution of the enzyme and stored at 4°C in this concentrated form.

The final recovery of enzyme activity was typically 30%, yielding a total of between 60-96 mg of protein at a specific activity of 65 units/mg. A purity check using SDS polyacrylamide gel electrophoresis by the method of Weber and Osborn (1969) revealed a major band and several very minor secondary bands, visible only on over-loaded gels, indicating that the preparation was essentially pure.

Routine Procedures

Standard Buffer:

The standard buffer used throughout this investigation, with the exception of the pH study on the rate of reaction of enzyme with DTNB, is 0.02 M Tris-Cl, 1.0 mM EDTA pH 7.8.

Enzyme Assay:

A unit of enzyme is defined as the amount of enzyme which is necessary to catalyse the reaction:

acetyl-CoA + oxaloacetate = citrate + CoA

at the rate of 1 μmole product formed per minute under standard assay conditions.

The enzyme was routinely assayed by the method of Srere et al. (1963) involving the measurement with DTNB of the appearance of the free sulfhydryl group of coenzyme A.

Danson and Weitzman (1973) have shown that in the presence of both substrates, citrate synthase is not inactivated by DTNB. The standard concentrations of ACoA and OAA in the assay mixture were both 0.1 mM, and the concentration of DTNB was 0.04 mM. The assays done in the presence of NADH were done at a concentration of 0.1 mM, and KCl at a concentration of 0.1 M. All assays were done in standard buffer, at a temperature of 21°C in a Gilford Recording Spectrophotometer, Model 2400-2.

Enzyme Concentration:

The subunit molecular weight of E. coli citrate synthase was taken to be 47,000 grams/mole, as determined by equilibrium ultracentrifugation and polyacrylamide gel electrophoresis techniques. (Tong and Duckworth, 1975).

The concentration of citrate synthase in solutions was determined from the optical density at 278 nm, using $E_{1\ cm}$ (1%) = 9.76 $\pm$ .20 (Tong and Duckworth, 1975). All protein concentrations are in terms of subunit concentration.

Modification of Citrate Synthase by DTNB or 4,4'PDS

To a protein solution of known concentration was added 10 to 50 µl of a freshly made stock solution of DTNB or 4,4'-PDS to make a final volume of 0.60 mls. The final concentration of 4,4'-PDS was usually 0.01 or 0.05 mM, while the final concentration of DTNB was typically 0.1 mM. The recorder on the Gilford was started at the moment of mixing and the progress of the reaction was observed by measuring the change in optical density at 324 nm ($\Delta\varepsilon_M$ = 19,800) in the case of 4,4'-PDS and 412 nm ($\Delta\varepsilon_M$ = 13,600) in the case of DTNB. At the end of the reaction, the

blank value was determined by adding the same aliquot of reagent to a blank of buffer and measuring the change in optical density. This value was subtracted from all absorbance values when converting these absorbance values into groups reacted.

Kinetic Analysis of Reaction of Enzyme with Sulfhydryl Reagents

The rate analysis of the reaction of DTNB with citrate synthase was carried out in one of two ways. Initially the reaction curves were analyzed by the method of Guggenheim (1926) in order to obtain the rate constant for the initially reacting group. This entails obtaining a series of values for groups reacted at times T within the first half-life of the reaction. Skipping a time period of 1½ half-lives another series of groups reacted is then determined for the same time intervals as in the first set of values. A plot of the natural logarithm of the differences between pairs of values from the two sets against time yields a straight line with a negative slope equal to the pseudo first order rate constant for the reaction. The virtue of this procedure is that no final end-point value for the reaction is needed; such a value is difficult to determine in this case since further sulfhydryl groups continue to react slowly after the initial one is modified.

A second method was used involving fitting the reaction profile to the following rate equation, allowing meaningful kinetic information to be obtained only for the most reactive class of sulfhydryl groups.

$$\text{groups reacted at time } t = N\,(1 - e^{-kt}) + M \cdot t$$

where N is the number of groups reacting quickly with a pseudo first order rate constant k

M is a zero order rate constant representing slowly reacting sulfhydryl groups of unknown size.

This was done manually by drawing a straight line of slope M through the data at long times, and extrapolating this back to t = 0 to obtain N. The rate constant k could then be obtained as the negative slope of a plot of ln (M·t + N- groups reacted at time t) against t. The data consistently yielded straight lines over the time period associated with the modification of the first group.

Kinetic analysis of the reaction of citrate synthase with 4,4'-PDS, when required, could be performed utilizing a standard first order plot. The slope of the straight line obtained from the plot of ln (groups reacted at infinity - groups reacted at time t) vs time is equal to the negative pseudo first order rate constant. Choosing a value of 2.0 for the number of groups reacted at infinity yielded satisfactory straight lines.

The analysis of the rate of reaction of the enzyme modified to differing extents by IAA with DTNB and 4,4'-PDS was carried out using the GENLSS program (DeTar, 1972) modified to run on a IBM 370/65 machine by Dr. J. L. Charlton. This involved setting initial parameters to the following equation and allowing the program to adjust them in order to obtain the best fit to the data. The equation used was:

$$\text{groups reacted at time } t = N_1 (1 - e^{-k_1 t}) + N_2 (1 - e^{-k_2 t})$$

where N_1 is the total number of groups reacting with rate constant k_1

N_2 is the total number of groups reacting with rate constant k_2.

In the case of DTNB, where only 1 group/subunit reacts in the presence of 0.1 M KCl, N_2 and k_2 were set to zero and these parameters were not adjusted.

Separation of Modified Enzyme from Reagent

Enzyme modified to the specified extent was separated from excess modifying reagent by passing it through a G-25 Sephadex (coarse) column (1.0 x 52 cm), eluting with standard buffer. Fractions containing protein were identified by OD at 278 nm. Using radioactive IAA as a model reagent, it was shown that a clear separation between protein and reagent was achieved.

Reactivation of Modified Citrate Synthase by DTT

A protein solution of known concentration was made up and assayed in the presence and absence of NADH. DTNB or 4,4'-PDS was then added to the appropriate concentration and the reaction was monitored as before. At appropriate intervals, samples were removed and assayed. After the reaction seemed complete as judged by lack of change in activity, 200 µl of the reaction mixture was diluted into 1.0 ml buffer and another 200 µl into 1.0 ml buffer containing 0.1 mM DTT, giving a final DTT: reagent ratio of 6:1. Samples of both mixtures were removed at appropriate times and assayed.

Modification of Citrate Synthase by IAA

A small aliquot of radioactive IAA was added to a protein solution

of known concentration to give a final protein concentration of 23.7 μM, a final IAA concentration of 1.23 mM and thus a final IAA/protein ratio of 52:1. The reaction mixture was allowed to react in the dark at room temperature. Measurements before and after the reaction indicated a constant pH of 7.8, enhancing the selective reaction of IAA with cysteines. After the specified reaction times, the samples were passed through a Sephadex G-25 column in the dark and the protein isolated. Degree of incorporation was calculated based on OD_{278} of protein fraction and counts generated by a suitable aliquot in the standard counting procedure.

Radioactive Counting Procedure

An aliquot (10 to 200 μℓ) of the sample was introduced into a clean scintillation vial followed by 5 mls of scintillation cocktail; the cocktail consisted of

7.0 grams	PPO
0.36 grams	POPOP
100 mls	Bio-Solv formula BBS-3
900 mls	toluene

the sample was mixed and placed in a Packard Tri-Carb Liquid Scintillation Spectrometer and allowed to cool. Counting was carried out so as to maximize counts in the ^{14}C channel. A blank of the same aliquot of buffer was added to another 5 ml of scintillation cocktail and counted at the same time as the sample in question.

Experiments with N-dansylaziridine

As suggested by Scouter et al. (1974), initial experiments were

tried using N-dansylaziridine cellulose, which was synthesized in the following manner: 10.89 mg N-dansylaziridine was dissolved in 1.0 mls absolute ethanol and mixed with 0.20 grams Whatman CF-11 cellulose to form a smooth paste (upon standing the mixture would separate into a liquid and solid layer). Air was passed over the mixture overnight and a dry powder was obtained in the morning.

To test the reaction of N-dansylaziridine cellulose with the enzyme, a suitable aliquot of enzyme (1.0 mls) was added to 3.45 mg of N-dansylaziridine cellulose and shaken well on a side arm continuous shaker. At appropriate intervals, a sample was removed and fluorescence at 485 nm and enzyme activity were measured.

Since there seemed to be no reaction between the N-dansylaziridine cellulose and the enzyme, I tested the effect of the N-dansylaziridine reagent itself. A protein solution was made (1.05 mls total; 17.2 μM) and the fluorescence emission spectrum was determined, as read against a blank of buffer, over the wavelength range 400 to 550 nm, with excitation at 345 nm.

To this solution was added 3.51 mg N-dansylaziridine. The compound did not dissolve, but the mixture was shaken for approximately 22 hours at room temperature. The supernatant after a brief centrifugation was put on a Sephadex G-25 column and the protein isolated. A fluorescence emission spectrum was obtained as above.

RESULTS

RESULTS

I) Characteristics of DTNB Reaction with Citrate Synthase

i) Reaction with DTNB

Figure 1 illustrates the modification of our preparation of *E. coli* citrate synthase with 0.1 mM DTNB. The reaction consists of a rapid phase, involving 1 equivalent of reagent per subunit reacting over a period of about 20 minutes, followed by a slow phase in which one or more other equivalents react.

The initial phase of the reaction was studied in the presence of KCl, a potent, apparently allosteric activator of citrate synthase. Figure 2 represents the reaction of the enzyme in the absence and in the presence of 100 mM KCl, the amount needed for fully allosteric activation. Two effects should be noted. KCl causes the initial phase of the reaction to proceed at a greater rate, as well as virtually eliminating any secondary reaction. Thus, in the presence of KCl, we can selectively modify the first reacting cysteine.

The dependence of the rate of the rapid phase of the reaction upon KCl concentration is seen in Figure 3. The effect is saturable by KCl, and the shape of the saturation curve is apparently hyperbolic, with a half-maximal KCl concentration of 110 $\pm$ 20 mM.

The rate of the reaction between citrate synthase and DTNB was studied as a function of DTNB concentration both in the absence and presence of KCl. These data are presented in Figure 4. The rate of the

Fig. 1: Modification of citrate synthase by 0.1 mM DTNB.

Groups reacted calculated from ΔOD_{412} with a monomer molecular weight of 47,000 g/mole. Protein subunit concentration was 19.7 μM. pH of reaction mixture was 7.8.

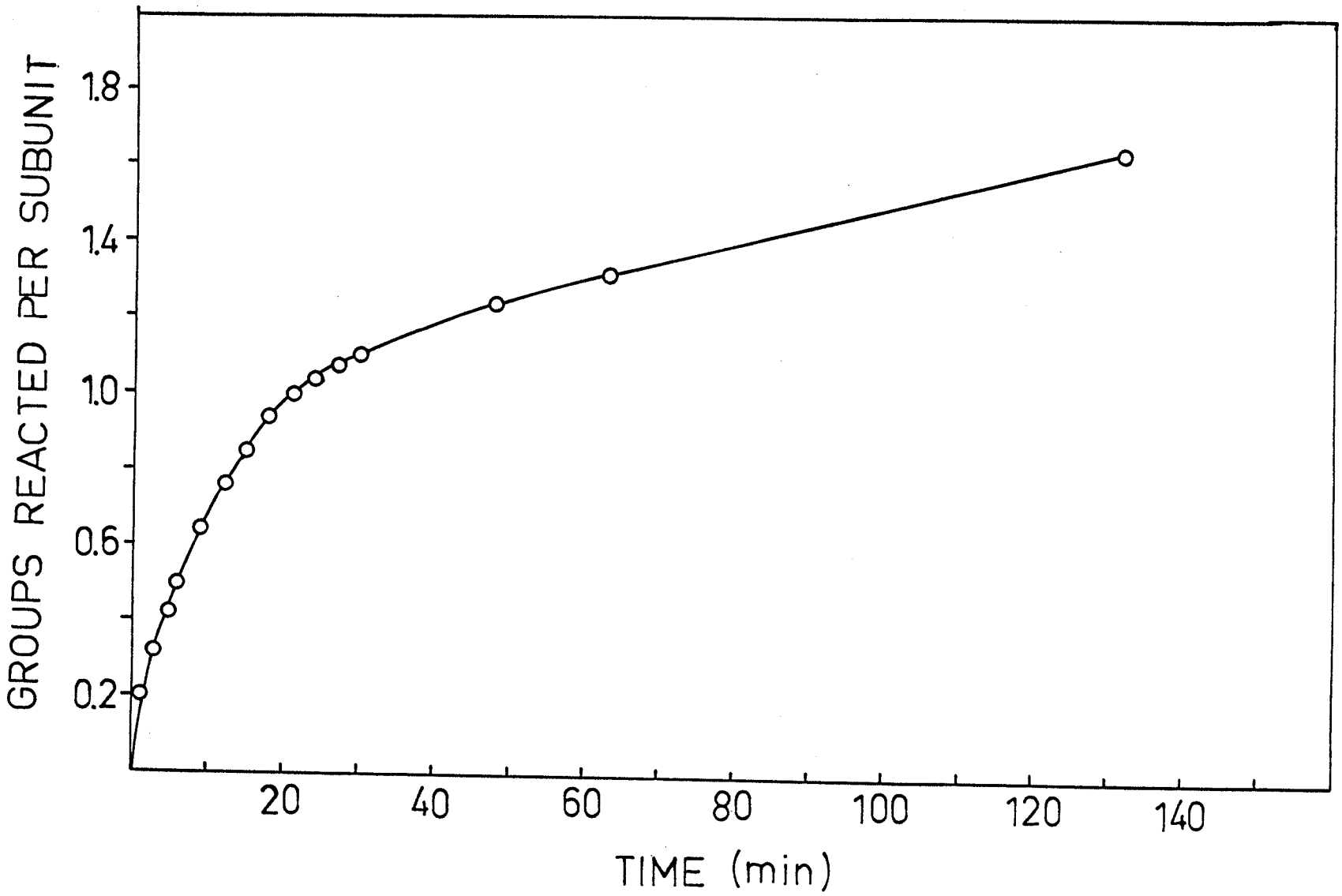

GROUPS REACTED PER SUBUNIT
1.8
1.4
1.0
0.6
0.2
20
40
60
80
100
120
140
TIME (min)

Fig. 2: Modification of citrate synthase by 0.1 mM DTNB in the presence and absence of KCl.

(o-o) 100 mM KCl (•-•) no KCl.

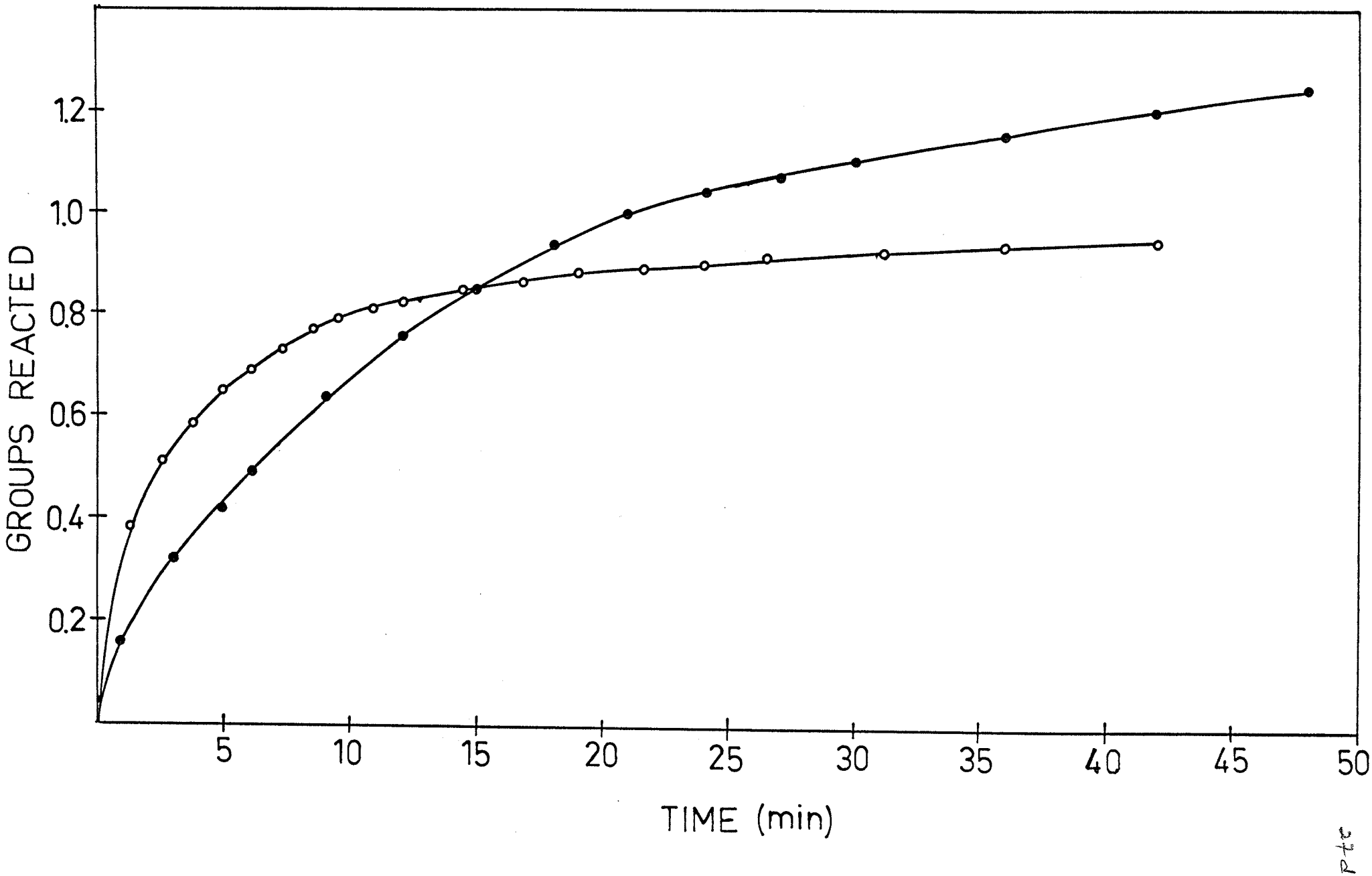

GROUPS REACTED
1.2
1.0
0.8
0.6
0.4
0.2
5
10
15
20
25
30
35
40
45
50
TIME (min)

Fig. 3: Rate of reaction of citrate synthase with 0.1 mM DTNB as a function of KCl concentration.

pH of reaction mixture was 7.8.

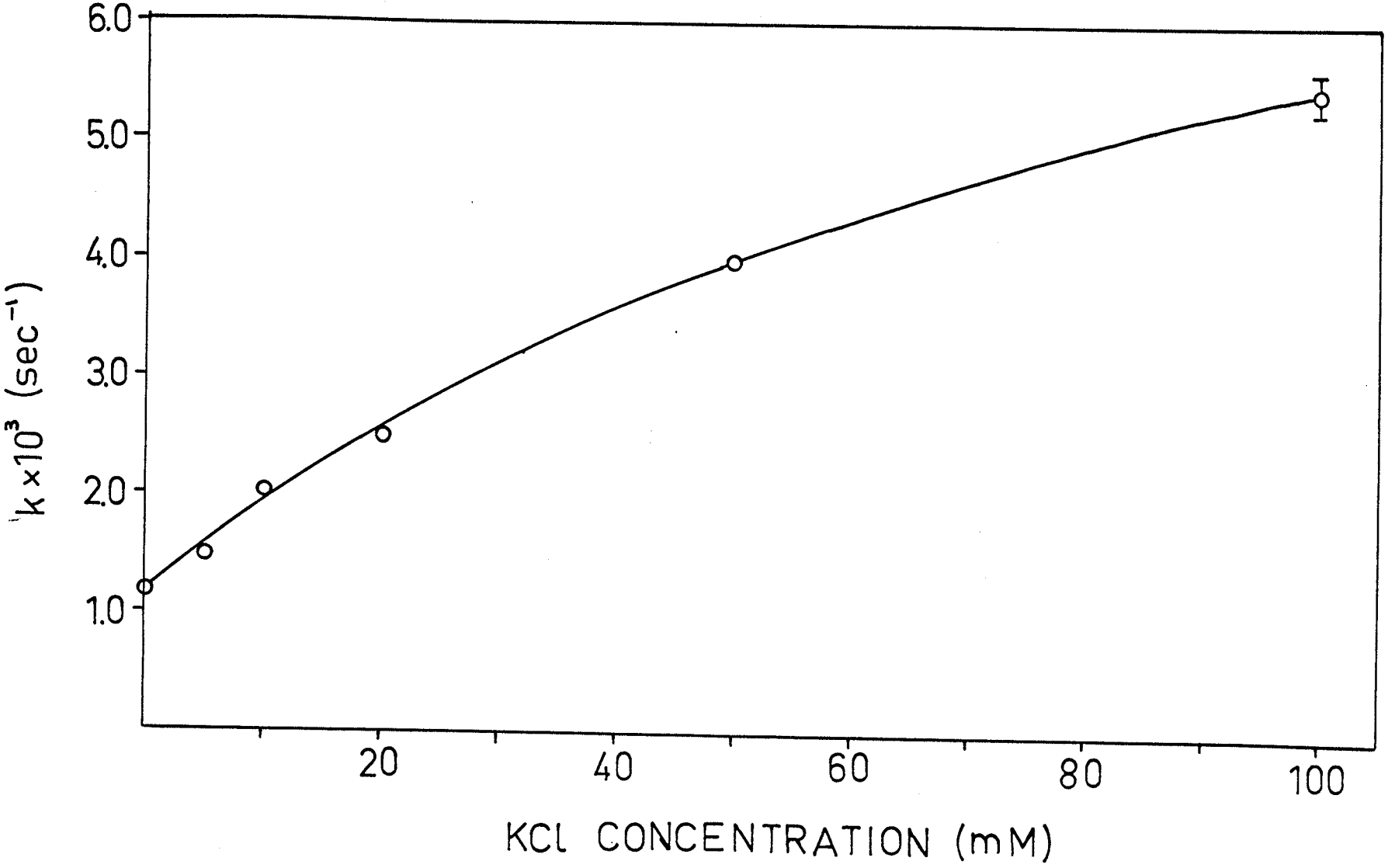
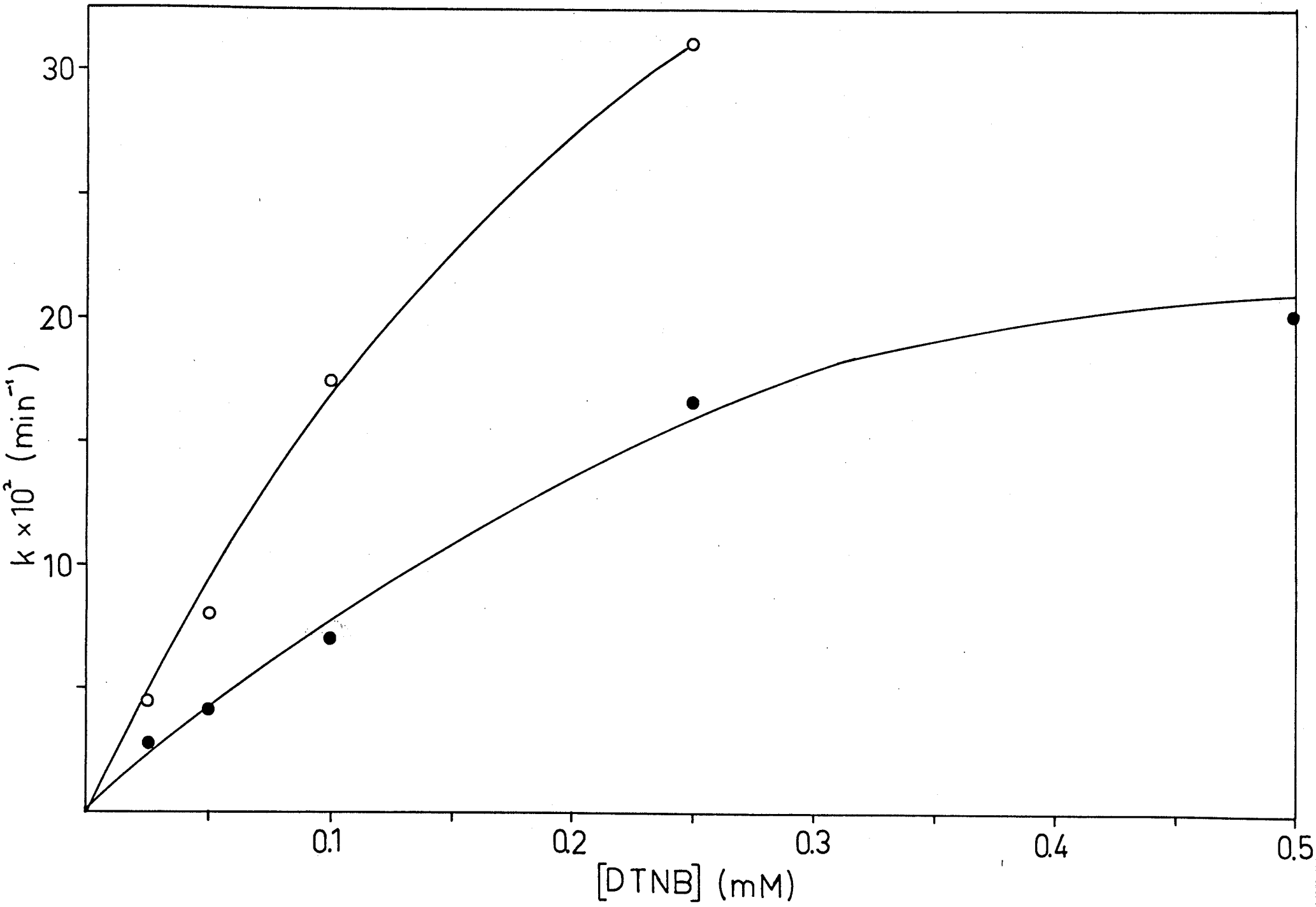

6.0
5.0
4.0
3.0
2.0
1.0
$k \times 10^3$ (sec^{-1})
20
40
60
80
100
KCl CONCENTRATION (mM)

Fig. 4: Rate of reaction of citrate synthase with DTNB as a function of DTNB concentration in the absence and presence of KCl.

