NADP+-LINKED ISOCITRATE DEHYDROGENASE: A STUDY OF THE CATALYTIC AND REGULATORY PROPERTIES OF THE ENZYME

PEGGY M. HOLLAND

$NADP^+$-LINKED ISOCITRATE DEHYDROGENASE: A STUDY OF THE CATALYTIC AND REGULATORY PROPERTIES OF THE ENZYME

A thesis

by

Peggy M. Holland

Submitted in partial fulfillment of
the requirements for
the degree of Master of Science

Department of Biochemistry
Memorial University of Newfoundland

August, 1971

ACKNOWLEDGEMENTS

The work for this thesis was carried out in the Department of Biochemistry of Memorial University of Newfoundland for which I am grateful to Professor L.A.W. Feltham.

Special thanks are due to Dr. C. Little for his overall supervision of the work.

Thanks are also due to Mrs. H. Kennedy for typing the thesis and my sister Rita for help in drawing the figures.

I am grateful to the National Research Council of Canada for awarding a scholarship.

ABBREVIATIONS

The following abbreviations have been used:

ICDH	-	isocitrate dehydrogenase
SH	-	sulfhydryl
pCMB	-	p-chloromercuribenzoate
NEM	-	N-ethylmaleimide
DTNB	-	5,5'-dithiobis(2-nitrobenzoic acid)
DNA	-	deoxyribonucleic acid
RNAse	-	ribonuclease
Tris	-	tris(hydroxymethyl)amino-methane
OAA	-	oxaloacetic acid
PPO	-	2,5 diphenyloxazole
POPOP	-	2,2'p phenylenebis(5 phenyloxazole)
LAHPO	-	linoleic acid hydroperoxide
MDH	-	malate dehydrogenase
LDH	-	lactate dehydrogenase
AMP	-	adenosine 5'monophosphate
ADP	-	adenosine 5'diphosphate
ATP	-	adenosine 5'triphosphate
DPNase	-	NAD nucleosidase (E.C. 3.2.2.5)
TCA cycle	-	tricarboxylic acid cycle

ABSTRACT

NADP-isocitrate dehydrogenase is believed to catalyze at least 3 distinct chemical reactions, to be inhibited allosterically by folate and to be inhibited in a concerted manner by mixtures of glyoxalate and oxaloacetate. Modification of the enzyme from pig heart mitochondria by various chemical reagents and X-irradiation has been used to attempt to differentiate between the sites responsible for the five apparent enzymic functions. Consistent with the views of other workers, the present results suggest that the 3 catalytic activities involve overlapping active sites with a sulfhydryl group in the region of total overlap. Furthermore, the site or sites involved in binding the inhibitors for the concerted inhibition seem separate from the catalytic sites, suggesting that this is an allosteric inhibition. The alleged allosteric inhibition of the enzyme by folate has been examined and suggested to be a non-specific effect without regulatory significance.

Evidence is also given suggesting that like the pig liver cytosol enzyme, the pig heart mitochondrial enzyme can catalyze the NADPH linked reduction of oxaloacetate. However, this activity is very low relative to the isocitrate dehydrogenase activity.

Sulfhydryl or methionine modification can cause inactivation of the enzyme. However, sulfhydryl modification seemed sufficient to explain the inactivation of the enzyme upon X-irradiation. The yields (G-values) for inactivation of the enzyme and for destruction of the 4 most radiosensitive sulfhydryl groups were 0.5 and 2.0 respectively, suggesting that isocitrate dehydrogenase is quite a radiosensitive enzyme possessing some of the most radiosensitive of enzyme sulfhydryl groups. The high chemical reactivity of these groups was further implied by the high sensitivity of the enzyme to various sulfhydryl reagents. Unlike most allosteric enzymes, the regulatory properties of isocitrate dehydrogenase were not more radiosensitive than the catalytic properties.

The results are discussed in terms of