(O-O) 100 mM KCl (●-●) no KCl.


30
20
10
k x10² (min⁻¹)
0.1
0.2
0.3
0.4
0.5
[DTNB] (mM)

modification reaction is saturable with DTNB, suggesting that the reagent first binds to the enzyme, then reacts with a susceptible sulfhydryl group. Analysis of the two curves with a hyperbolic curve fitting program demonstrates that the presence of KCl causes a dramatic increase in the V_{max} for the reaction, and a slight increase in K_m. In the absence of KCl, the K_m is 0.31 $\pm$ .08 mM, while in the presence of 100 mM KCl, it rises to 0.40 $\pm$ .12 mM. The maximal velocity of the reaction, however, is increased from 0.34 $\pm$ .05 min^{-1} to 0.82 $\pm$ .18 min^{-1} when KCl is present.

These data, along with the effect of KCl on the modification of the initial cysteine of citrate synthase (Figure 2), strongly suggest that KCl is causing the enzyme to undergo considerable conformational changes, such that the reactive cysteines in the molecule are becoming more susceptible to DTNB modification.

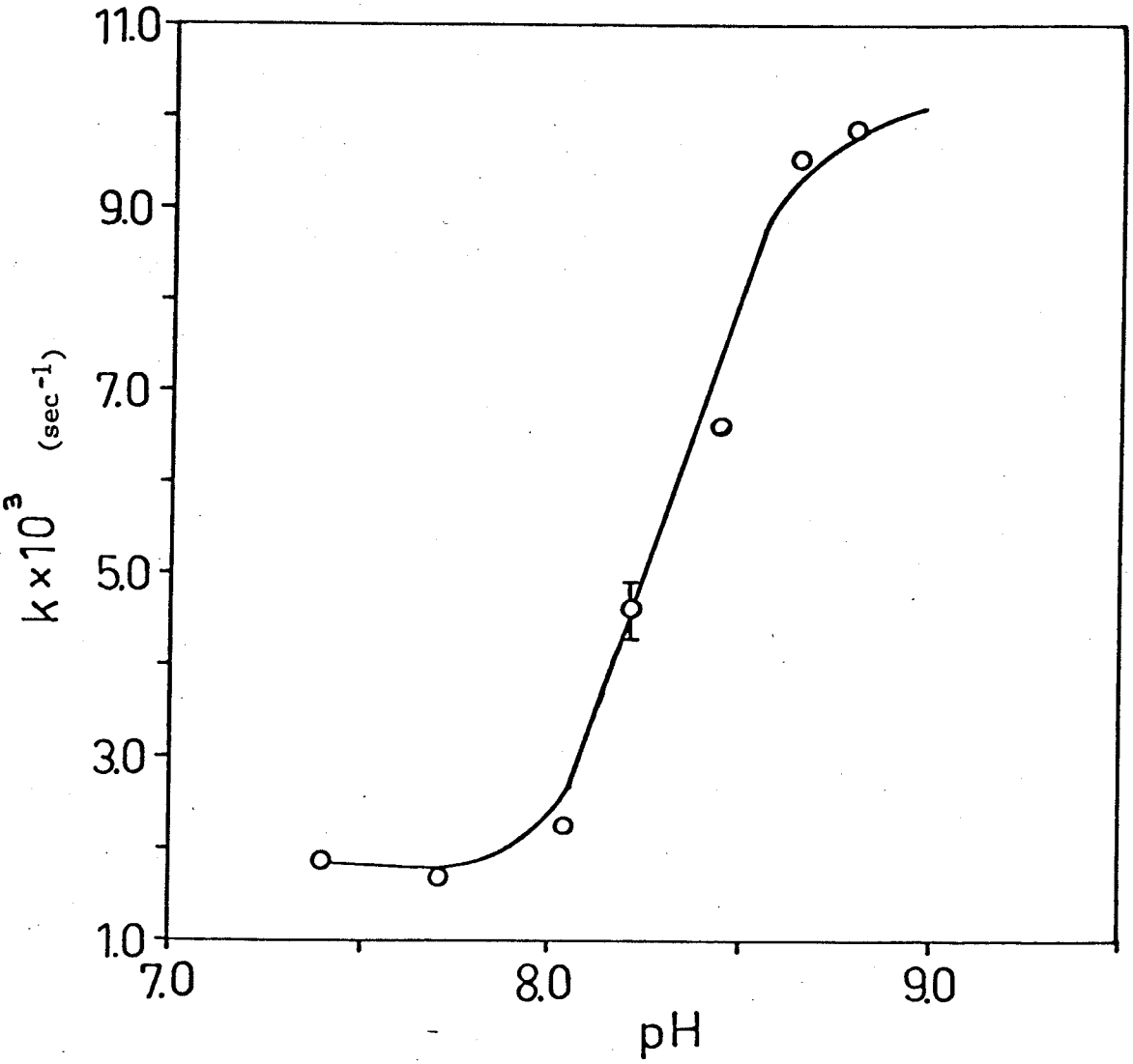
The pH dependence of the DTNB reaction in the presence of KCl is shown in Figure 5. As expected, the titration curve indicates that the reaction rate seems to depend on the basic form of a sulfhydryl group, with an apparent pKa of 8.3 $\pm$ .2. Webb (1966) has suggested that a sulfhydryl group near a positively charged group will exhibit a pKa of 7.2 to 8.5.

ii) Effect of substrates and NADH

The effect of the two substrates and allosteric inhibitor NADH upon the reaction of DTNB with the initially reacting sulfhydryl group of citrate synthase was studied. A particular difficulty arose in the study involving ACoA, in that this compound as received from the manufacturer

Fig. 5: Rate of reaction of citrate synthase with 0.1 mM DTNB in the presence of 100 mM KCl as a function of pH.

The buffer used to obtain the noted pH was .02 M Tris-Cl in all cases.


11.0
9.0
7.0
5.0
3.0
1.0
$k \times 10^3$ (sec^{-1})
7.0
8.0
9.0
pH

contains a significant contaminant which is very reactive with DTNB. This is probably CoASH (with a free SH) or, less probably, the disulfide CoAS-SCoA. The preparation was purified by passing it through a short column of Thiopropyl - Sepharose 6B, but still 2.5% of the total ACoA reacted almost instantly with DTNB. Consequently, the curve presented in Figure 6 for ACoA represents the difference between the curve generated by the protein in the presence of ACoA and the curve generated by an ACoA blank.

As Figure 6 illustrates, the substrate ACoA and inhibitor NADH have a profound effect on the DTNB reactivity of the enzyme, while the other substrate OAA has little or no effect. Qualitatively it can be suggested that ACoA and NADH block the modification of the first or rapidly reacting sulfhydryl, but that secondary modification proceeds at essentially the same rate as the control.

iii) Effect of DTNB modification on kinetics of citrate synthase

In order to investigate the effect of DTNB modification on the functional characteristics of citrate synthase, the KCl and ACoA saturation curves were determined for enzyme modified to the extent of 1 group/subunit. Figure 7a, represents the results with KCl and Figure 7b the results with ACoA.

Table 1 represents a comparison of kinetic parameters of the modified enzyme with those of the unmodified enzyme (H. W. Duckworth, unpublished data). It is important to note that the modification causes a change in the allosteric properties of the enzyme, as suggested by the lowering of the Hill coefficients for both ACoA and KCl, as well as the

Fig. 6: Reaction of citrate synthase with DTNB in the presence of substrates and NADH.

The control reaction (O - O) represents reaction of enzyme at a concentration of 17.1 μM with 0.1 mM DTNB.

The reaction in the presence of OAA (● - ●) was done at a OAA concentration of .1 mM, with a enzyme concentration of 6.3 μM and a DTNB concentration of 0.1 mM.

The reaction in the presence of ACoA (⊘ - ⊘) has been corrected for reaction due to an impurity in the acetyl-CoA, as explained in the text. The enzyme (91 μM) was incubated with 0.20 mM DTNB in standard buffer containing 1.50 mM acetyl-CoA. The blank reaction depleted the concentration of DTNB from 0.2 mM to 0.162 mM, so the corresponding progress curve for citrate synthase in the absence of ACoA for this experiment should be slightly greater than the control curve shown.

The reaction in the presence of NADH (⊘ - ⊘) was done at a NADH concentration of 40 μM, using an enzyme concentration of 17 μM and 0.1 mM DTNB.

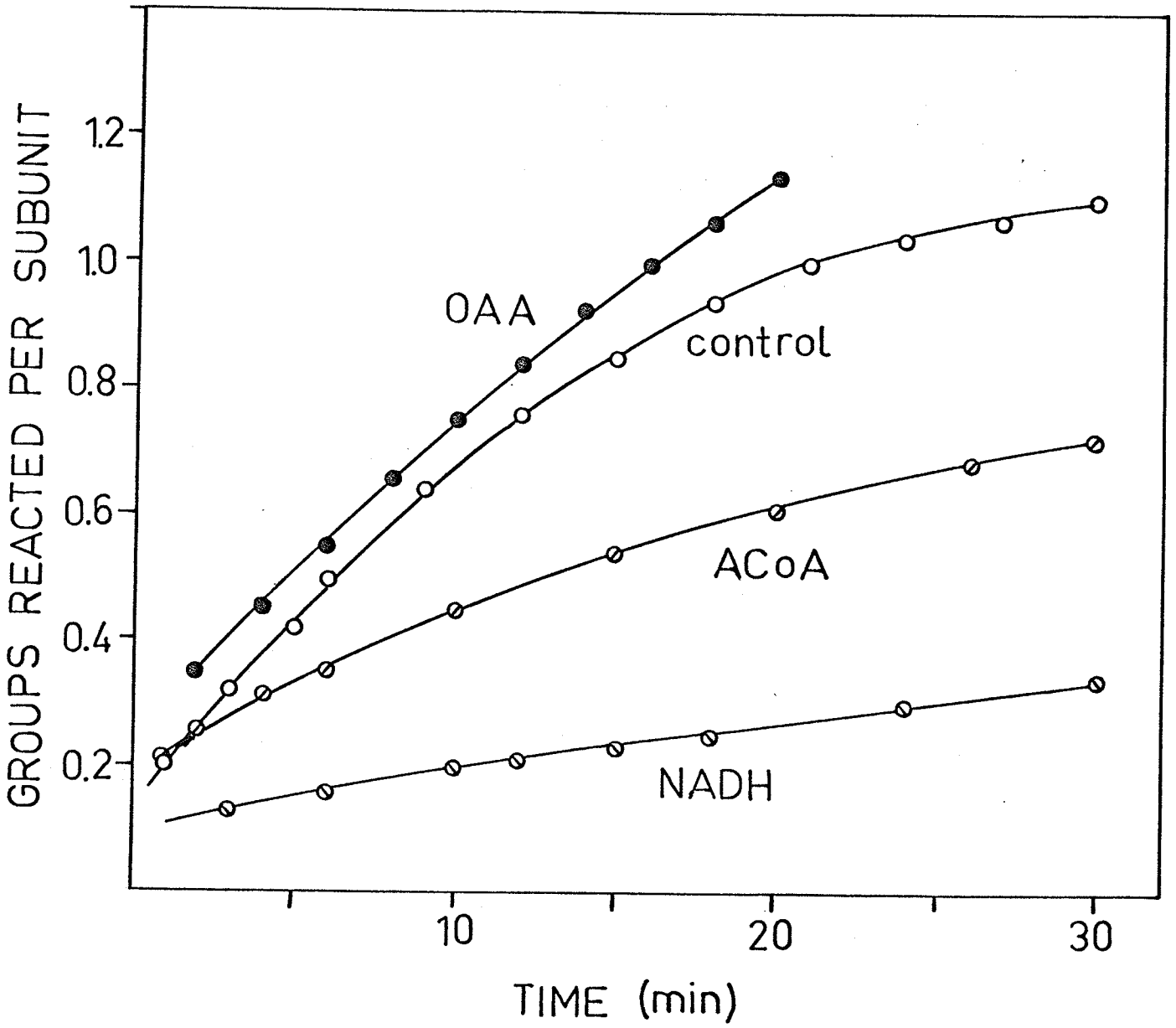

GROUPS REACTED PER SUBUNIT
1.2
1.0
0.8
0.6
0.4
0.2
OAA
control
ACoA
NADH
10
20
30
TIME (min)

Fig. 7a: The KCl saturation characteristics of citrate synthase modified to the extent of 1 group per subunit by DTNB.

In the Lineweaver-Burk plot, Δvel refers to the observed velocity at a particular KCl concentration minus the velocity of the reaction in the absence of KCl. In the Hill plot, $\checkmark$ refers to the observed velocity minus the velocity at 0 KCl over the maximal velocity minus the observed velocity minus the velocity at 0 KCl.

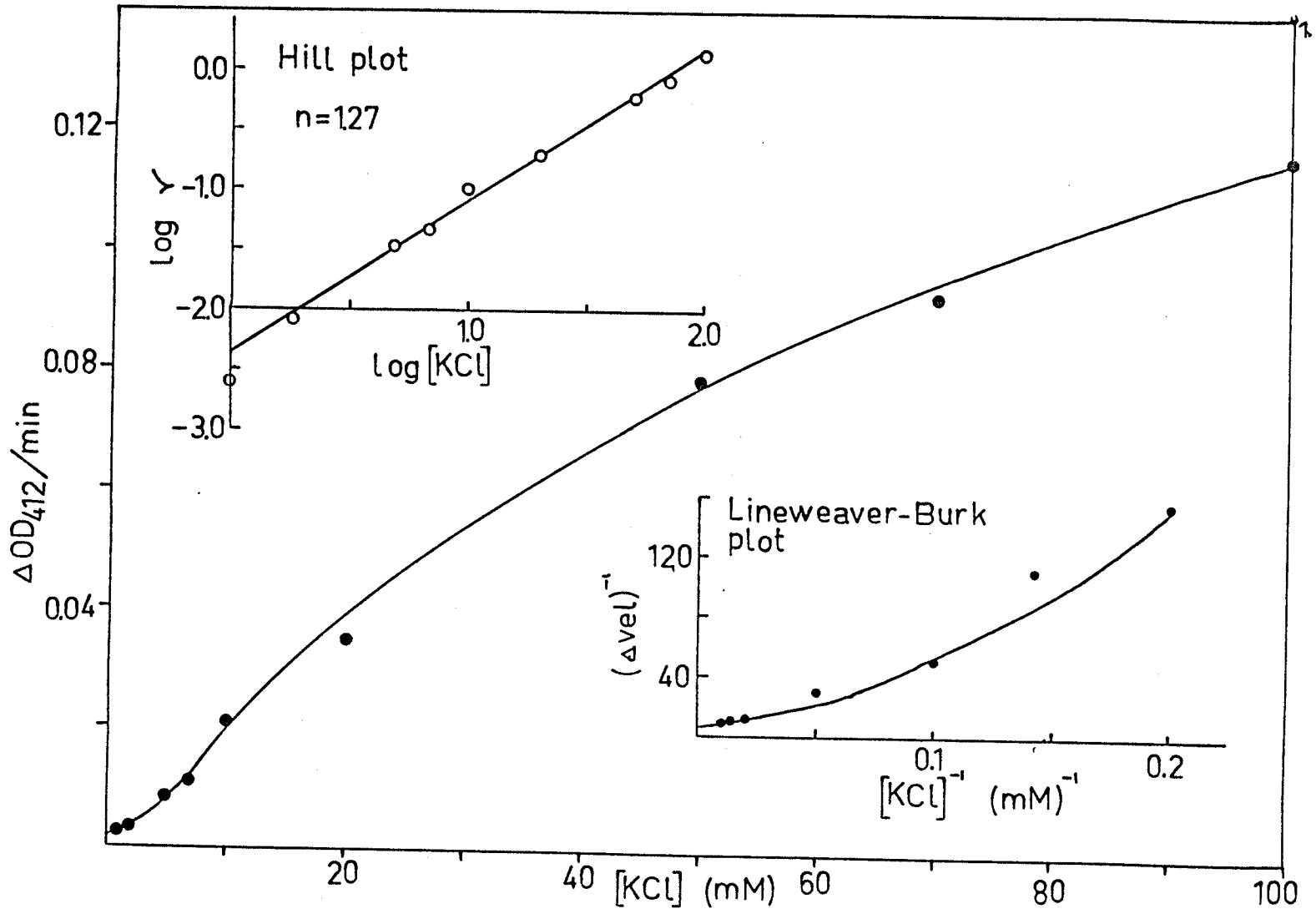

Hill plot
n=1.27
Log Y
0.0
-1.0
-2.0
-3.0
1.0
2.0
log [KCl]
Lineweaver-Burk plot
(Δvel)⁻¹
120
40
0.1
0.2
[KCl]⁻¹ (mM)⁻¹
ΔOD412/min
0.12
0.08
0.04
20
40
[KCl] (mM)
60
80
100

Fig. 7b: The ACoA saturation characteristics of citrate synthase modified to the extent of 1 group per subunit by DTNB.

In the Hill plot, ν refers to the observed velocity at a particular concentration of ACoA over the maximal velocity for the reaction minus the observed velocity.

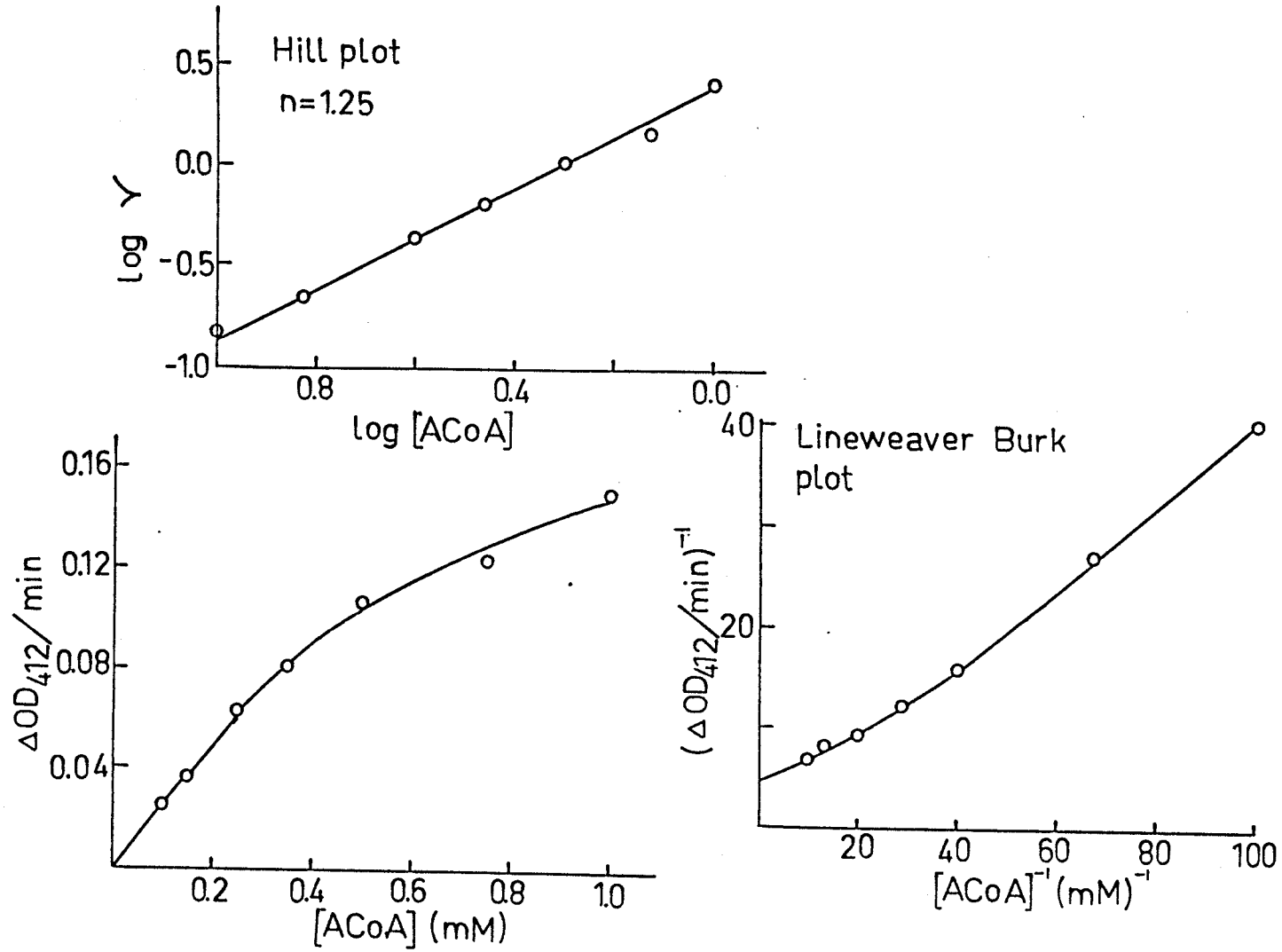

Hill plot
n=1.25
Log Y
0.5
0.0
-0.5
-1.0
0.8
0.4
0.0
log [ACoA]
ΔOD412/min
0.16
0.12
0.08
0.04
0.2
0.4
0.6
0.8
1.0
[ACoA] (mM)
Lineweaver Burk
plot
(ΔOD412/min)⁻¹
40
20
20
40
60
80
100
[ACoA]⁻¹ (mM)⁻¹

Table 1: Kinetic Parameters for DTNB-Modified Citrate Synthase

	acetyl-CoA		KCl		$\frac{V_{0.1\ M\ KCl}}{V_{control}}$
	$S_{0.5}$	n^a	$A_{0.5}$	n^a	
unmodified	1.2 mM	1.9	30 mM	1.6	90 ± 10
modified	0.48 mM	1.25	77 mM	1.27	58

[a]Calculated according to Hill (1910).

In all measurements, OAA was at 0.10 mM. No KCl was included in measurements of the acetyl-CoA saturation curve. In measurements of activation by KCl, acetyl-CoA was at 0.10 mM.

change in affinities of the enzyme for these compounds. A possible mode of action to explain the effects of modification could be a shift in the allosteric equilibrium. This is considered in more detail in the Discussion.

iv) Effect of DTNB modification on NADH inhibition of citrate synthase

Figure 8 represents my studies on the functional characteristics of citrate synthase upon modification with DTNB. Initially, before modification, the enzyme is inhibited approximately 80% by 0.1 mM NADH. After addition of DTNB to achieve a final concentration of 0.1 mM, the modification reaction proceeds. It is clear that as the reaction approaches 1 thiol group/subunit modified, the enzyme looses considerable NADH sensitivity. In this case, there is no loss of standard activity, i.e. activity in the absence of allosteric effectors. Further thiol groups will continue to react, lowering enzyme activity overall.

Figure 9 represents another experiment, investigating the possibility of reversing the DTNB modification by incubating the modified enzyme with DTT. Initially we have modification of approximately 1 group/subunit, with a concomitant large inactivation of the enzyme, as well as loss of NADH sensitivity. Whether or not standard activity loss is seen during modification seems to depend on the particular enzyme preparation used. In the previous experiment, a somewhat aged enzyme preparation with a lower specific activity was used, and here a fresh batch was the subject of investigation. It is possible to conceive of a very slow transition from a vulnerable, DTNB-sensitive, high activity form of the enzyme, to a lower activity form in which the enzyme is more resistant to DTNB

Fig. 8: Effect of modification of citrate synthase with 0.1 mM DTNB on NADH inhibition.

Standard activity is activity of enzyme in absence of any effectors. NADH activity is activity in presence of 0.1 mM NADH.

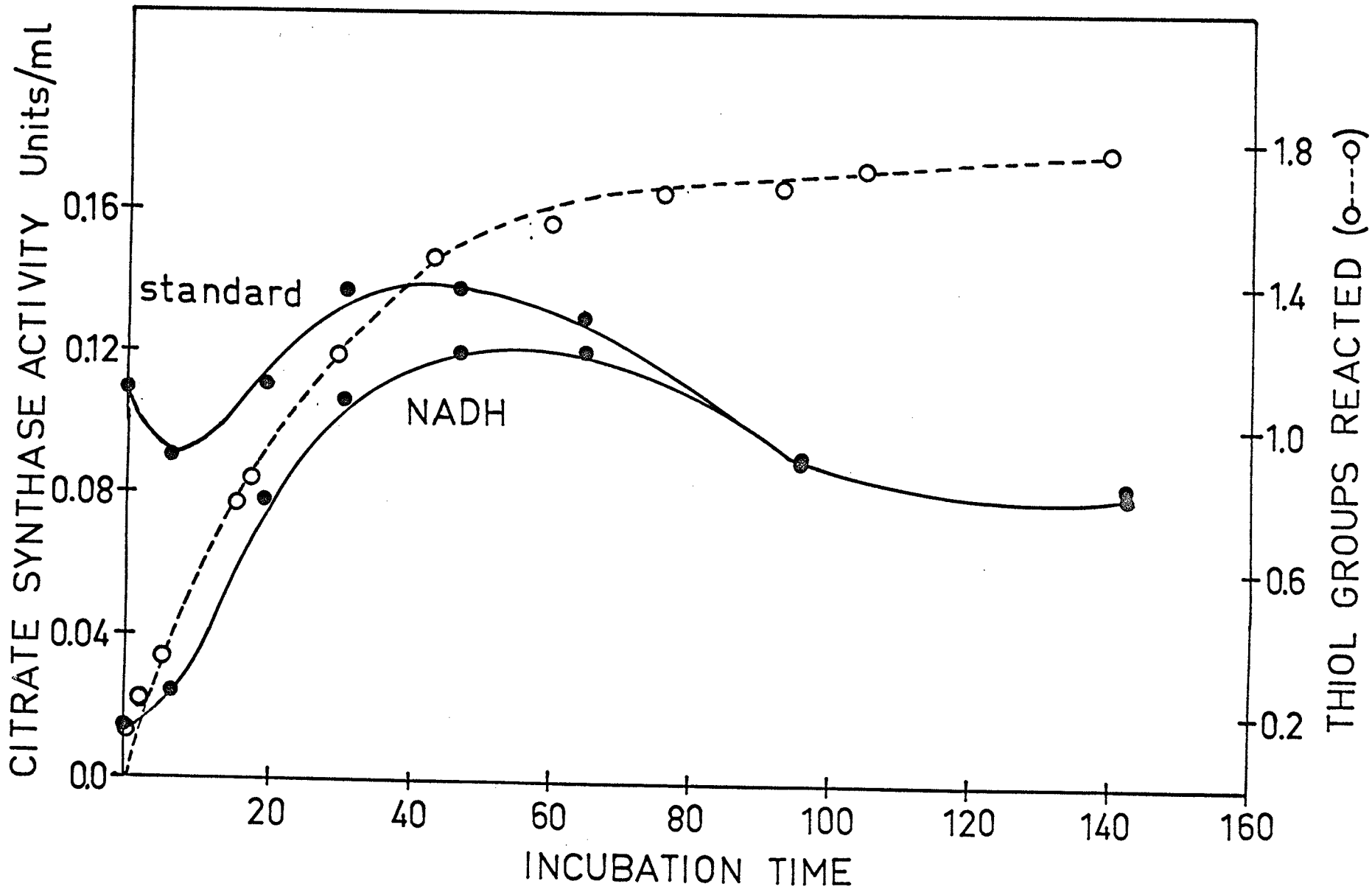
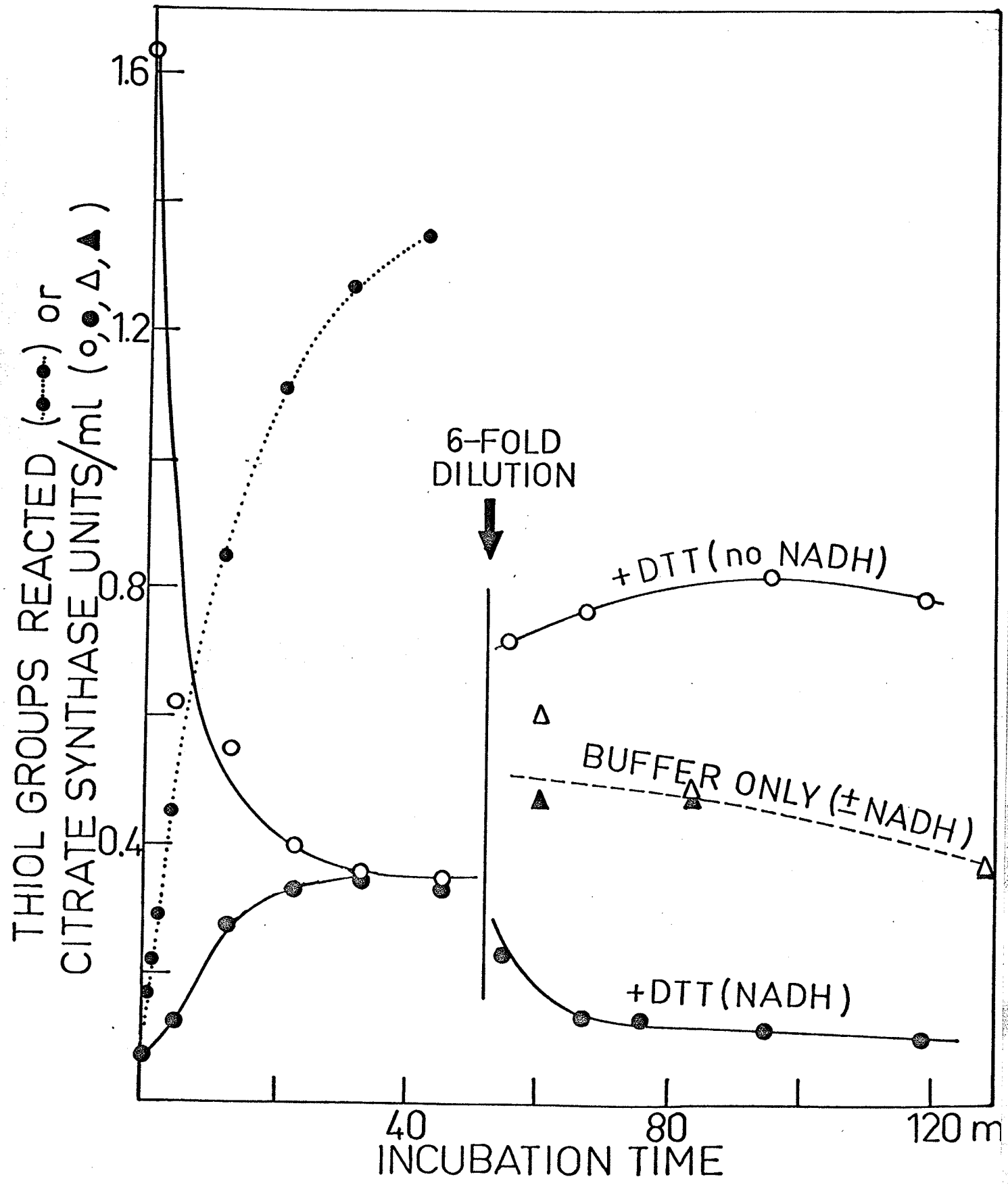

CITRATE SYNTHASE ACTIVITY Units/ml
0.0
0.04
0.08
0.12
0.16
standard
NADH
20
40
60
80
100
120
140
160
INCUBATION TIME
THIOL GROUPS REACTED (o---o)
0.2
0.6
1.0
1.4
1.8

Fig. 9: Effect of DTT on activity of DTNB-modified citrate synthase.

Enzyme (24.6 μM) was incubated with 0.10 mM DTNB and assayed (without KCl) (○-○) and in the presence of 0.1 mM NADH (●-●). The course of the DTNB reaction was monitored at 412 nm (●...●). After 48 mins, samples were diluted into buffer (triangles) or buffer containing 0.1 mM dithiothreitol. Assays with (●, ▲) and without (○, △) NADH were carried out on these diluted samples.


1.6
1.2
0.8
0.4
THIOL GROUPS REACTED (●---●) or
CITRATE SYNTHASE UNITS/ml (○,●,△,▲)
6-FOLD
DILUTION
+DTT (no NADH)
BUFFER ONLY (±NADH)
+DTT (NADH)
40
80
120 m
INCUBATION TIME

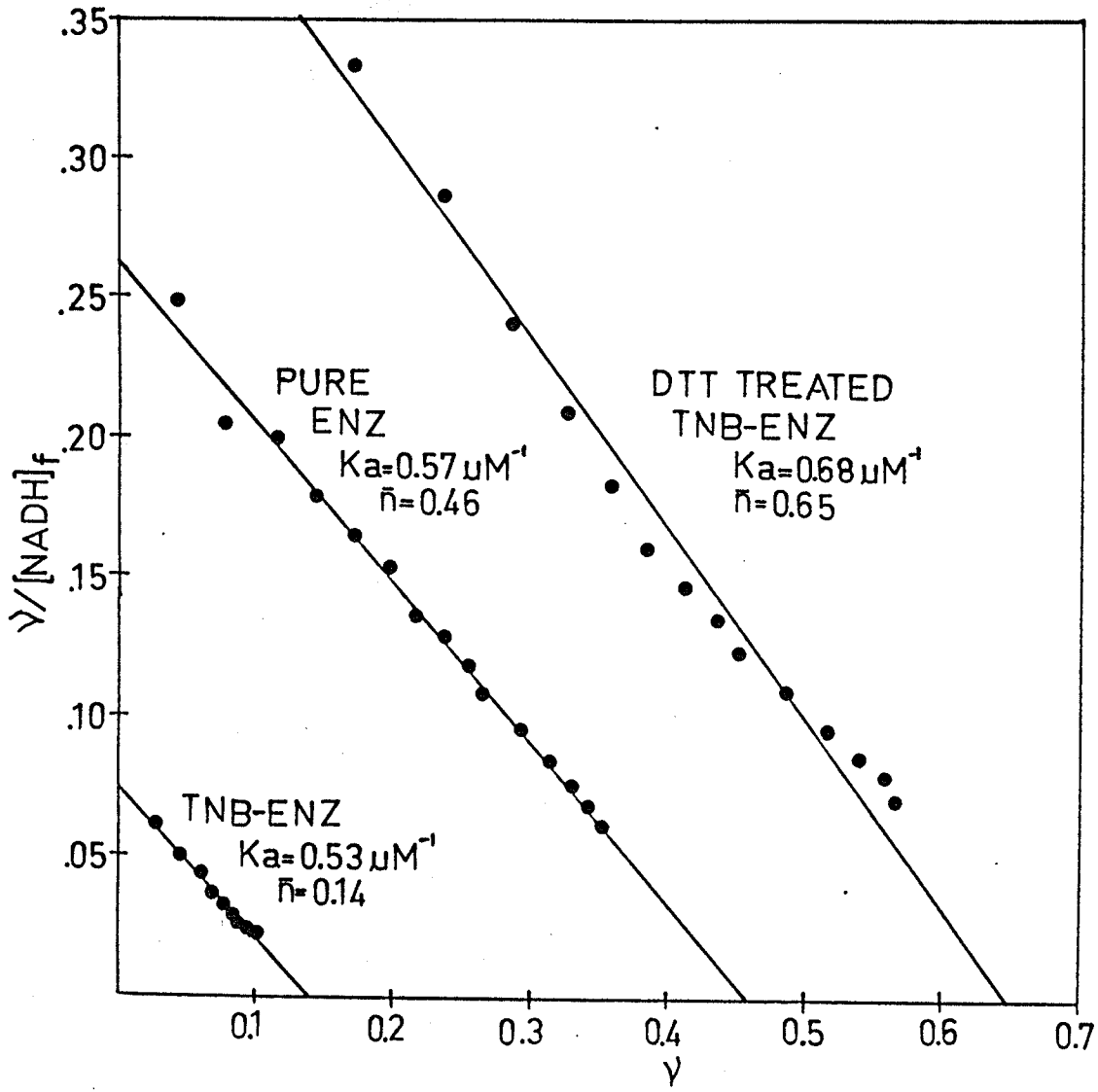
inactivation. In all cases, however, NADH sensitivity is lost as 1 group/subunit is modified with DTNB. After modification we have diluted equal aliquots of the reaction mixture into buffer containing dithiothreitol as well as buffer alone. The results are clear cut. Upon exposure to DTT, the DTNB-modified enzyme rapidly recovers activity as well as its sensitivity to NADH inhibition. The enzyme in buffer only, serving as the control in this experiment, continues to exhibit approximately the same activities and insensitivity to NADH as an inhibitor. Thus the functional characteristics of the enzyme have been largely regenerated by the reversal of the DTNB modification.

v) Effect of DTNB modification on NADH binding

Duckworth and Tong (1976) have described an elegant method for measuring NADH binding to citrate synthase based on the enhancement of NADH fluorescence when this nucleotide is bound to the enzyme. Figure 10 represents Scatchard plots for the binding of NADH to pure enzyme, to enzyme modified by DTNB to the extent of 1 group per subunit, and to modified enzyme that has been treated with DTT. The values quoted for pure enzyme are in good agreement with those found by Duckworth and Tong (1976). It is evident that the modified enzyme binds NADH with approximately the same association constant as pure unmodified enzyme, but with far fewer sites. The amount of NADH binding that remains after modification of 1 group per subunit varies slightly in different experiments but it is always small. The modified enzyme treated with DTT, on the other hand, shows almost the same affinity for NADH but more sites per subunit. The increase in the number of sites over that of the pure enzyme

Fig. 10: NADH binding to citrate synthase, citrate synthase modified to the extent of 1 group per subunit by DTNB and modified enzyme treated with DTT.

The data are presented in the form of a Scatchard plot, where the x-intercept represents the number of binding sites for the molecule and the negative slope of the line represents the association constant. In this figure, ν represents the concentration of NADH bound over the total concentration of enzyme in solution.


.35
.30
.25
.20
.15
.10
.05
ν/[NADH]f
PURE
ENZ
Ka=0.57 μM⁻¹
n̄=0.46
DTT TREATED
TNB-ENZ
Ka=0.68 μM⁻¹
n̄=0.65
TNB-ENZ
Ka=0.53 μM⁻¹
n̄=0.14
0.1
0.2
0.3
0.4
0.5
0.6
0.7
ν

may represent a reduction of partially oxidized groups,present from the initial isolation of the enzyme, or difficulties in controlling precisely the pH of the protein solution, since the number of NADH sites seen is dependent on the pH of the environment (Duckworth and Tong, 1976). The conclusion from this work, however, is that the loss of the ability of the enzyme to bind NADH is directly responsible for the loss of NADH inhibition during modification.

II) Characteristics of 4,4'PDS Reaction with Citrate Synthase

i) Reaction with 4,4'PDS

Figure 11 represents the reaction of my preparation of E. coli citrate synthase with 0.05 mM 4,4'PDS in the presence and absence of KCl. In the absence of KCl, the reagent reacts with approximately 2.0 groups/subunit over a period of 40 minutes with an apparent pseudo first order rate constant of 0.086 $\pm$ .005 min^{-1}. The presence of 100 mM KCl appears to increase the rate of reaction of the initial group reacting (k = 0.12 $\pm$ .01) while decreasing the rate of reaction of the second group (k = 0.045 $\pm$ .005) and causing an overall decreased extent of reaction.

ii) Effect of NADH on modification reaction

The modification of citrate synthase by 4,4'PDS has been investigated in the presence of 32 μM NADH, a concentration 20 times the dissociation constant for this molecule (Duckworth and Tong 1976). Figure 12 represents these results. It is evident that NADH inhibits the reaction to some extent, yet it does not affect the overall extent of reaction. Since NADH binds to the enzyme in a reversible manner, eventually all reactive

Fig. 11: Modification of E. coli citrate synthase by 0.05 mM 4,4'PDS in the presence and absence of 100 mM KCl.

In this figure (●-●) represents the reaction in the presence of 100 mM KCl and (○-○) the reaction in its absence.

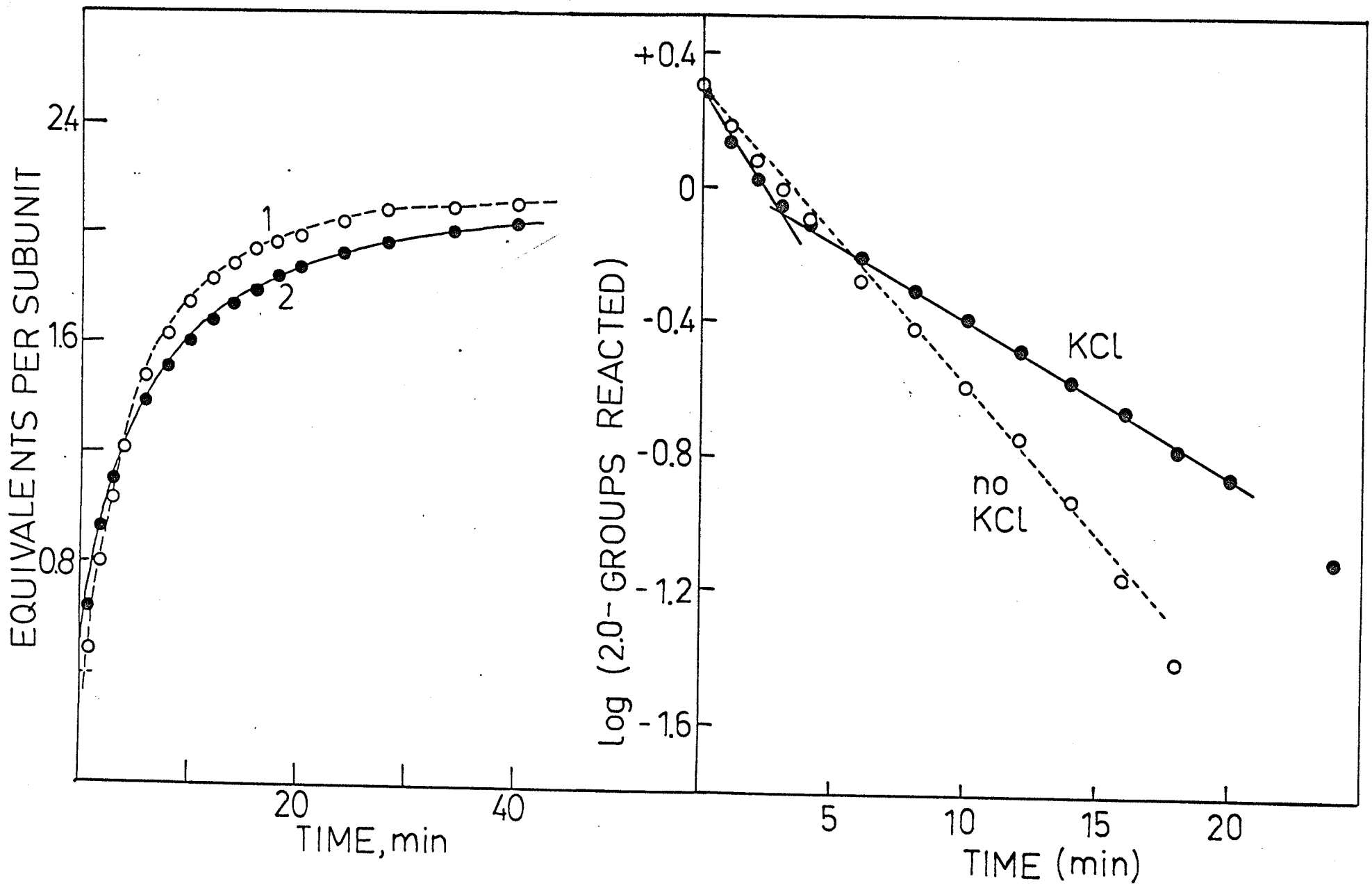
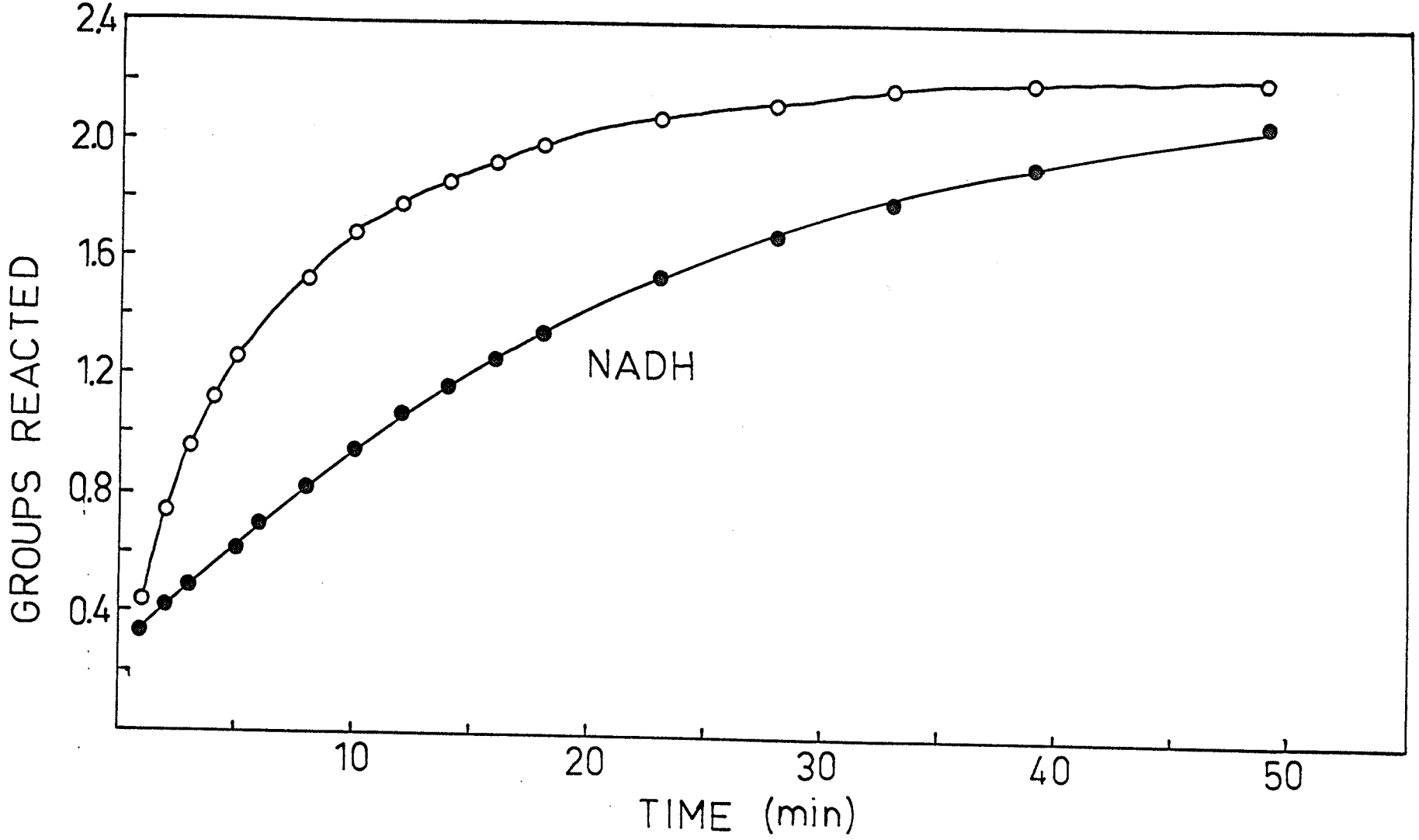

EQUIVALENTS PER SUBUNIT
2.4
1.6
0.8
1
2
20
40
TIME, min
Log (2.0- GROUPS REACTED)
+0.4
0
-0.4
-0.8
-1.2
-1.6
KCl
no KCl
5
10
15
20
TIME (min)

Fig. 12: Reaction of citrate synthase with 0.05 mM 4,4'PDS in the presence of 32 μM NADH.

In this figure, (○-○) represents the reaction in the absence of NADH, and (●-●) in its presence.


2.4
2.0
1.6
1.2
0.8
0.4
GROUPS REACTED
NADH
10
20
30
40
50
TIME (min)

sulfhydryls will react with the reagent in the presence of NADH, only more slowly because of the steric blocking effect of the NADH.

iii) Effect of modification on NADH binding

The NADH binding properties of enzyme modified to the extent of 1.0 ± 0.2 groups/subunit by 4,4'PDS have been investigated and the results are shown in Table 2.

It is evident that modification of the enzyme to the total extent of 1.0 groups/subunit results in loss of NADH binding sites, as well as a greater Kd for the sites remaining. This parallels the finding of the loss of NADH binding sites for the DTNB modification.

iv) Effect of modification on enzyme activity

Wright and Sanwal's (1971) observation that 4,4'PDS causes a rapid inactivation of the enzyme has been confirmed in my investigation. Figure 13 represents the effect on activity of citrate synthase of modification with 0.1 mM 4,4'PDS.

v) Effect of KCl and NADH on rate of inactivation by 4,4'PDS

Further studies on the inactivation of citrate synthase by 4, 4'PDS have been carried out using 0.01 mM reagent concentration, in order to slow down the reaction to the point that a large number of points could be taken.

Figure 14 represents the rate of inactivation of the enzyme by 4,4'PDS in the absence and presence of 0.1 M KCl. As is evident from the

Table 2: NADH-Binding Parameters for 4,4'PDS-Modified Citrate Synthase

	Kd (μM)	number of sites
unmodified	0.78 ± .04	0.58 ± .01
modified	1.57 ± .04	0.065 ± .005

Fig. 13: The effect of modification of E. coli citrate synthase by 0.1 mM 4,4'PDS on enzyme activity.

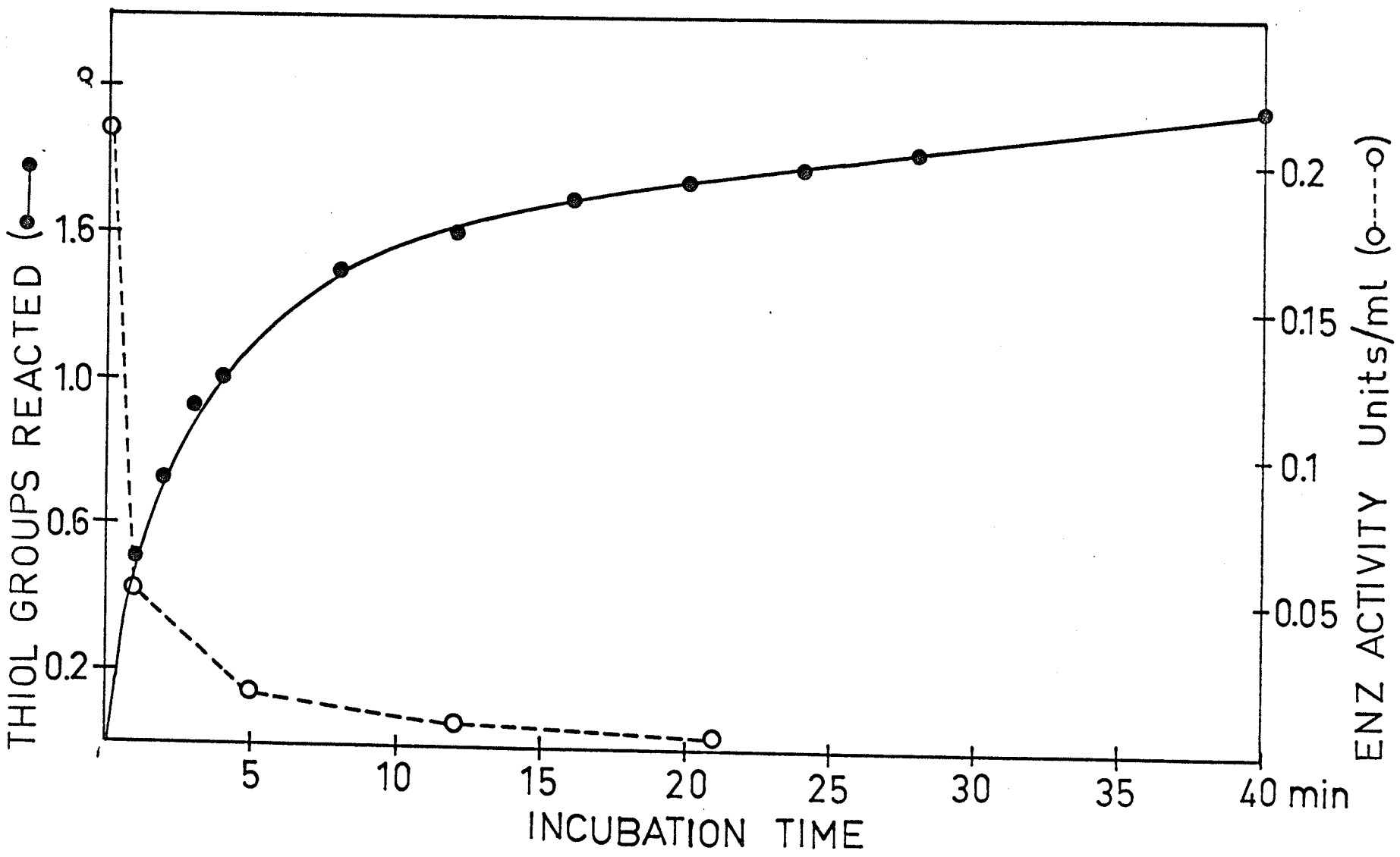
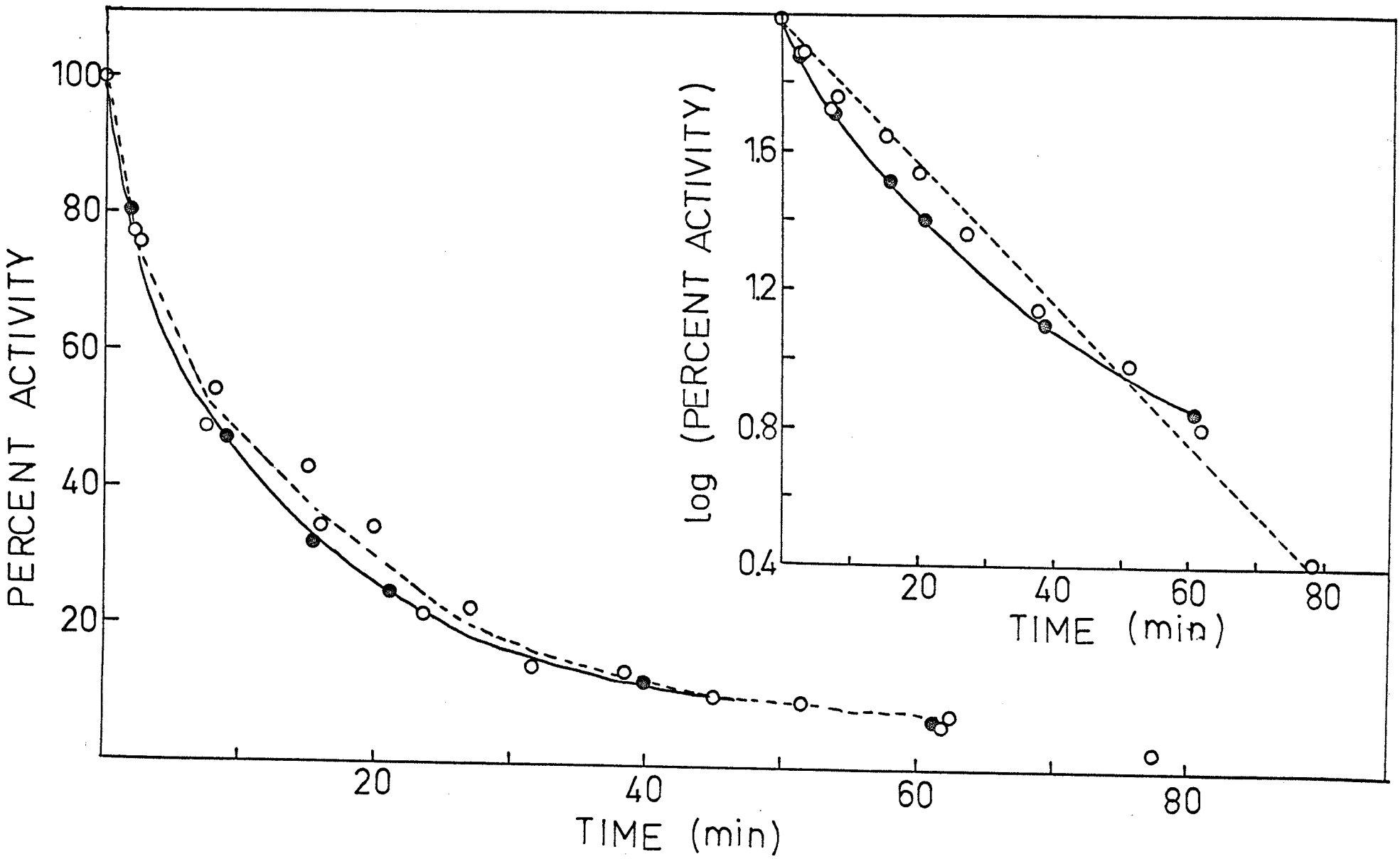

THIOL GROUPS REACTED (●—●)
ENZ ACTIVITY Units/ml (o---o)
INCUBATION TIME
0
1.6
1.0
0.6
0.2
0.2
0.15
0.1
0.05
5
10
15
20
25
30
35
40 min

Fig. 14: Inactivation of citrate synthase by 0.01 mM 4,4'PDS in the presence and absence of 100 mM KCl.

In this figure, (O-O) represents the activity of the enzyme upon incubation with 4,4'PDS and (●-●) the activity upon incubation with 100 mM KCl and 4,4'PDS.


PERCENT ACTIVITY
100
80
60
40
20
20
40
60
80
TIME (min)
Log (PERCENT ACTIVITY)
1.6
1.2
0.8
0.4
20
40
60
80
TIME (min)

first order plots, KCl enhances the rate of inactivation slightly.

The rate of inactivation of the enzyme was also measured in the presence of NADH. Figure 15 represents the results at two different concentrations of NADH. The inactivation of citrate synthase by 4,4'PDS is definitely inhibited by the presence of NADH. The higher concentration of NADH may protect the enzyme to a greater extent, although the rate of inactivation is almost identical to that of the lower concentration.

vi) Effect of DTT on inactivation of citrate synthase by 4,4'PDS

The effect of DTT on the inactivation of citrate synthase by 4,4'PDS has also been studied, and the results are presented in Figure 16.

Addition of a 5-fold molar excess of DTT quickly causes the enzyme to regain approximately 60% of its original activity. Further addition of DTT does not result in any further regeneration of enzyme activity.

III) Reciprocal Blocking Experiments

With a view to obtaining some information on the sulfhydryl groups that react with DTNB and 4,4'PDS respectively, reciprocal blocking experiments have been carried out as follows.

A protein sample was reacted to the extent of 0.95 $\pm$ .05 groups/subunit in 0.1 M KCl with DTNB, then passed through a G-25 Sephadex column to stop the reaction. The isolated, modified protein was then allowed to react with 0.05 mM 4,4'PDS, as was a control of unmodified protein.

Fig. 15: Inactivation of citrate synthase by 0.01 mM 4,4'PDS in the presence and absence of NADH.

In this figure, (O-O) represents the activity of the enzyme upon incubation with 4,4'PDS, (●-●) the activity upon incubation with 4,4'PDS and 32 µM NADH, and (⊗-⊗) the activity of the enzyme upon incubation with 4,4'PDS and 160 µM NADH.

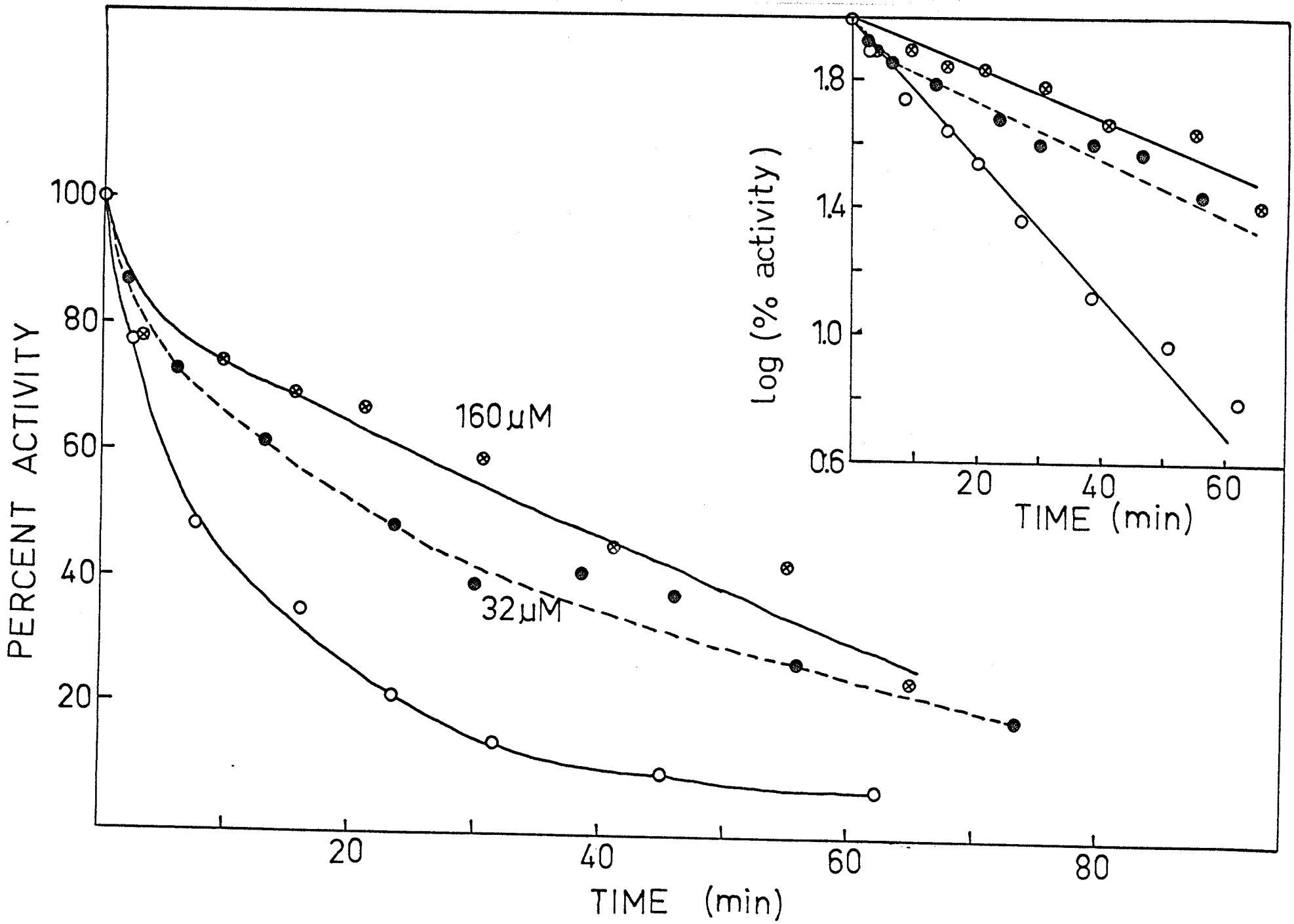
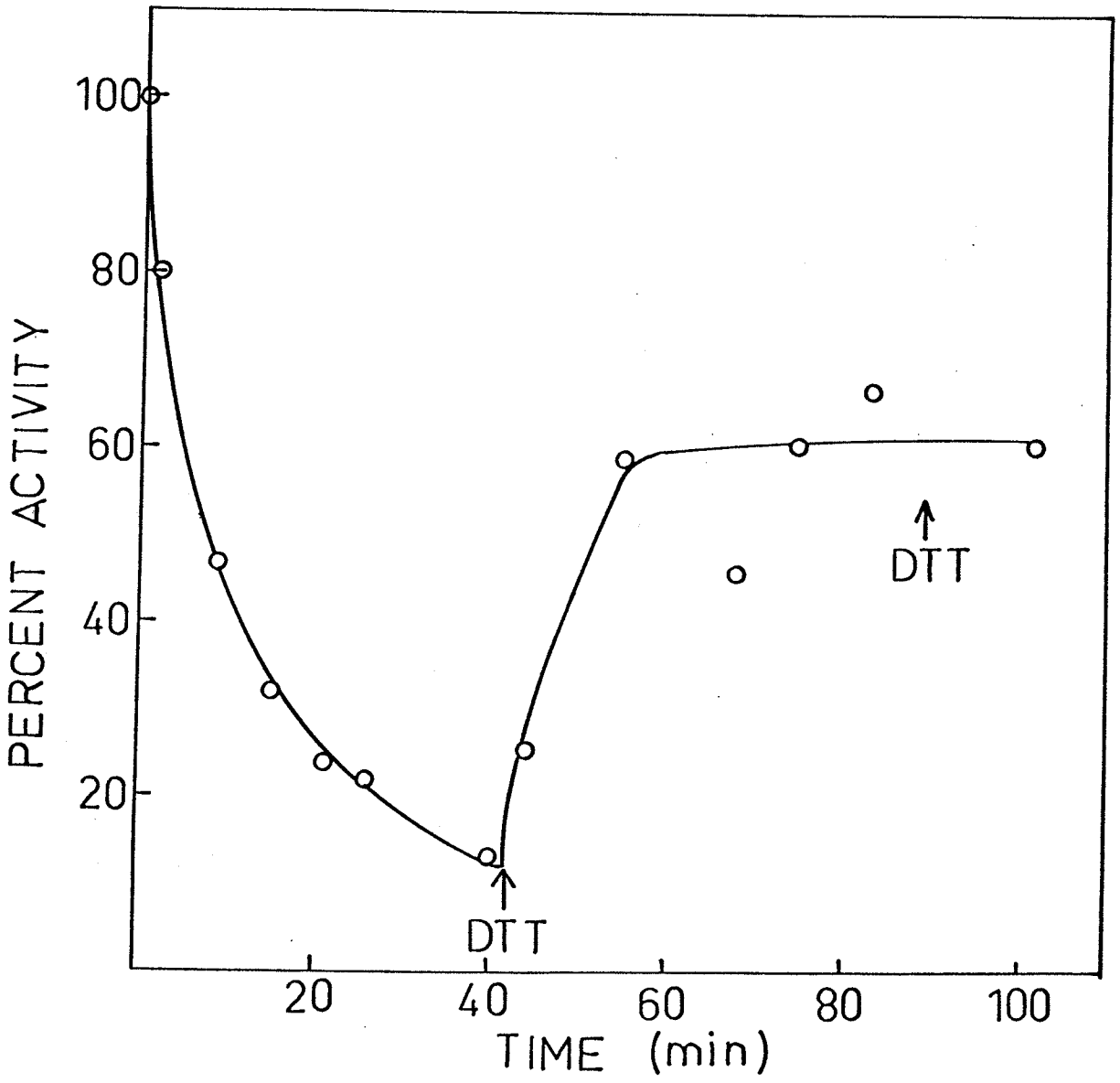

PERCENT ACTIVITY
100
80
60
40
20
160 µM
32 µM
20
40
60
80
TIME (min)
Log (% activity)
1.8
1.4
1.0
0.6
20
40
60
TIME (min)

Fig. 16: The effect of DTT on the inactivation of citrate synthase by 0.01 mM 4,4'PDS.

Enzyme was incubated with 0.01 mM 4,4'PDS and loss of activity monitered as before. At 42 minutes, DTT was added to a final concentration of 0.05 mM, and assays continued. At 89 minutes, another aliquot of DTT was added to achieve a final DTT concentration of 0.11 mM.


100
80
60
40
20
PERCENT ACTIVITY
DTT
DTT
20
40
60
80
100
TIME (min)

The results are displayed in Figure 17.

The protein modified by DTNB has lost approximately .80 groups/subunit that could react with 4,4'PDS. This suggests that DTNB reacts with one of the two sulfhydryls that are susceptible to modification by 4,4'PDS.

The reverse experiment was also carried out. A protein sample was reacted with slightly less than an equivalent amount of 4,4'PDS in 0.1 M KCl. This led to protein modified to the extent of 1.0 group/subunit, as diagrammed in Figure 18. The modified protein was isolated and subjected to modification by 0.1 mM DTNB, along with a control of pure unmodified protein. Figure 19 represents the results.

It is evident that at long times, the protein has lost approximately 0.9 groups/subunit that react with DTNB. This suggests that the first group that reacts with 4,4'PDS is the same group that reacts with DTNB.

IV) Effect of NADH Analogues on Enzyme Activity and Sulfhydryl Reactivity

With a view towards gaining a better understanding of the interaction of NADH with the enzyme, I have initiated a series of experiments designed to study the effect of NADH analogues on enzyme activity and modification by sulfhydryl reagents.

i) Adenosine diphosphate ribose

Figure 20 represents an enzyme activity saturation curve for

Fig. 17: Reaction of E. coli citrate synthase and citrate synthase modified to the extent of 0.95 groups per subunit with DTNB with 0.05 mM 4,4'PDS.

In this figure, curve 1 refers to the reaction of unmodified enzyme with 0.05 mM 4,4'PDS and curve 2 refer to the reaction of enzyme previously modified to the extent of 0.95 groups/ subunit by DTNB in 100 mM KCl with 4,4'PDS. Both reactions were done in the presence of 100 mM KCl.

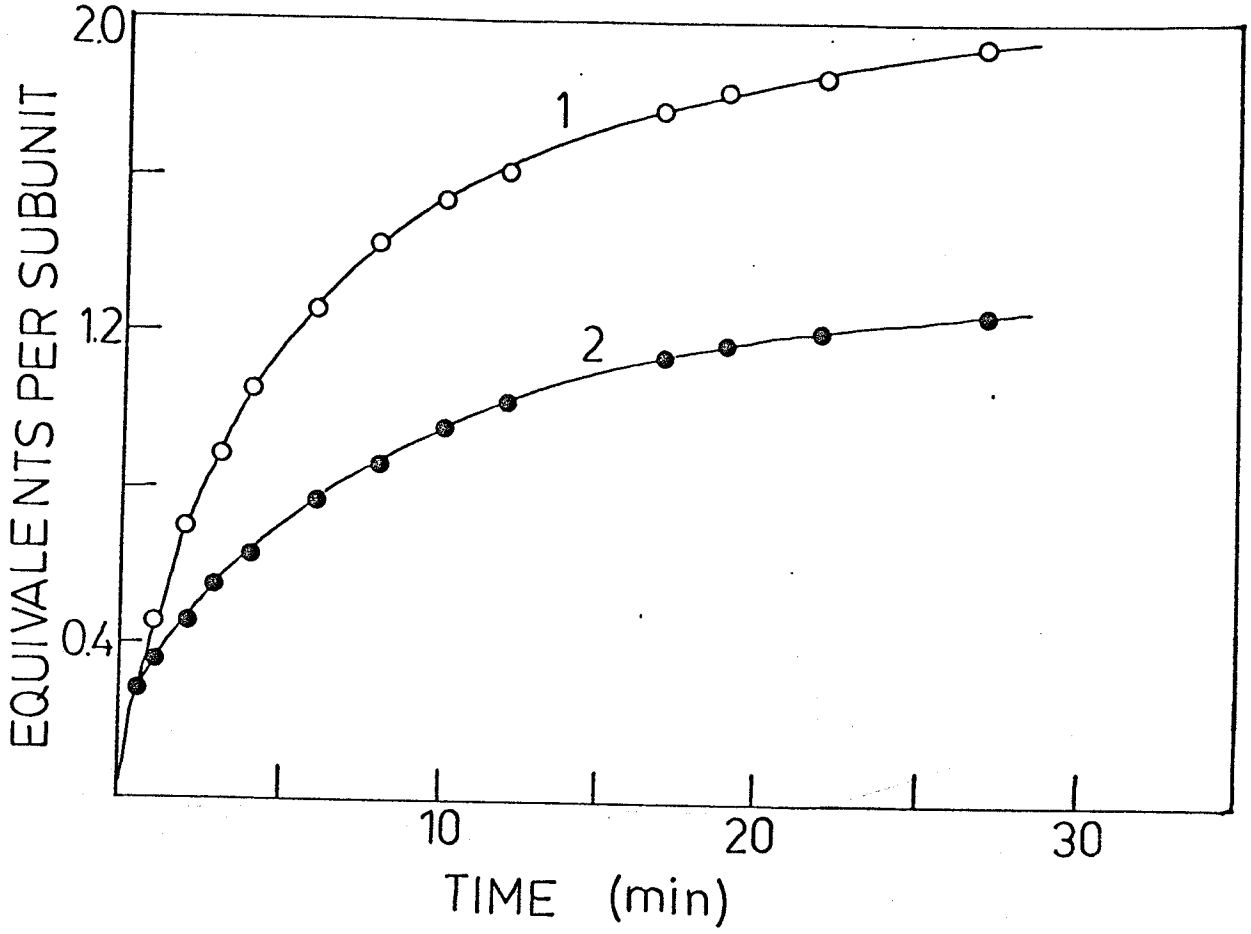
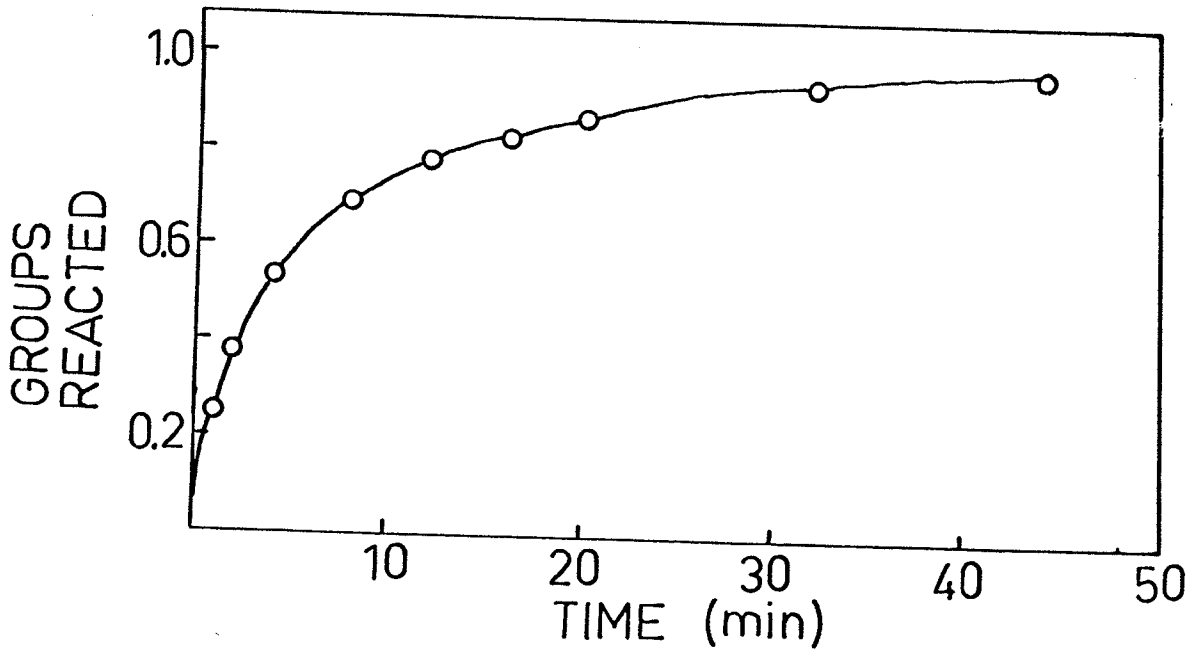

2.0
1.2
0.4
EQUIVALENTS PER SUBUNIT
1
2
10
20
30
TIME (min)

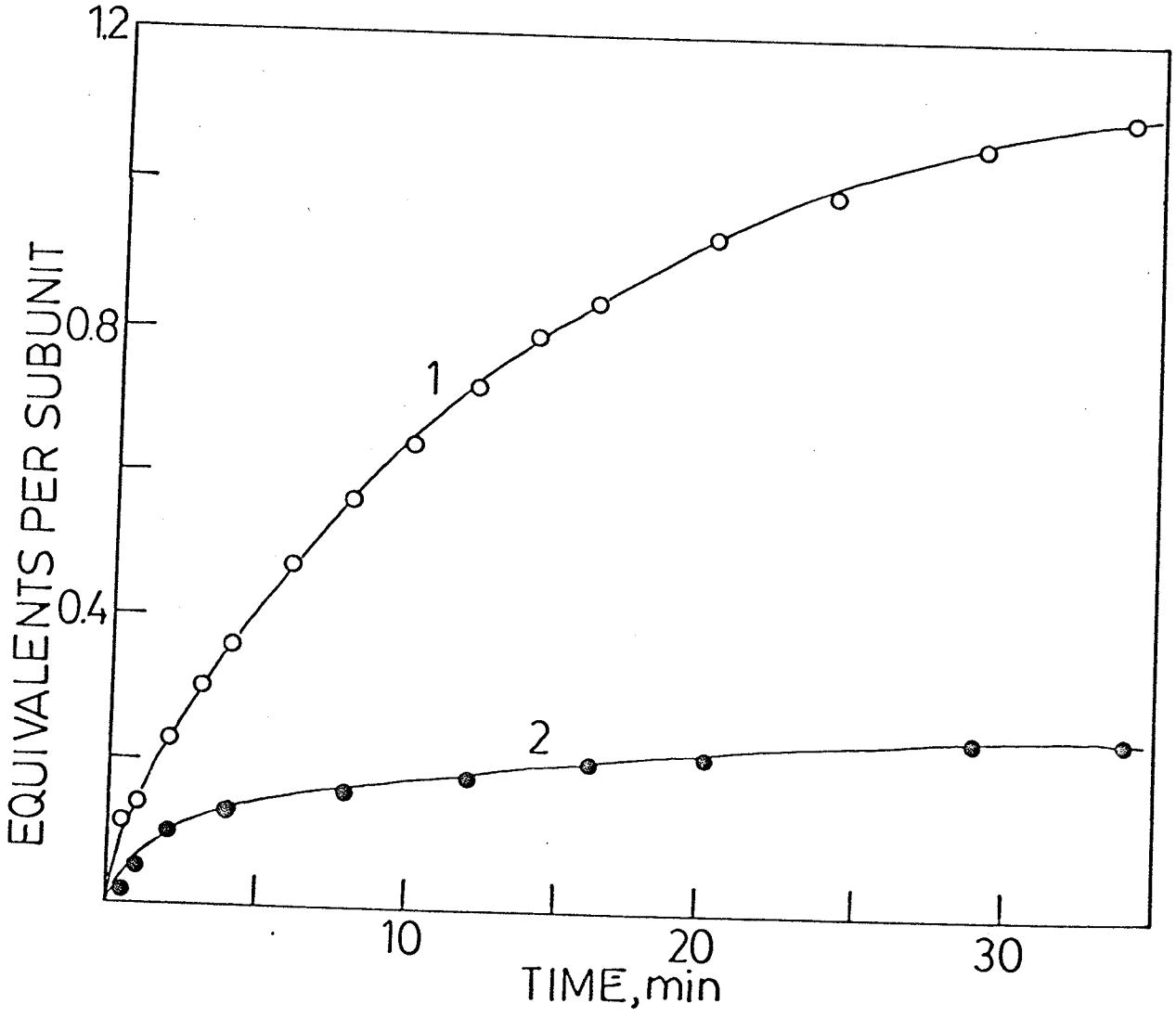
Fig. 18: Modification of E. coli citrate synthase with an equivalent amount of 4,4'PDS.

Enzyme (19.7 μM) was incubated in the presence of 100 mM KCl with 19.1 μM 4,4'PDS.

Fig. 19: Reaction of citrate synthase and citrate synthase previously modified to the extent of 1 group per subunit by 4,4'PDS with 0.1 mM DTNB.

Curve 1 refers to the reaction of unmodified citrate synthase with 0.1 mM DTNB in 100 mM KCl, and curve 2 refers to the reaction of previously 4,4'PDS modified enzyme (as above) with 0.1 mM DTNB in 100 mM KCl.


1.0
0.6
0.2
GROUPS REACTED
10
20
30
40
50
TIME (min)


1.2
0.8
0.4
EQUIVALENTS PER SUBUNIT
1
2
10
20
30
TIME, min

ADPR. As is evident, ADPR strongly activates the enzyme in a sigmoidal fashion.

NADH binding to citrate synthase in the presence of ADPR has been studied, and the results are displayed in Figure 21(a) in the form of Scatchard plots. Figure 21(b) gives a value for the Ki of ADPR of 150 $\pm$ 20 μM.

The effect of ADPR on DTNB and 4,4'PDS reactivity has been investigated and the results are displayed in Figure 22. In both cases, ADPR significantly blocks the reactivity of DTNB and 4,4'PDS with the enzyme. ADPR also significantly protects the enzyme from inactivation by modification with 4,4'PDS, as diagrammed in Figure 23.

All of these points suggest that ADPR acts as a true competitive inhibitor of NADH and binds to the NADH site. Rather than inhibiting the enzyme, however, it activates it, a completely different functional effect than that of NADH.

In order to investigate any possibility that 5'AMP and ribose-5-phosphate, being both halves of ADPR, may have a synergistic effect on enzyme activity, a series of assays have been carried out with them separately and together. Table 3 presents those data.

It is evident that the activity effects of the two compounds are directly additive and that they are not acting synergistically.

ii) 2'5' and 3'5' adenosine diphosphates

The effect of the adenosine diphosphates, 2'5'ADP and 3'5'ADP on

Fig. 20: The effect of ADPR on the activity of E. coli citrate synthase.

In the Scatchard plot, the term Δ(ΔOD/min) refers to the velocity of the reaction at a specific concentration of ADPR minus the velocity at zero concentration of ADPR. In the Hill plot, Δv refers to the same quantity.

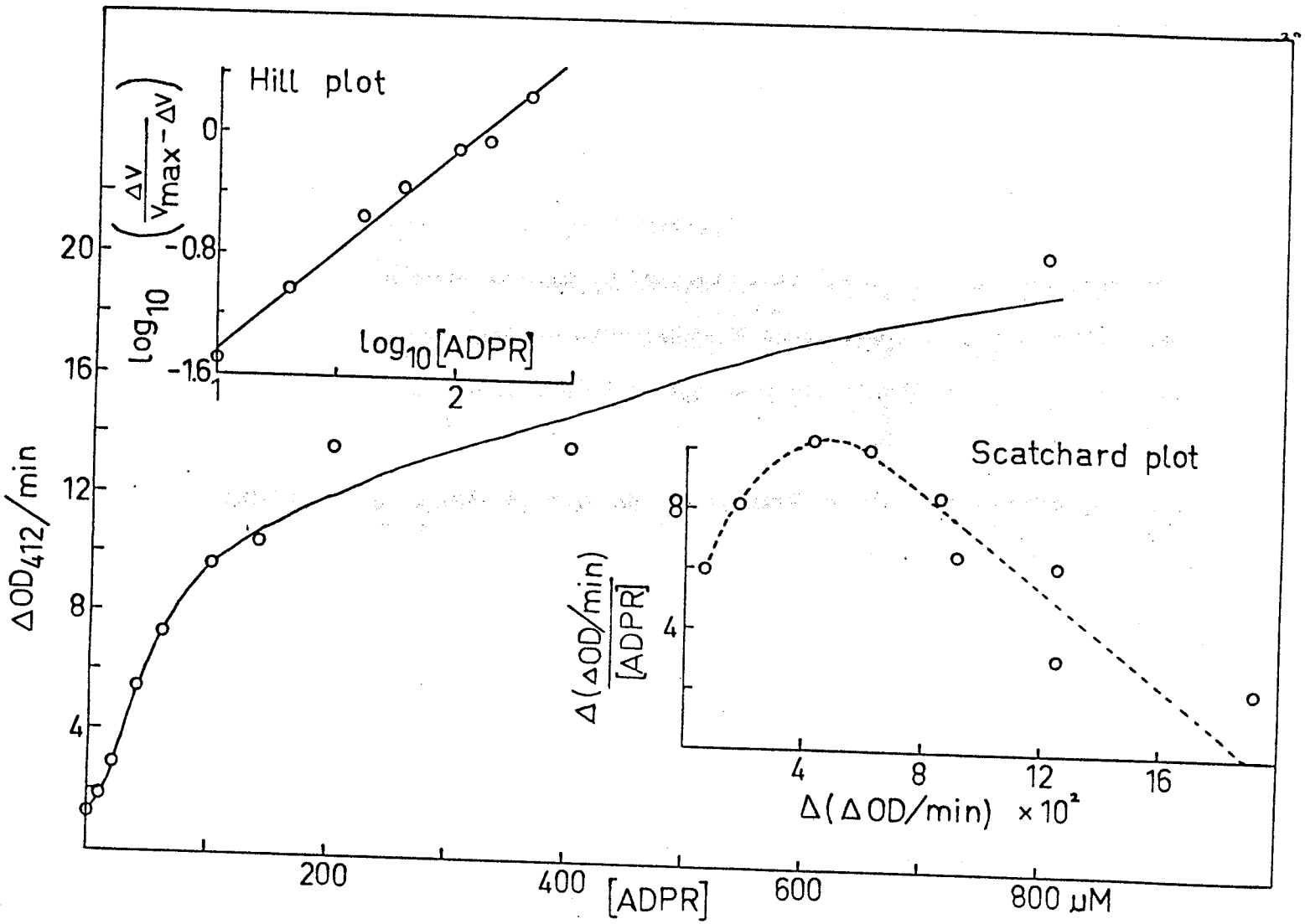

Hill plot
$\log_{10}\left(\frac{\Delta v}{v_{max}-\Delta v}\right)$
0
-0.8
-1.6
1
2
$\log_{10}$[ADPR]
Scatchard plot
$\Delta\left(\frac{\Delta OD/min}{[ADPR]}\right)$
8
4
4
8
12
16
$\Delta(\Delta OD/min) \times 10^2$
$\Delta OD_{412}/min$
20
16
12
8
4
200
400
600
800 µM
[ADPR]

Fig. 21: NADH binding to *E. coli* citrate synthase in the presence of ADPR.

a) In this figure, the data are presented in the form of Scatchard plots. Curve 1 represents the binding of NADH to the enzyme in the absence of ADPR, curve 2 in the presence of 0.25 mM ADPR, and curve 3 in the presence of 0.5 mM ADPR.

b) A plot of dissociation constants vs ADPR concentration will yield the K_i for this compound using the relationship:

$$Kd^{obs} = Kd\ (1 + \frac{[ADPR]}{K_i})$$

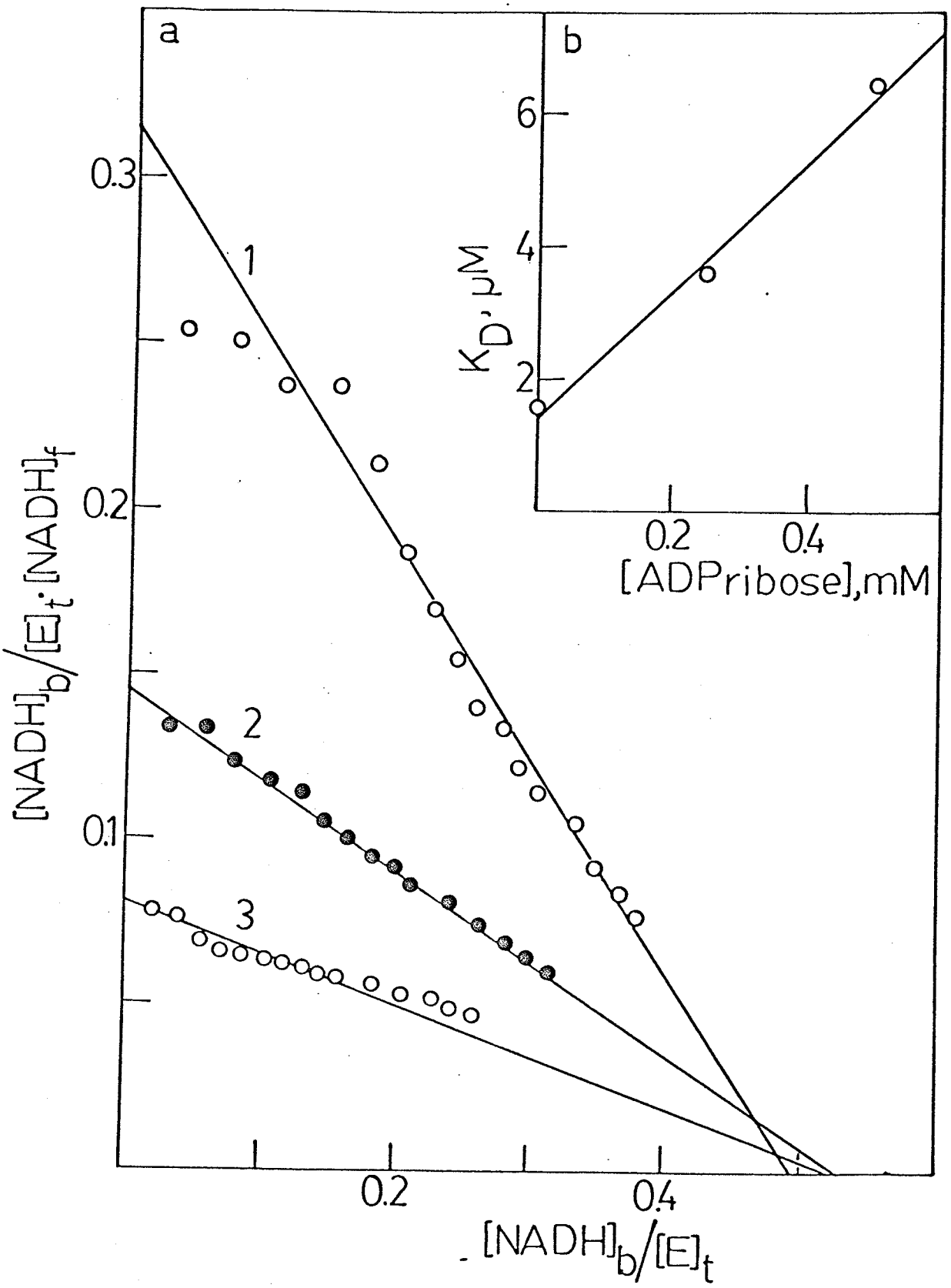

a
b
0.3
0.2
0.1
1
2
3
0.2
0.4
[NADH]b/[E]t
[NADH]b/[E]t·[NADH]f
6
4
2
KD, μM
0.2
0.4
[ADPribose],mM

Fig. 22: a) Modification of E. coli citrate synthase with 0.1 mM DTNB in the presence and absence of ADPR.

In this figure, (O-O) refers to the reaction of the enzyme in the absence of ADPR with 0.1 mM DTNB and (●-●) to the reaction with 0.1 mM DTNB in the presence of 2.94 mM ADPR.

b) Modification of E. coli citrate synthase with 0.05 mM 4,4'PDS in the presence and absence of ADPR.

In this figure (O-O) refers to the reaction of the enzyme with 0.05 mM 4,4'PDS in the absence of ADPR, and (●-●) refers to the reaction in the presence of 2.94 mM ADPR of enzyme and 0.05 mM 4,4'PDS.

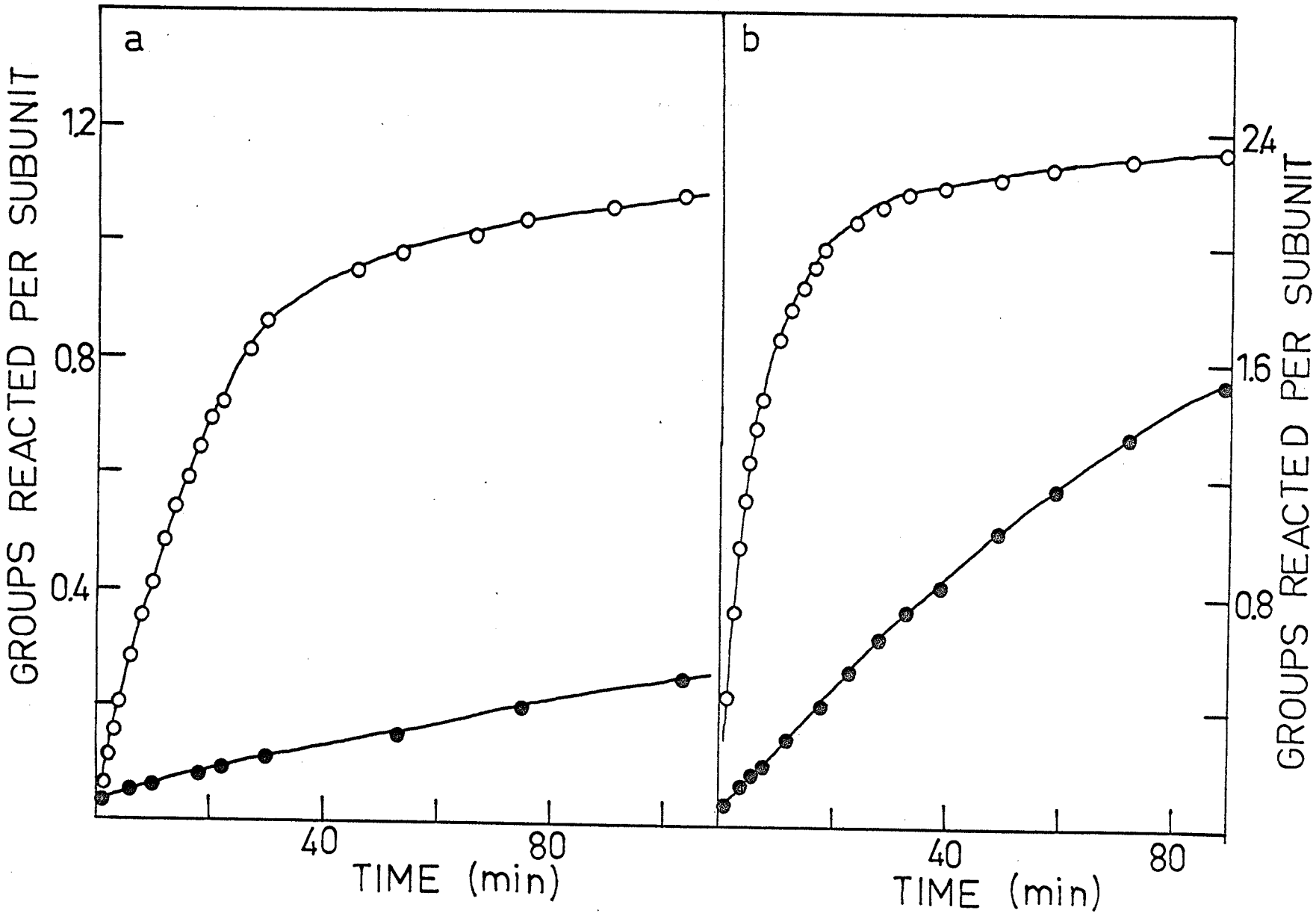

a
b
GROUPS REACTED PER SUBUNIT
1.2
0.8
0.4
40
80
TIME (min)
2.4
1.6
0.8
GROUPS REACTED PER SUBUNIT
40
80
TIME (min)

Fig. 23: Inactivation of E. coli citrate synthase by 0.01 mM 4,4'PDS in the presence and absence of ADPR.

In this figure, (O - O) refers to the rate of inactivation of the enzyme by 0.01 mM 4,4'PDS in the absence of ADPR, and (● - ●) refers to the rate of inactivation in the presence of 2.94 mM ADPR.

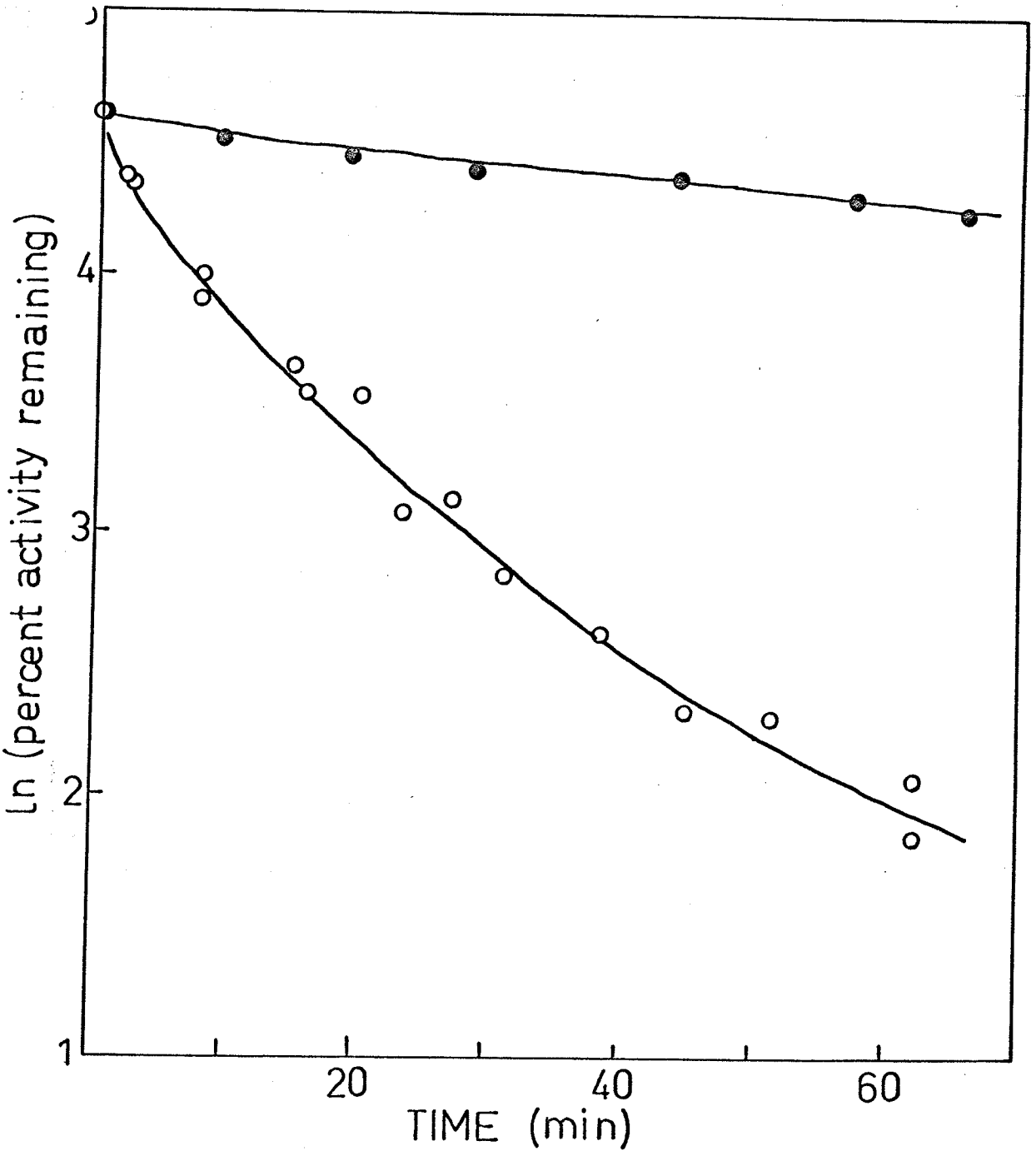

Ln (percent activity remaining)
4
3
2
1
20
40
60
TIME (min)

Table 3: Effect on Activity of Citrate Synthase of Ribose-5-Phosphate and 5'AMP

	Enzyme activity[a] (ΔOD_{412}/min x 10^3)	Percent Activity
standard	3.3	100
1.0 mM Ribose-5-Phosphate	4.1	124
1.0 mM 5'AMP	16.4	498
1.0 mM Ribose-5-Phosphate + 1.0 mM 5'AMP	20.7	628

[a]All assays were done in the absence of KCl, with ACoA at 20 µM and OAA at 0.10 mM.

enzyme activity have been studied. Spot checks at two concentrations (0.2 and 1.0 mM) indicate that both of these compounds have very little effect on enzyme activity (Table 4).

Table 5 represents the effect of 2'5'ADP and 3'5'ADP on NADH binding. As is evident, these compounds alter the Kd for the binding of NADH, but have no effect on the number of sites seen.

iii) Adenosine 5'monophosphate and adenosine 3'monophosphate

In investigating the effects of 5'AMP on the enzyme, I have found that the activity is significantly enhanced in its presence. Figure 24 presents these data in the form of a saturation curve as well as a Scatchard plot. The activation appears to be slightly sigmoidal with an overall activation of 4 fold.

Table 6 represents the results of activity studies with 3'AMP. As is evident, 3'AMP inhibits the enzyme slightly, indicating that the position of the phosphate group on the nucleotide is very important in determining its influence on the activity of the enzyme.

Figure 25 represents the effect of both these monophosphate nucleotides on the modification of citrate synthase by 0.1 mM DTNB. Both of these compounds exert the same effect of blocking the initial modification of a sulfhydryl group. Comparing this diagram with Figure 6, it can be seen that the protection offered the enzyme by 3'AMP and 5'AMP is essentially identical with the protection resulting from the presence of NADH at the concentrations used - all 20 times their respective Kd values.

Table 4: Effect of 2'5' and 3'5' ADP on Citrate Synthase Activity

	Enzyme Activity[a] (ΔOD_{412}/min x 10^2)
standard	3.6 ± 0.2
0.2 mM 2'5' ADP	3.9 ± 0.2
0.2 mM 3'5' ADP	3.8 ± 0.2
1.0 mM 2'5' ADP	3.6 ± 0.2
1.0 mM 3'5' ADP	3.8 ± 0.2

[a]All assays were done in the presence of 100 mM KCl, with ACoA at 20 μM and OAA at 0.1 mM.

Table 5: Effect of 2'5' and 3'5' ADP on NADH Binding of Citrate Synthase

	Kd (μM)	number of sites
standard	1.38 ± 0.10	0.60 ± .02
0.25 mM 2'5' ADP	3.58 ± 0.09	0.57 ± .01
0.25 mM 3'5' ADP	6.4 ± 0.2	0.64 ± .02

Fig. 24: The effect of 5'AMP on enzyme activity of E. coli citrate synthase.

In this figure, Δ relative velocity used in the Scatchard plot refers to the velocity at a particular concentration of 5'AMP minus the velocity at zero concentration of 5'AMP.

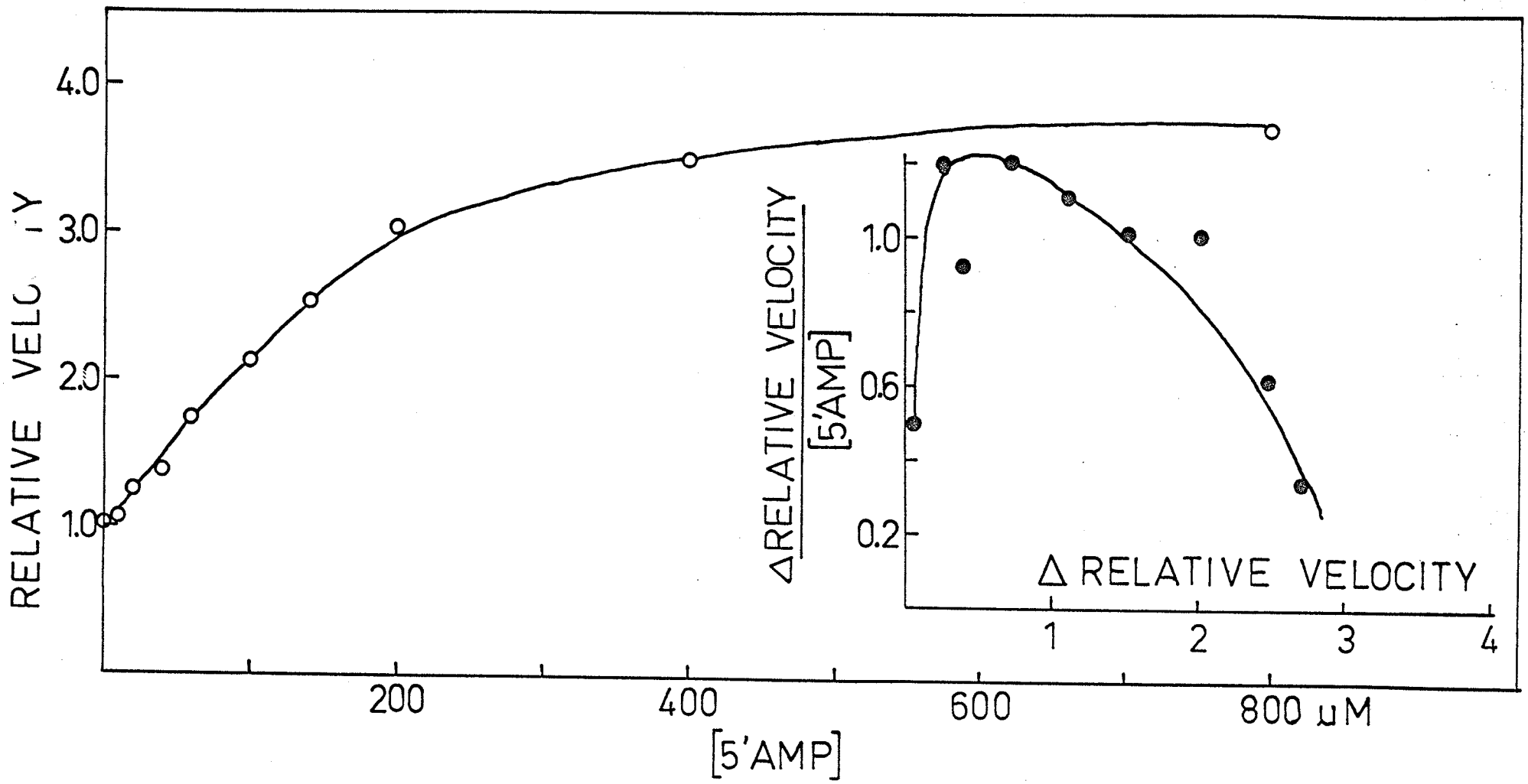

RELATIVE VELOCITY
4.0
3.0
2.0
1.0
200
400
600
800 µM
[5'AMP]
ΔRELATIVE VELOCITY / [5'AMP]
1.0
0.6
0.2
Δ RELATIVE VELOCITY
1
2
3
4

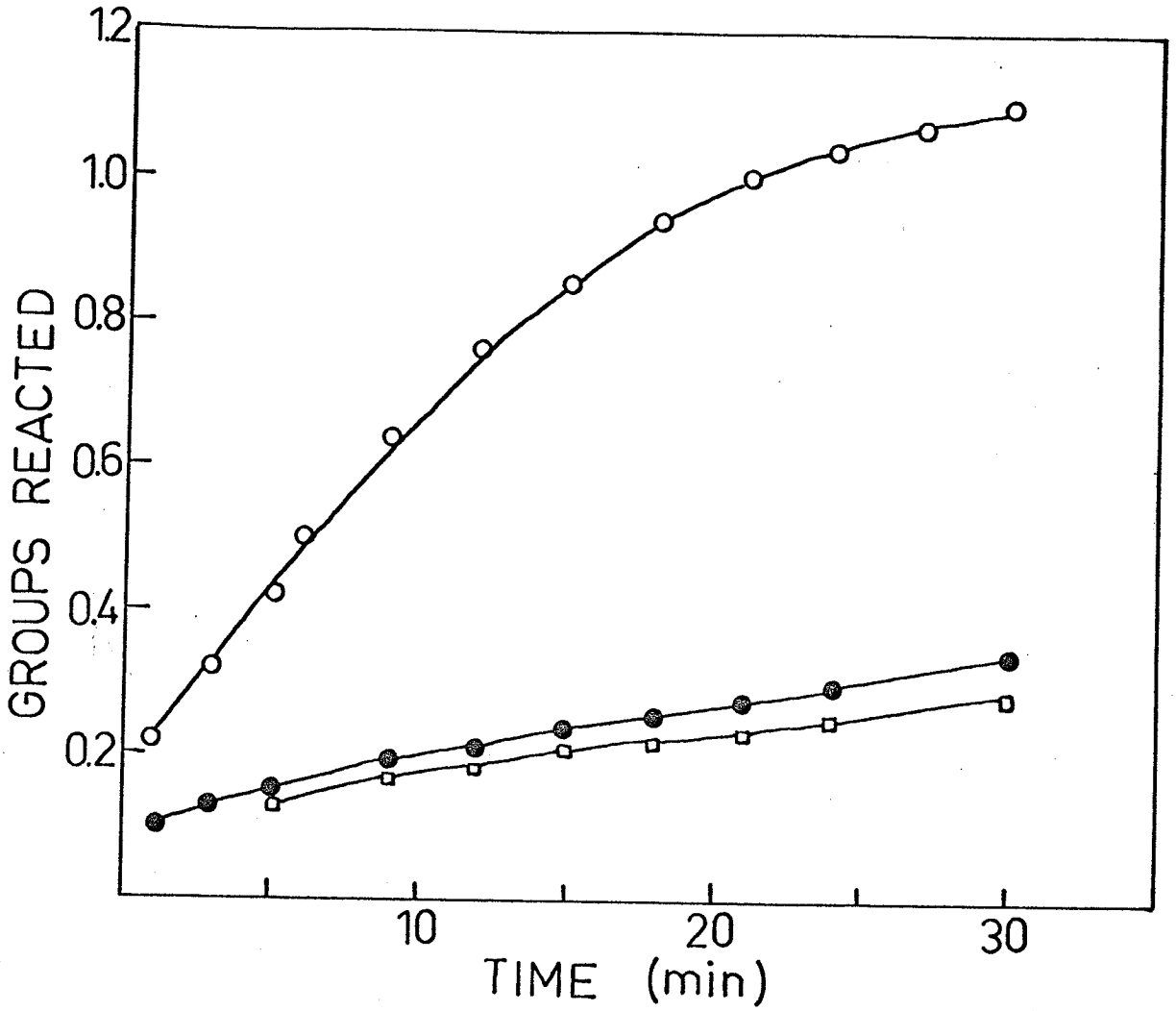
Table 6: Effect of 3'AMP on Citrate Synthase Activity

	Enzyme Activity[a] (ΔOD_{412}/min x 10^2)	Percent Activity
standard	3.6 ± 0.2	100
0.2 mM 3'AMP	2.4 ± 0.2	67
1.0 mM 3'AMP	2.1 ± 0.2	58

[a]In all assays, KCl was at 0.1 M, ACoA was at 20 µM and OAA was at 0.1 mM.

Fig. 25: The modification of E. coli citrate synthase by 0.1 mM DTNB in the presence and absence of 3'AMP and 5'AMP.

In this figure, (O - O) refers to the modification of the enzyme in the absence of nucleotides by 0.1 mM DTNB, (□ - □) refers to the modification in the presence of 1.3 mM 3'AMP and, (● - ●) refers to the modification of the enzyme in the presence of 1.6 mM 5'AMP. The concentrations choosen were all 20 times their respective K_ia's (Duckworth and Tong, 1975).


1.2
1.0
0.8
0.6
0.4
0.2
GROUPS REACTED
10
20
30
TIME (min)

The modification of citrate synthase by .05 mM 4,4'PDS is also significantly blocked by the presence of 3'AMP and 5'AMP. Figure 26 represents these data. Again the effects of both compounds are essentially identical, and both come very close to completely mimicking the effects of NADH (cf Figure 12). Thus, although the two isomers have differing effects on enzyme activity, they both are good analogues for NADH in blocking sulfhydryl reactivity.

Both NADH and 5'AMP protect citrate synthase from inactivation by 0.01 mM 4,4'PDS. It is evident from the results presented in Figure 27 that the rate and extent of inactivation in the presence of 5'AMP are very close to the results obtained in the case of 160 μM NADH.

All of the data above suggest that the sulfhydryl modified by DTNB and initially by 4,4'PDS resides in the adenylate binding portion of the NADH binding site, since the adenosine monophosphates afford it such good protection.

iv) Reduced nicotinamide adenine dinucleotide phosphate

Another possible NADH analogue, NADPH, was investigated as to its effect on enzyme activity. Figure 28 shows that NADPH activates the enzyme to some extent. Some difficulty was associated with this experiment because of the fact that the NADPH used was the tetrasodium salt. Faloona and Srere (1969) have shown (and I have confirmed) that citrate synthase is significantly activated by sodium ions. In light of this, all assays were done with a constant concentration of sodium ions, the sum of the sodium associated with the NADPH and added NaCl.

Fig. 26: The modification of E. coli citrate synthase by 0.05 mM 4,4'PDS in the presence and absence of 3'AMP and 5'AMP.

In this figure, (○-○) refers to the modification of the enzyme by 0.05 mM 4,4'PDS in the absence of nucleotides, (□-□) refer to the modification in the presence of 1.3 mM 3'AMP and (●-●) refer to the modification of the enzyme in the presence of 1.6 mM 5'AMP.

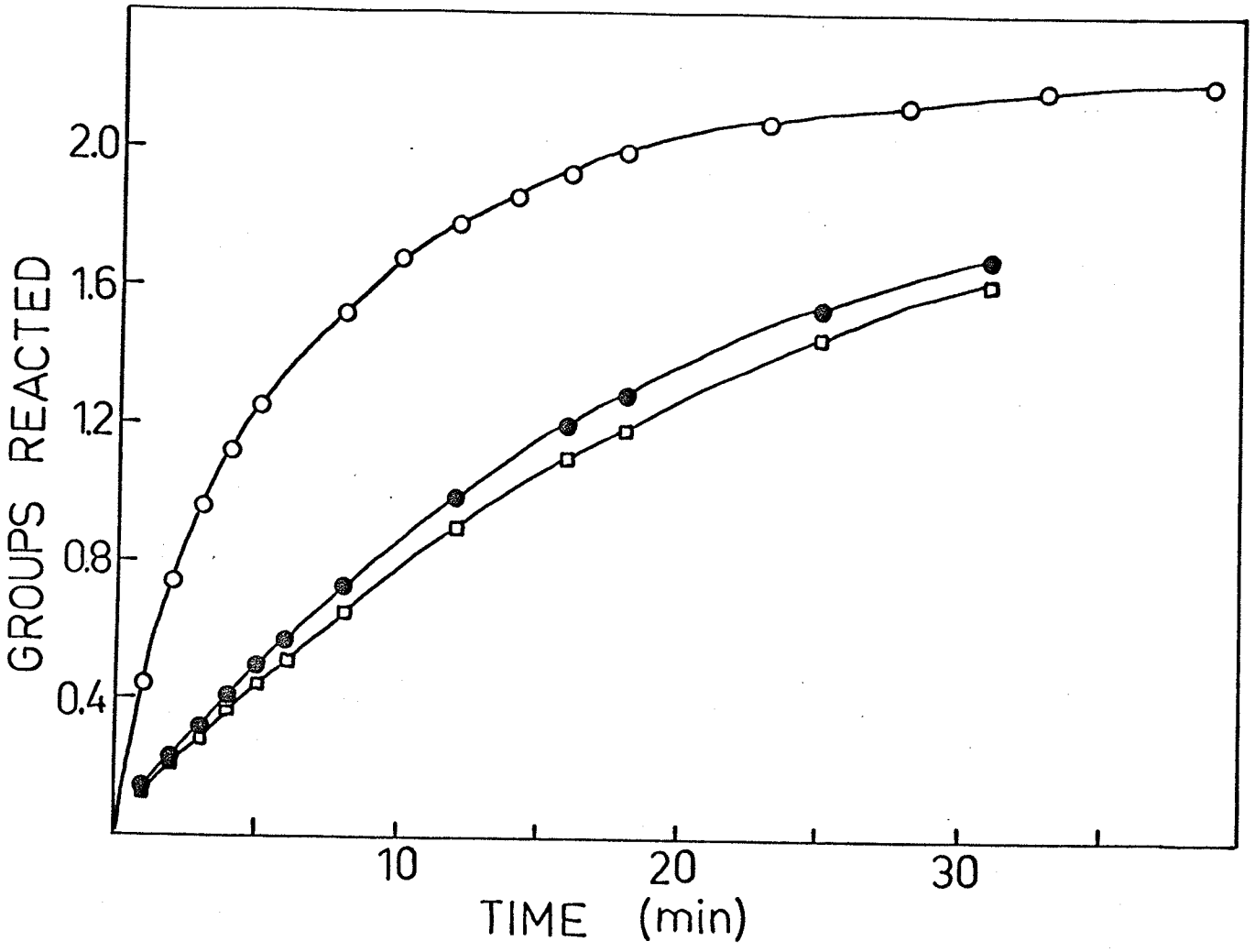

GROUPS REACTED
2.0
1.6
1.2
0.8
0.4
10
20
30
TIME (min)

Fig. 27: The inactivation of E. coli citrate synthase by 0.01 mM 4,4'PDS in the presence and absence of 5'AMP.

In this figure, (○ - ○) represents the activity of the enzyme as it is incubated with 0.01 mM 4,4'PDS and (□ - □) represents the activity of the enzyme incubated with 1.6 mM 5'AMP and 0.01 mM 4,4'PDS.

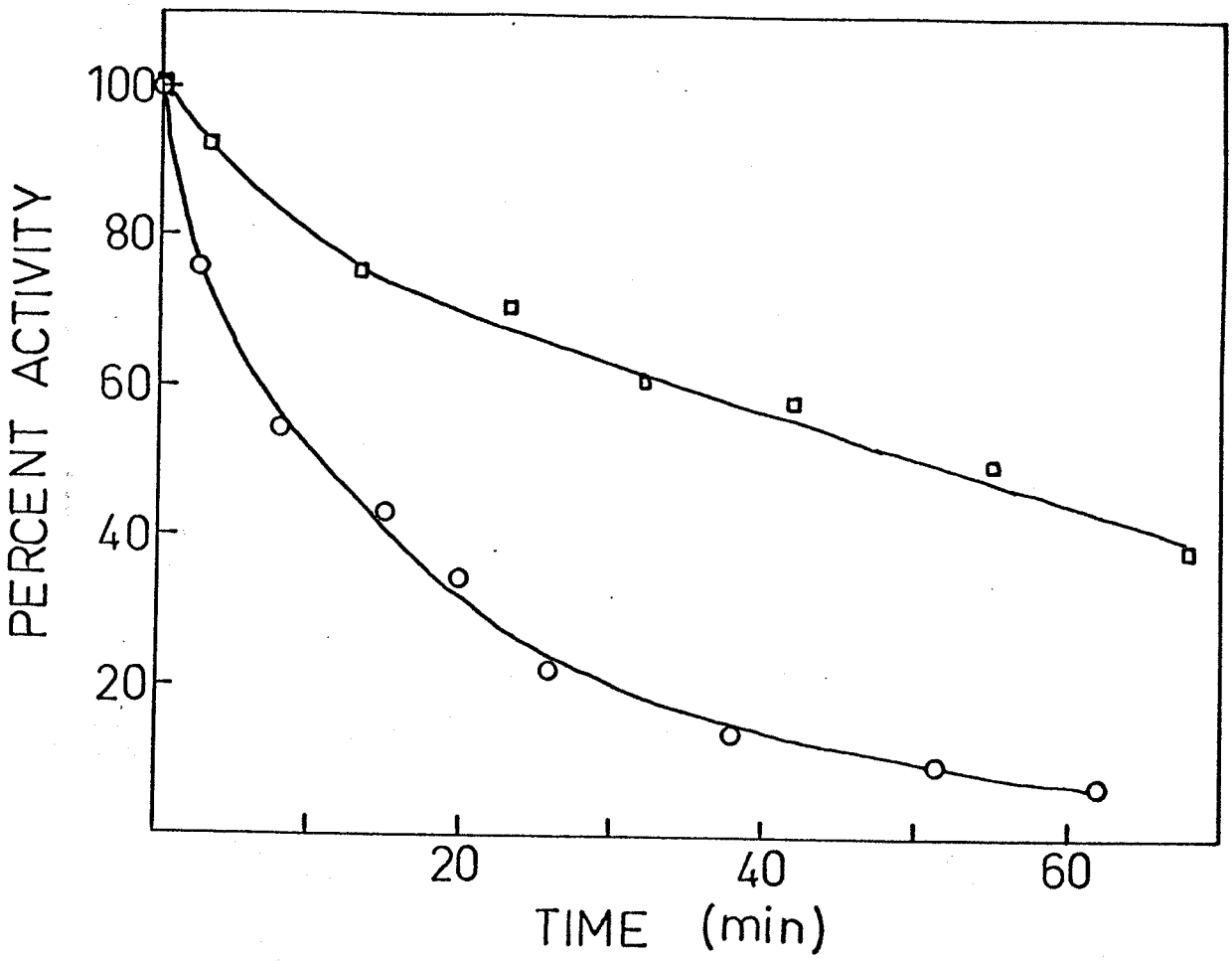
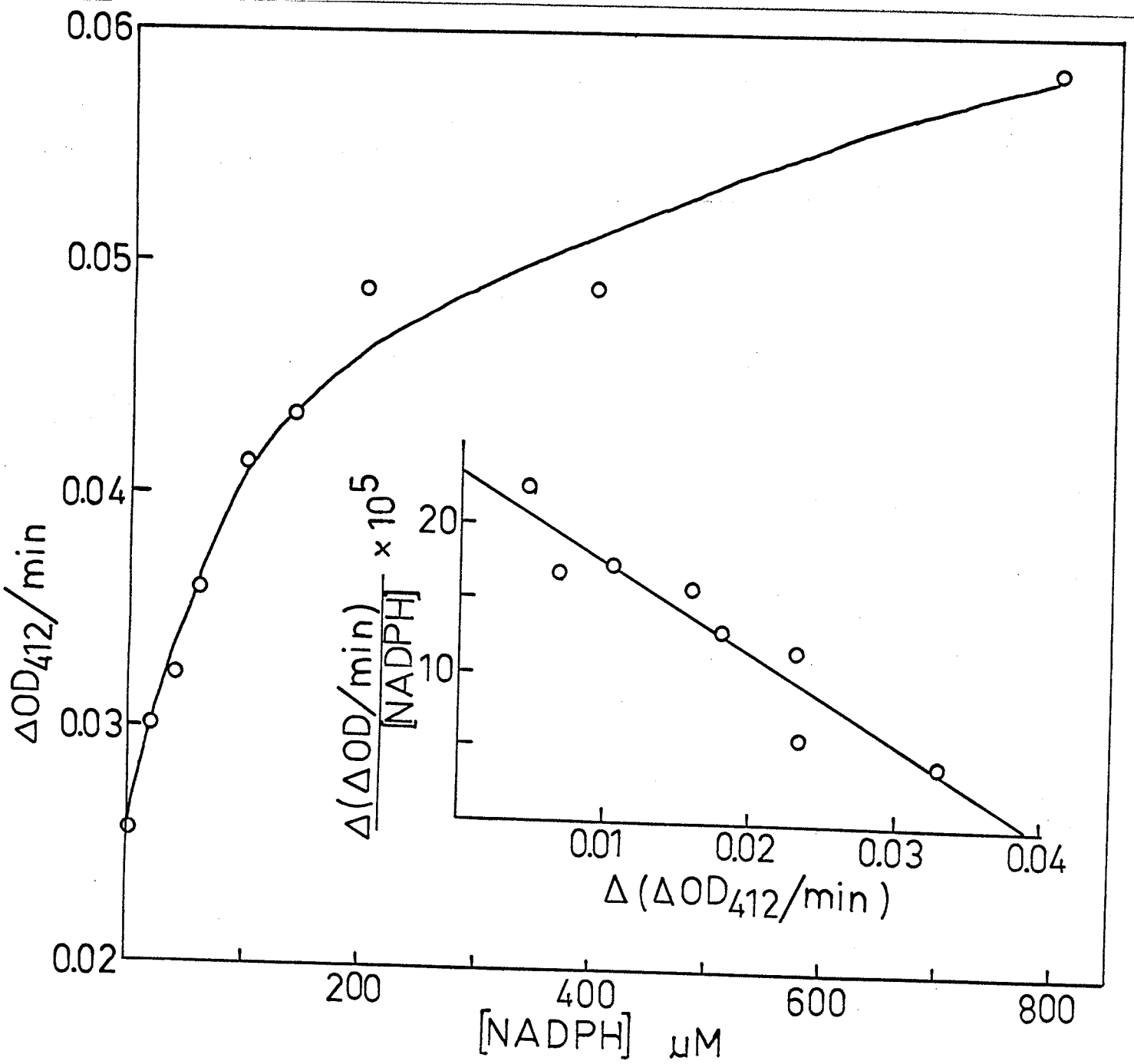

100
80
60
40
20
PERCENT ACTIVITY
20
40
60
TIME (min)

Fig. 28: The effect of NADPH on the activity of E.coli citrate synthase

In this figure, $\Delta(\Delta OD_{412}/min)$ refers to the velocity of the enzyme at various concentrations of NADPH minus the velocity at zero concentration NADPH.


0.06
0.05
0.04
0.03
0.02
ΔOD412/min
200
400
600
800
[NADPH] μM
Δ(ΔOD/min) / [NADPH] ×10^5
20
10
0.01
0.02
0.03
0.04
Δ (ΔOD412/min)

The results indicate an overall activation of approximately 2.3 fold in an apparently non-sigmoidal manner. The K_m of NADPH for this activation is 150 $\pm$ 30 μM. Thus, the difference of 1 phosphate group on the molecule produces a completely different functional effect on the enzyme: weak activation in the case of NADPH, and strong inhibition in the case of NADH.

The effect of NADPH on the reactivity of citrate synthase with DTNB is represented in Figure 29. As can be seen, the reaction is inhibited to some extent with NADPH present.

IV) Modification of Citrate Synthase with Iodoacetic Acid

A series of experiments has been designed to study the modification of citrate synthase with ^{14}C labelled iodoacetate. Since IAA will slowly inactivate the enzyme, as well as release NADH inhibition at a slightly faster rate (data not shown), my principal interest in the reagent has been as a radioactive label for the important sulfhydryl involved with NADH regulation, leading to possible isolation of the appropriate peptide. It is also possible to study the interaction of DTNB and 4,4'PDS with the modified protein, thus obtaining information concerning the hypothesis that DTNB and 4,4'PDS modify the same sulfhydryl group.

Figure 30 represents the time course for the modification of citrate synthase.

The NADH binding properties, DTNB and 4,4'PDS reactivities have been determined for several protein samples modified to differing extents

Fig. 29: Modification of E. coli citrate synthase with 0.1 mM DTNB in the presence and absence of NADPH.

In this figure, (O-O) refers to the modification of the enzyme in the absence of NADPH and (●-●) refers to the modification in the presence of 1.06 mM NADPH.

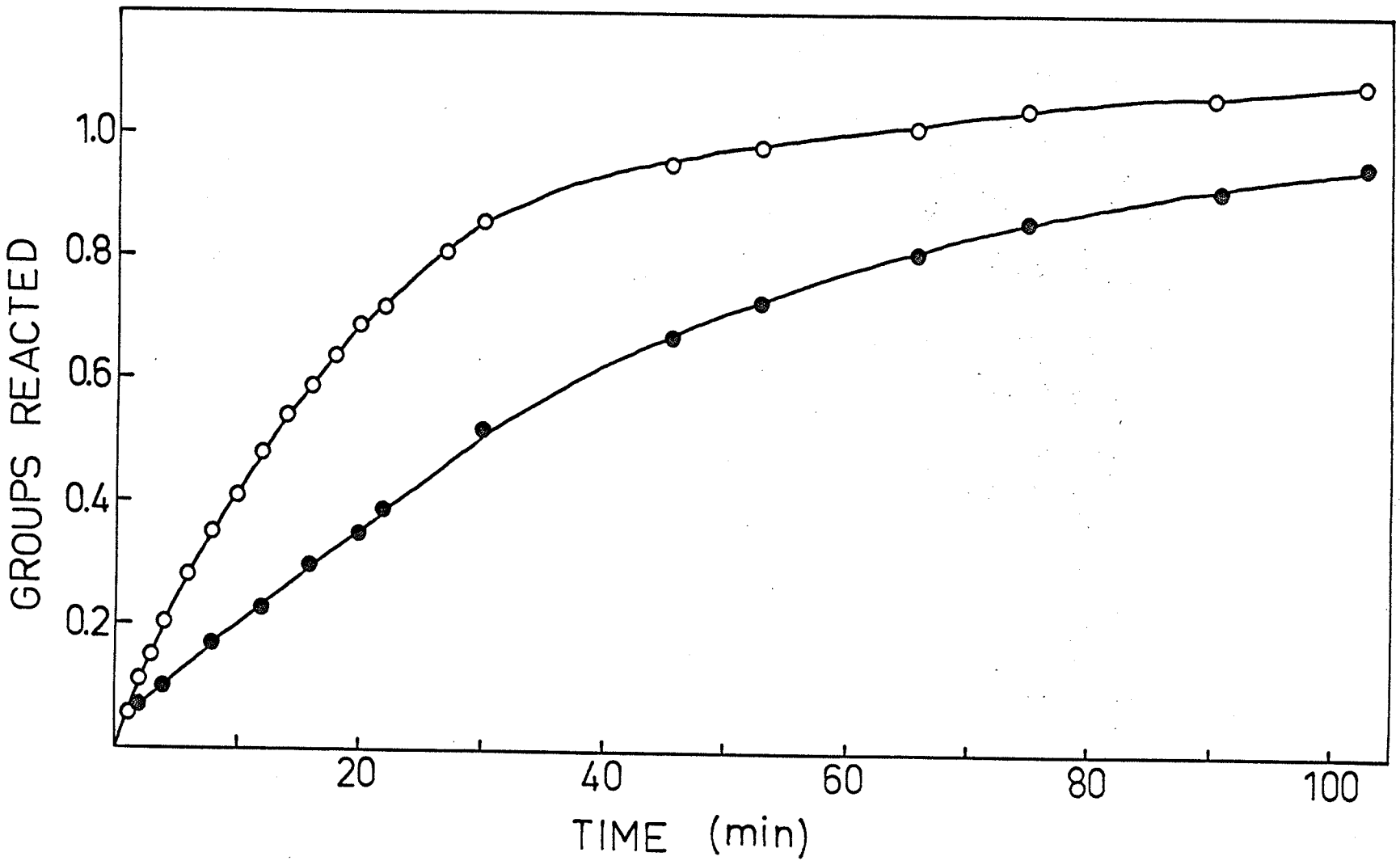
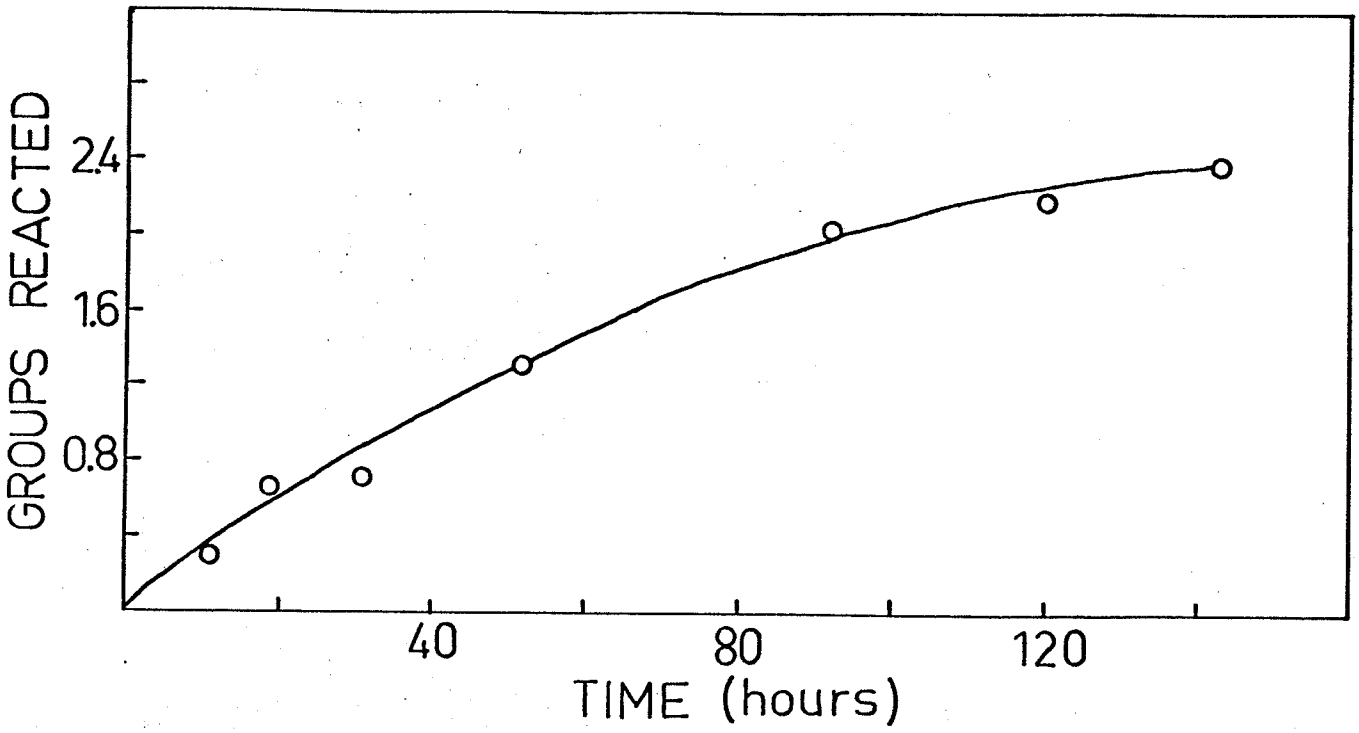

GROUPS REACTED
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
TIME (min)

Fig. 30: The time course for the modification of E. coli citrate synthase with IAA.

The modification was carried out as described in the Methods section.


GROUPS REACTED
2.4
1.6
0.8
40
80
120
TIME (hours)

by IAA. These results are tabulated in Table 7.

It is evident that more than 2 groups are susceptible to carboxymethylation. Assuming that a total of 3.0 groups react, kinetic analysis yields a first order process with an overall rate constant of $(1.12 \pm .05) \times 10^{-2}$ hr^{-1} for the carboxymethylation process. The loss of NADH binding sites is also a first order process yielding a rate constant of $(1.35 \pm .10) \times 10^{-2}$ hr^{-1}. Since these two rates are very nearly the same, it is clear that the sulfhydryl associated with the NADH site is not preferentially carboxymethylated by IAA.

Analysis of the reaction between the modified protein and DTNB or 4,4'PDS was carried out with the GENLSS curve fitting program (DeTar, 1972) using the equation described in Methods. One of the principal difficulties encountered with this method is the possibility that the program will converge on an answer that is not the unique best fit to the data. The answer will be the best fit associated with the initial parameters supplied by the user, but different initial parameters sometimes lead to a different and better fit.

In the case of DTNB, where only 1 group/subunit reacts in the presence of KCl, two parameters are adjusted by the program to produce the best fit. This leads to a unique fit regardless of any reasonable initial parameters used, and produced the values in Table 7. Analysis of the loss of DTNB reactivity upon carboxymethylation indicates that this is a first-order process yielding a rate constant of $(1.24 \pm .22) \times 10^{-2}$ hr^{-1}. It is encouraging to note that the loss of NADH binding sites and

Table 7

Binding of NADH, and reactivity towards DTNB and 4,4'-PDS, of citrate synthase modified to various extents with IAA

Time of reaction, hr	carboxymethyl groups incorporated per subunit	NADH binding		DTNB reaction		4,4'-PDS reaction			
						fast phase		slow phase	
		sites per subunit	$K_{D'}$ μM	groups reacting per subunit	k_{obs} min^{-1}	groups reacting per subunit	k_{obs} min^{-1}	groups reacting per subunit	k_{obs} min^{-1}
0	0	0.68 ±0.04	2.0 ±0.4	1.17 ±0.17[a]	0.23 ±0.04[a]	0.85 ±0.02[a]	1.11 ±0.03[a]	1.02 ±0.06[a]	0.095 ±0.015[a]
10.5	0.30	0.55	2.5	0.99 ±0.02[b]	0.23 ±0.01[b]	0.744[c]	1.02 ±0.01[b]	1.06 ±0.04[b]	0.097 ±0.003[b]
19.4	0.67	0.43	1.8	0.95 ±0.02	0.32 ±0.03	0.663	0.743 ±0.026	1.00 ±0.01	0.060 ±0.002
30.4	0.72	0.45	3.0	0.93 ±0.02	0.21 ±0.02	0.575	1.00 ±0.06	1.03 ±0.01	0.097 ±0.003
45.9	1.31	0.28	2.0	0.46 ±0.02	0.12 ±0.01	0.470	0.74 ±0.11	1.15 ±0.03	0.031 ±0.002
91.0	2.04	0.18	2.2	0.41 ±0.02	0.10 ±0.01	0.261	0.33 ±0.06	1.09 ±0.04	0.024 ±0.002
120.3	2.20	ND	ND	ND	ND	ND	ND	ND	ND
143.4	2.38 ±0.19	0.09	2.1	ND	ND	ND	ND	ND	ND

[a] Standard deviations sets of parameters obtained by GENLSS program from 3 separate kinetic runs.

[b] Errors in the rest of this column were assigned by GENLSS program for a fit to 1 kinetic run. For the significance of these errors, see DeTar (1972).

[c] Values in this column were calculated according to the text.

ND = not determined.

loss of DTNB reactivity upon modification with IAA proceed with essentially the same rate constant. This is further evidence that it is the modification of a susceptible sulfhydryl group by DTNB that leads to loss of NADH binding.

In the case of 4,4'PDS the situation becomes more complicated with the introduction of two other parameters, representing the modification of the second sulfhydryl group. Extensive experimentation with computer generated fits to the data indicates that the number of groups reacting with a slow rate constant does not change appreciably over the range of modification studied. However, there does not seem to be a unique solution associated with the number of groups reacting quickly. One solution is generated by assuming a reasonable value for all four parameters and allowing the computer to adjust all of them to fit the data. This can be referred to as the "unrestricted" solution. Evidence has been presented to suggest that the sulfhydryl group susceptible to DTNB modification is the first sulfhydryl group to react with 4,4'PDS. A consequence of this hypothesis is that the first group reacting with 4,4'PDS should disappear during carboxymethylation at the same rate as DTNB reactivity (or NADH binding sites). To test this hypothesis, I have calculated the number of groups initially reacting with 4,4'PDS for each sample of modified protein assuming that they will disappear with the same rate constant as the loss of DTNB reactivity; according to the following equation:

$$\text{initial groups reacted at time } t = 0.85\, e^{-.0124(t)}$$

where t = time of incubation with IAA

0.85 = initial number of groups reacting at zero time

0.0124 = rate constant for disappearance of DTNB groups upon carboxymethylation

Then by fixing this parameter in the computer program, a "restricted" fit can be generated by allowing the computer to adjust the other three parameters to fit the data. This calculated initial value, and the computer generated three other values are the ones displayed in Table 7. Figure 31 allows judgement of the success of these two fits (the "unrestricted" and the "restricted") in reproducing the data. It is evident that the restricted fit closely approximates the data; thus the carboxymethylation results are consistent with the earlier finding that DTNB and 4,4'PDS react with the same sulfhydryl group.

V) Modification of Citrate Synthase with other Sulfhydryl Reagents

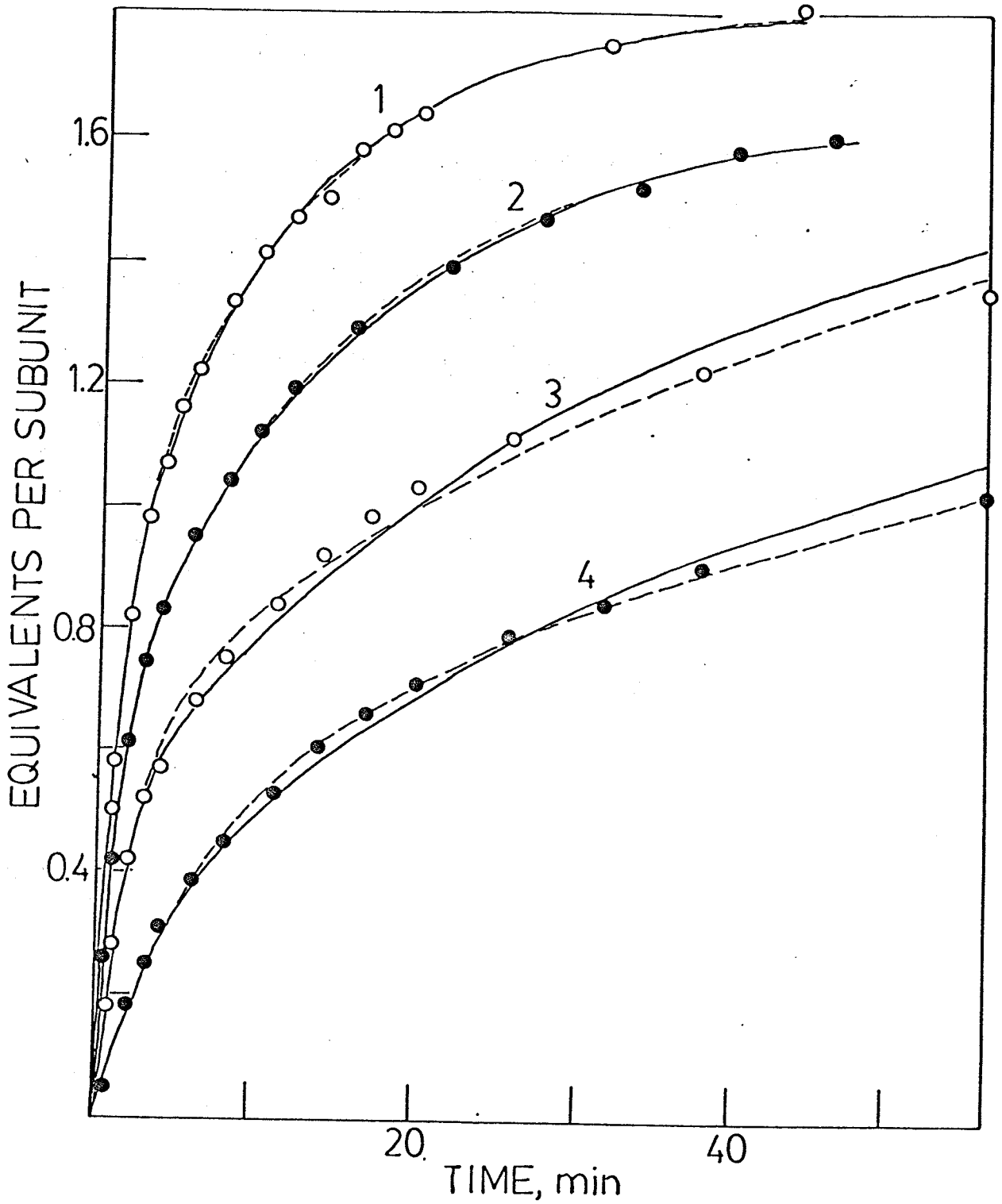
i) N-dansylaziridine

In response to a published report of a fluorescent reagent with a high specificity for sulfhydryls (Scouter, Lubcher, and Boughman, 1974) I have investigated the reaction between N-dansylaziridine and E. coli citrate synthase.

Initial experiments entailed incubating citrate synthase with 5% N-dansylaziridine - cellulose and monitoring any change in fluorescence at 485 nm with excitation at 345 nm, or change in enzyme activity in the presence and absence of NADH. After a reaction time of 60 minutes, no change in fluorescence or enzyme activity was found. In order to check the activity of the reagent, the same incubation was carried out with

Fig. 31: Reaction of 0.05 mM 4,4'PDS with carboxymethylated E. coli citrate synthase.

Curves 1-4 are for samples containing 0, 0.67, 1.31 and 2.04 carboxymethyl groups per subunit, respectively (see Table 7). The solid lines were calculated using Equation (2) and represent the "restricted" fit and the broken lines were calculated using Equation (2) and represent the "unrestricted" fit. For further details, see the text.


EQUIVALENTS PER SUBUNIT
1.6
1.2
0.8
0.4
1
2
3
4
20
40
TIME, min

1 mg/ml bovine serum albumin since this protein has been shown to react with the reagent under these conditions. Over a period of 100 minutes, a linear increase in fluorescence was observed. Therefore it was concluded that citrate synthase did not react with N-dansylaziridine - cellulose.

Figure 32 represents the results of an experiment incubating citrate synthase with N-dansylaziridine. It is evident that the fluorescent spectrum has changed dramatically upon incubation with N-dansylaziridine, hopefully indicating an interesting modification of the enzyme.

ii) 2,2'PDS

A very brief investigation of the reactivity of 2,2'PDS with citrate synthase consisted of modifying the enzyme with 1.0 mM reagent. Figure 33 represents the results. Note that these results, comparable with 4,4'PDS as to extent of incorporation and rate of reaction, were obtained with a concentration 20 times greater than the 4,4'PDS work. These seems to exist a significant difference in the reaction of citrate synthase with the two PDS isomers.

iii) Iodoacetamide

Modification of a 8.40 μM solution of citrate synthase with a 20 molar excess of iodoacetamide leads to an initial loss of approximately 40% of enzyme activity over a period of 10 minutes, then practically no loss of enzyme activity over the next 150 minutes. NADH inhibition remains virtually unchanged, with the enzyme 96% inhibited at 0 minutes and 94% inhibited at 150 minutes. Thus I conclude that the enzyme dis-

Fig. 32: Fluorescence emission spectrum of E. coli citrate synthase before and after incubation with N-dansylaziridine.

In this figure, (● - ●) represents the emission spectrum of the native enzyme, and (O - O) the emission spectrum after incubation with N-dansylaziridine as described in Methods. Excitation was at 345 nm. Photometer readings for (O - O) were multiplied by scalor factor of 6.2 in order to correct for differing protein concentrations in the samples.

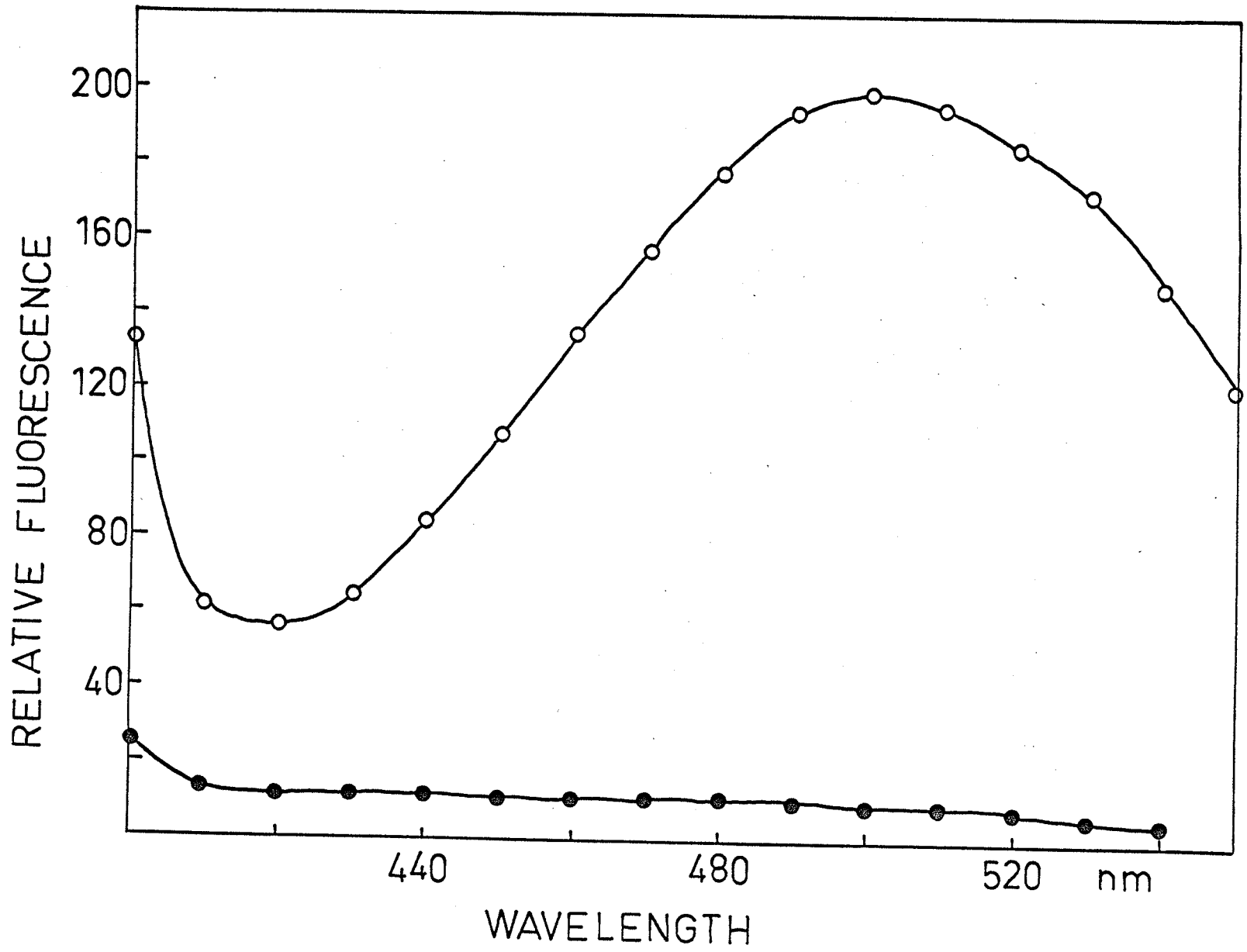
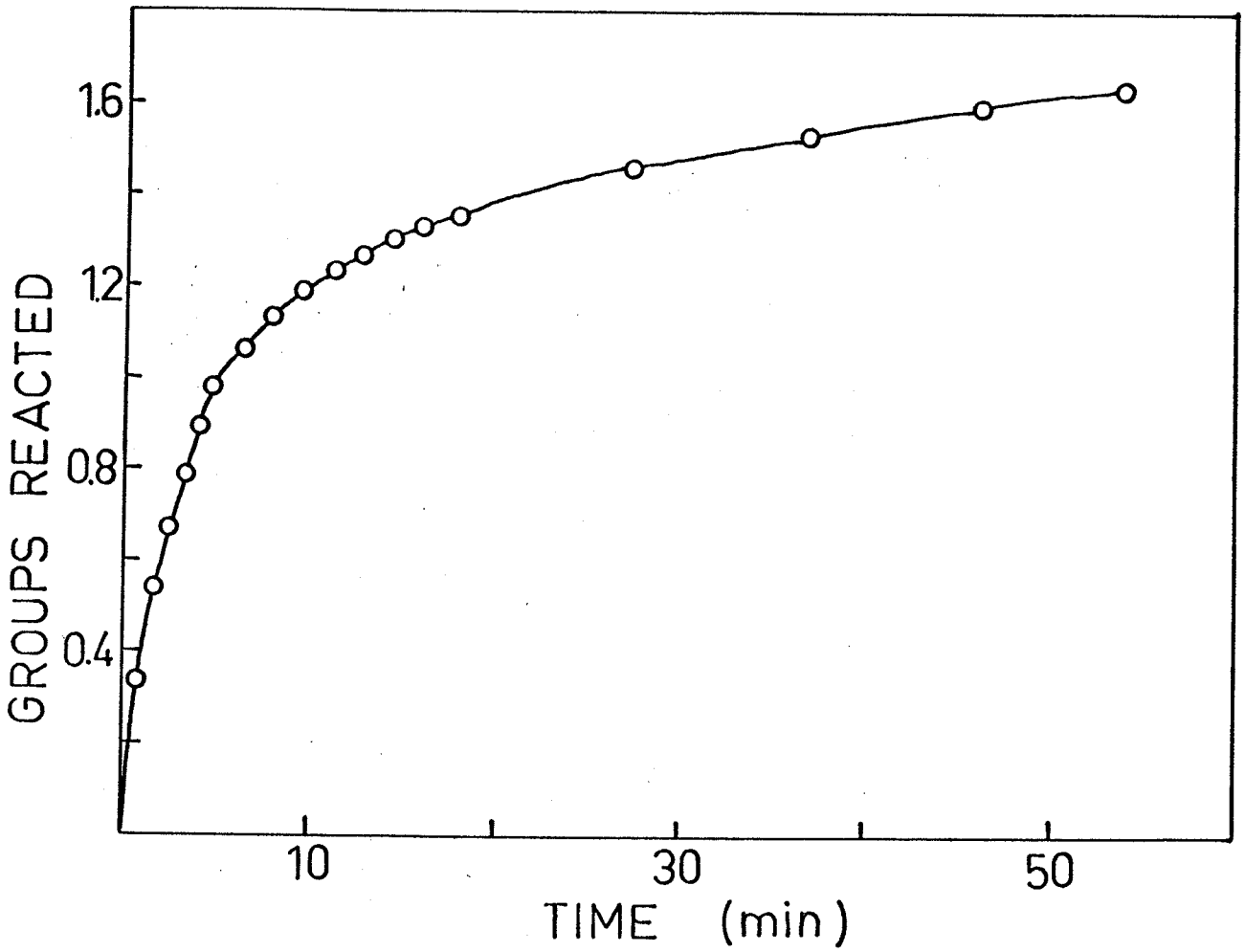

200
160
120
80
40
RELATIVE FLUORESCENCE
440
480
520
nm
WAVELENGTH

Fig. 33: Reaction of E. coli citrate synthase with 1.0 mM 2,2'PDS.


GROUPS REACTED
1.6
1.2
0.8
0.4
10
30
50
TIME (min)

plays differing effects in its reactions with IAM and IAA.

iv) 2-Nitro-5-thiocyanatobenzoic acid (TNB-CN)

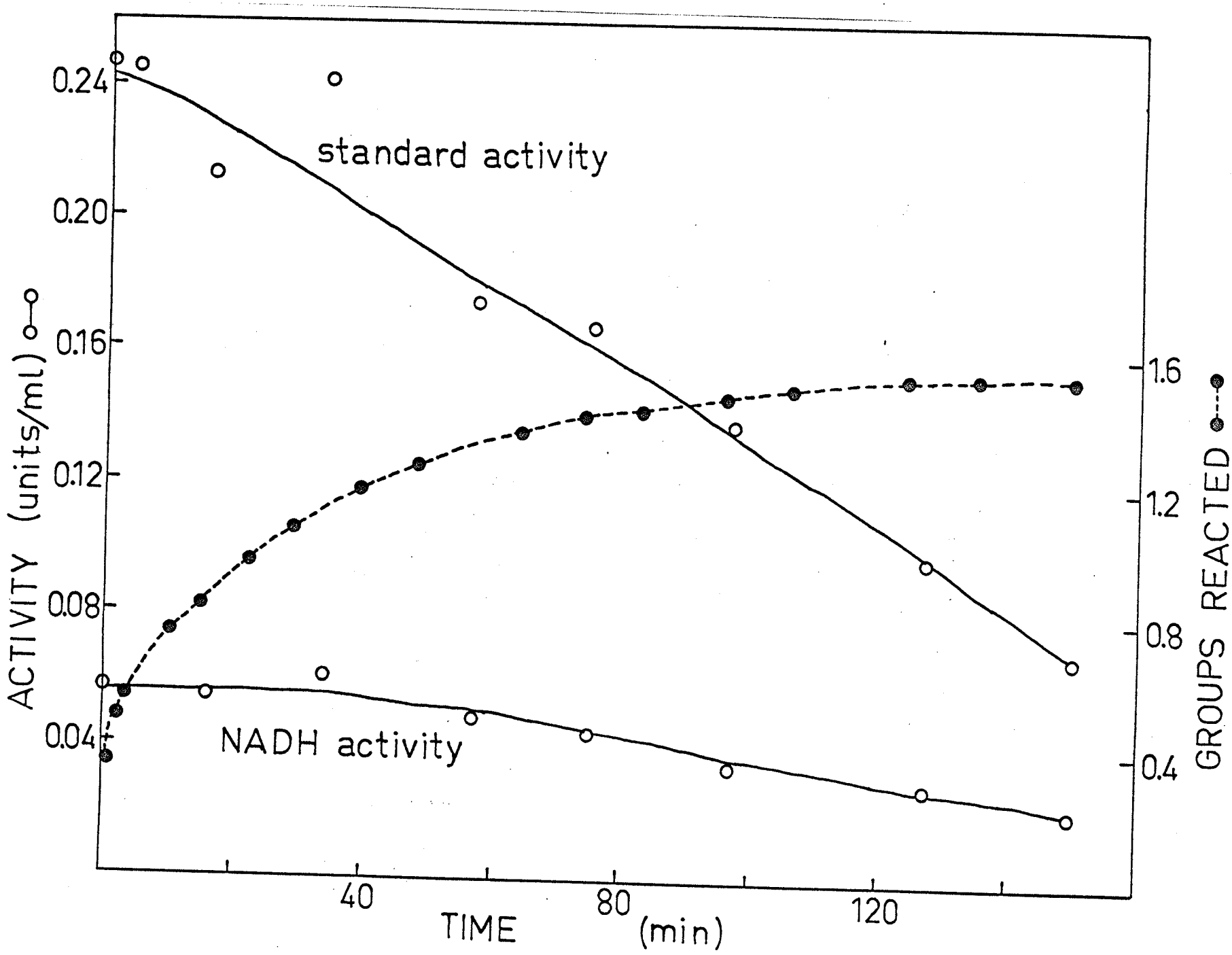
Degani et al. (1971) have described the modification of protein thiol groups using the reagent TNB-CN. Figure 34 represents the results of my studies with reagent on the functional properties of citrate synthase. The modification reaction was done in the presence of 23.8 mM KCN in order to ensure that the reaction proceeds in the direction of CN incorporation (see Price, 1976). A blank consisting of TNB-CN and KCN was run simultaneously in order to correct for reaction between these two substances. An assay was done in the absence of OAA in order to correct for any change in absorbance due to reaction between KCN and DTNB. This pseudo activity was subtracted from all subsequent activity values.

It is evident that as the reaction proceeds, the enzyme is losing its sensitivity to NADH inhibition at a faster rate than its activity. This parallels in a qualitative way the effect of modification with DTNB.

The findings concerning the modification of E. coli citrate synthase with N-dansylaziridine, 2,2'PDS, iodoacetamide and 2-nitro-5-thiocyanatobenzoic acid were not pursued beyond the point described in this thesis.

Fig. 34: Effect on E. coli citrate synthase enzyme activity and NADH inhibition of incubation with 0.1 mM TNB-CN.

An enzyme solution was made (13.7 μM) and KCN (23.8 mM) and TNB-CN (0.1 mM) were added. At suitable intervals, aliquots were removed and subjected to a standard assay (O-O) and an assay containing 0.1 mM NADH (O-O). The progress of the reaction was observed by the change in optical density at 412 nm (●---●).


0.24
0.20
0.16
0.12
0.08
0.04
ACTIVITY (units/ml)
standard activity
NADH activity
1.6
1.2
0.8
0.4
GROUPS REACTED
40
80
120
TIME
(min)

DISCUSSION

DISCUSSION

The most interesting aspect of this investigation has been the effect of sulfhydryl modification on the functional aspects of E. coli citrate synthase. Modification by DTNB leads to loss of some enzyme activity, but more importantly, the enzyme becomes almost completely insensitive to the effects of its allosteric inhibitor, NADH. This observation led to the further study of the reaction of DTNB with citrate synthase and its interaction with the NADH site.

The DTNB saturation curve and pH rate profile for the reaction indicate that the reagent first binds to the enzyme, then reacts with the basic form of a susceptible sulfhydryl group which exists in an environment near a positively charged group (Webb, 1966). Since the reaction between DTNB and citrate synthase is markedly inhibited in the presence of NADH (Fig. 6) and modification of the enzyme eliminates NADH binding, it is evident that the sulfhydryl modified by DTNB is closely associated with the NADH site on the enzyme. Modification of the enzyme with another sulfhydryl reagent, 4,4'-PDS leads to very rapid inactivation of the enzyme, so that it is difficult to follow any effects of modification on NADH inhibition directly. The reaction between enzyme and 4,4'-PDS is strongly inhibited by NADH however, and modification to the extent of 1 group/subunit also leads to loss of NADH binding to the enzyme. These data strongly suggest that the modification of the initial sulfhydryl group by 4,4'-PDS not only inactivates the enzyme, but as in the case of DTNB, it is strongly associated with the NADH - binding properties of the enzyme.

These similar effects of modification by two different reagents have led to investigations indicating that the reagents are reacting with the same sulfhydryl group. Enzyme modified to the extent of 1.0 ± 0.1 groups/subunit with DTNB loses 0.80 groups that would normally react with 4,4'-PDS (Fig. 17). This number is in good agreement with 0.85, the number of groups reacting quickly with 4,4'-PDS in the unmodified case (as determined by computer fitting of PDS modification profiles; see Table 7, zero modification by IAA and discussion below). In a reciprocal experiment, modification of 1.0 ± 0.1 groups/subunit with 4,4'-PDS eliminates 0.9 groups/subunit that react with DTNB in the control. Since modification by one reagent effectively blocks the reaction of the enzyme with the other, the simplest conclusion is that the two reagents are reacting with the same sulfhydryl group. The rapid phases of both reactions are stimulated by 0.1 M KCl and treating the enzyme with IAA causes its reactivity towards both reagents, and its ability to bind NADH, to disappear at similar rates.

Although the two reagents are reacting with the same sulfhydryl group and producing similar effects on NADH binding to the enzyme, their effects on enzyme activity are very different. The effects of DTNB modification on activity have been reported by several workers. Initially Danson and Weitzman (1973) reported that the enzyme lost 38% of enzyme activity when modified to the extent of 1 group/subunit (recalculated using our MW of 47,000 g/mole) by DTNB over a period of 2 mins. and a total of 65% of enzyme activity was lost after 2.50 groups had been modified in a period of 60 minutes. Over the same time, the enzyme was being completely desensitized to NADH inhibition. In a recent paper, Danson and

Weitzman (1977) report that two of eight reactive sulfhydryl groups per tetramer of the native enzyme (MW of 230,000) reacted rapidly with DTNB with a loss of 25% enzyme activity but no change in the NADH inhibition. Subsequent modification of the 6 remaining groups resulted in a further loss of 60% of activity and complete desensitization to NADH. Wright and Sanwal (1971) have reported a slow loss of enzyme activity to 10% of the original activity as 2.6 groups/subunit (47,000 g/mole) are modified over a period of 6 hours. Faloona and Srere (1969) found that after 2 hours incubation with DTNB, reaction of enzyme with DTNB was complete with only a 20-40% loss of activity. My own finding that enzyme activity loss is not reproducible between preparations is consistent with the variable findings of others.

The reagent 4,4'-PDS on the other hand, reacting with sulfhydryl groups with the same kind of mechanism, results in complete inactivation of the enzyme as 1 group/subunit is modified. Since the rate of inactivation is higher in the presence of KCl, which enhances the reactivity of the first group and lowers that of the second group reacting with 4,4'-PDS, it would seem that the inactivation is associated with the more reactive group. Therefore modification of this single sulfhydryl is responsible for all the effects, since it has been shown that the more reactive group is also implicated with the loss of NADH binding.

These several effects can be interpreted in light of the allosteric model of Monod et al (1965). Two conformational states of the enzyme are proposed - a R or active state which binds ACoA selectively and catalyses

the reaction, and a T state which binds NADH selectively and has little or no affinity for ACoA. The easiest way to account for the effects of modification is to suggest that the susceptible sulfhydryl group exists in or near the NADH binding site. Modification introduces a bulky structure preventing the approach of NADH, thus eliminating NADH binding. Heterotropic interactions between the ACoA (active) and NADH (allosteric) sites have been demonstrated by the observations that NADH increases the sigmoidicity of ACoA saturation curves (Wright and Sanwal 1971) and that NADH binding is weakened by ACoA in a cooperative manner (Duckworth and Tong 1976). In this light, the effect of ACoA on the DTNB reaction rate (Fig. 6) and the effect of DTNB modification on the Hill number for the ACoA and KCl saturation curves (Table 1) are regarded as further examples of heterotropic interactions between allosteric and active sites. The effects of the two reagents DTNB and 4,4'-PDS on enzyme activity may be explained by suggesting that they have differing orientations within the NADH site. Occupation of a "critical" cleft within the site by the 1,4-dihydronicotinamide ring of NADH or the 4-pyridylmercapto ring of PDS may prevent the transition from the T (inactive) state to the R (active) state. Since saturation of the catalytic rate is sigmoid (Faloona and Srere 1969; Wright and Sanwal 1971), while NADH binding is hyperbolic (Duckworth and Tong 1976), it may be concluded that the T state predominates in the absence of effectors, and that an allosteric transition must take place in order for the enzyme to become active. Modification by DTNB, on the other hand, introduces the negatively charged 3-carboxy, 4-nitrothiophenoxy group into the site, which may occupy a noncritical region of

the site. This group may be orientated away from the critical region by hydrogen bonding involving its nitro group, or by an ion pair interaction between its carboxyl group and a cationic group elsewhere in the site, presumably present to bind the pyrophosphate moiety of NADH. The 4-pyridylmercapto group, which is uncharged at neutral pH, would not be subject to this influence.

Further evidence for the hypothesis that the sulfhydryl group susceptible to modification exists in or near the NADH binding site arises from my work with NADH analogues. Duckworth and Tong (1976) have shown that 5'AMP and 3'AMP compete with NADH for its binding sites on citrate synthase. My work has demonstrated that ADPR, 2'5', and 3'5' ADP also compete for NADH binding sites as measured by fluorescent enhancement. Support for the above suggestion results from the findings that ADPR, 5'AMP and 3'AMP all significantly block the reaction between citrate synthase and DTNB or 4,4'-PDS, leading to the speculation that the sulfhydryl which is modified lies in the adenylate binding portion of the NADH site. It is interesting to note that ADPR and 5'AMP activate the enzyme, suggesting that the inhibitory characteristics of NADH derive from the nicotinamide end of the molecule, and that partial occupation of the allosteric site may lead to heterotropic interactions at the active site that are opposite to that normally seen. 3'AMP has an inhibitory effect on the enzyme and has been shown to be competitive with ACoA (Duckworth and Tong 1976). This suggests that either it is binding at the active site and interacting heterotropically with the allosteric site to block the modification of the sulfhydryl group there, in much the same manner as ACoA, or else that

it can bind to both the active and allosteric sites.

It is apparent from this work that KCl, an allosteric activator, and ACoA, a substrate, act in different ways on the enzyme. Weitzman (1966) first showed that KCl altered the reactivity of the enzyme to DTNB by measuring its susceptibility to inactivation upon modification. Faloona and Srere (1969) have found that K^+ affects both the initial rate and the extent of reaction of the enzyme with DTNB, as well as the rate of inactivation upon modification. The work presented here confirms these findings. 100 mM KCl, the amount needed for full allosteric activation, enhances the rate of reaction of a quickly reacting sulfhydryl group, while virtually eliminating secondary reaction, in the case of DTNB, and slowing the rate of a slower reacting group in the case of 4,4'PDS. It is important to note that this is generally an opposite effect from that of ACoA, indicating that ACoA and KCl are not acting in exactly the same manner on the enzyme. The implication of this is that ACoA and KCl cannot be causing the same allosteric shift between the T and R state in the MWC model of allosteric behavior. More than two states must be postulated in order to account for the differing behavior of ACoA and KCl on sulfhydryl reactivity.

An alternative explanation concerning the method of action of DTNB modification must be considered. This hypothesis arose from consideration of Table 1, which suggests that the modified enzyme has undergone a significant conformational change such that the allosteric parameters for ACoA and KCl saturation have been altered. The lower $S_{0.5}$ and Hill values for ACoA and the lower Hill value for KCl suggest that the modified

enzyme now exists in a more R- like state. The higher KCl $S_{0.5}$ value for the modified enzyme, indicating that it binds less tightly, may be another manifestation of my above suggestion that ACoA and KCl act in different manners on the enzyme to produce an enhancement of activity. It may be that the DTNB modification occurs at another, physically separated site, far from the NADH binding site, and abolishes NADH binding because it causes a decisive conformational shift from T to R state. Modification in the presence of NADH might be inhibited due to burying of the sulfhydryl group in the course of an NADH induced allosteric shift. This last suggestion must be accompanied by the further assumption that the enzyme does not normally exist in the T state in the absence of effectors, but in some "intermediate" state between T and R. There are no other data to support this hypothesis; in fact, the hyperbolic binding curve of NADH to the enzyme (Duckworth and Tong 1976) argues against it. But the strongest objection to this argument is that since the modified enzyme has a Hill value for ACoA and KCl above 1.0, there must exist, after modification, a significant percentage of T state of the enzyme, or "intermediate" state capable of going to the T (NADH binding) state. If all the enzyme existed in R state, the Hill values would be 1.0, since ACoA or KCl would not induce an allosteric shift. If T state, or the potential for T state, exists, the modified enzyme must be able to bind NADH. Since I find little or no binding to the modified state, the only likely conclusion is that modification leads to a localized blocking at the NADH site, preventing binding, as well as some secondary conformational changes such that the allosteric parameters are perturbed.

The reaction between IAA and citrate synthase was investigated as another example of modification of the enzyme leading to alteration in its response to NADH. Although the enzyme is slowly inactivated by the reagent, modification leads to loss of NADH inhibition at a slightly faster rate. These preliminary findings prompted me to make a series of enzyme samples carboxymethylated to differing extents by IAA, subjecting them to analysis for loss of NADH binding sites. It was hoped that the slight selectivity exhibited in the functional effects would mean that the cysteine involved in the NADH binding site was being preferentially modified by ^{14}C-IAA, allowing easy isolation of the relevant peptide in any future sequence investigations. Kinetic analysis of the rate of modification by IAA indicates that the reagent reacts with an overall rate constant of $(1.2 \pm 0.05) \times 10^{-2}$ hr^{-1}. Since this is almost identical with the rate constant for loss of NADH binding ($1.35 \pm 0.10 \times 10^{-2}$ hr^{-1}), it is likely that the sulfhydryl associated with the NADH site is not preferentially carboxymethylated. Thus reaction with IAA will not lead to a specifically labelled NADH peptide directly. An alternative approach would be to label the reactive sulfhydryl with DTNB, then expose the modified protein to cold IAA. With all other reactive sulfhydryls blocked, the DTNB could be removed with DTT and the regenerated sulfhydryl labelled with hot IAA, allowing for easy identification of the important peptides.

This work has also allowed me to strengthen the hypothesis that the modification of the DTNB or 4,4'-PDS - sensitive sulfhydryl is responsible for the loss of NADH binding and that DTNB and 4,4'-PDS react with the same sulfhydryl group. The disappearance of NADH binding capacity

and the disappearance of DTNB- reactive groups during IAA modification proceed with pseudo first order rate constants that are essentially the same, within the precision of the measurements. This is consistent with the evidence already given that DTNB and NADH interact at or close to the same sulfhydryl group. Although my analysis of the rate of disappearance of 4,4'-PDS reactivity during IAA modification is somewhat artificial, in that I fixed the rate at the rate of DTNB disappearance, I think it demonstrates that the data are consistent with the hypothesis that DTNB and 4,4'-PDS are reacting with the same sulfhydryl group. Thus we have a situation in which the enzyme is modified by two different disulfide reagents, DTNB and PDS, as well as being modified by a different mechanism by IAA. All three reagents modify 1 particular sulfhydryl group involved in NADH regulation of the enzyme with varying degrees of specificity. In all cases the modification leads to loss of NADH binding, along with varying effects on enzyme activity. This susceptible sulfhydryl group may well be very useful as a marker for the NADH site in the primary sequence of the enzyme when future work on its sequence is done.

Weitzman and Jones (1968) have conducted a regulatory survey of citrate syntheses from a large number of bacterial genera in order to ascertain the prevalence of NADH inhibition of the enzyme, as well as the incidence of AMP reactivation of the NADH inhibited enzyme, first (mistakenly) reported for E. coli citrate synthase by Weitzman (1967). Subsequent publication (Weitzman and Danson, 1976) identified these results as arising from contamination of the E. coli culture with Acinetobacter lwoffi, a Gram-negative strict aerobe. Results of the survey (Weitzman

and Jones, 1968) classify bacteria into two major groups i) citrate synthase that is sensitive to NADH inhibition, which seem to exist in Gram-negative bacteria and ii) Gram-positive bacteria citrate synthase which exhibit no NADH inhibition. The Gram-negative bacteria can also be subdivided on the basis of whether or not their NADH inhibition can be reversed by the action of AMP. E. coli is an example not showing AMP reactivation of the NADH inhibited enzyme, while in A. lwoffi, the NADH inhibition of citrate synthase is relieved by AMP. In this case, the subdivision coincides with the metabolic distinction between the two groups, one being facultative anaerobes, and the other strictly aerobic, respectively. A rationale for such a division has been suggested by Weitzman and Jones (1968) based on the metabolic pathways of energy production used by these two classes of bacteria. The facultative anaerobes, as typified by E. coli, are well endowed with glycolytic enzymes, some of which are sensitive to activation by AMP or ADP in a manner consistent with a role in the regulation of the energy charge of the cell. Thus the major source of energy production in these bacteria is fermentation alone, without use of the citric acid cycle, and since "control points" already exist, they do not need energy control sites at "later" points in the metabolism. The strict aerobes, on the other hand, are absolutely dependent on the citric acid cycle for energy production. Thus, activation of their citrate synthases by AMP (and ADP) may be an example of "low-energy signals" activating a key enzyme in the pathway primarily responsible for energy production.

My findings that adenylates bind to the allosteric site of the

E. coli enzyme suggests a closer similarity than has been suspected between this enzyme and the citrate synthases of the strictly aerobic Gram-negative bacteria. Weitzman (1967) found that the *A. lwoffi* enzyme exhibited a sigmoidal rather than a hyperbolic dependence of the degree of inhibition on NADH concentration and noncompetitive, rather than competitive, NADH inhibition with respect to acetyl-CoA. My results suggest that the NADH binding sites for the two types of enzymes are very similar, and that the different functional effects of AMP arise from shifts in the allosteric equilibrium of citrate synthase which stem from differing subunit interactions. These allosteric effects would entail differences both in L, the allosteric constant and c, the ratio of dissociation constants for the binding of AMP to the R and T forms of the enzyme (using the terminology of Monod, Wyman and Changeux, 1965). Nevertheless, this type of arrangement would be economical from an evolutionary standpoint, since one subunit "type" would be able to serve a number of situations, depending on the energy requirements of a particular species.

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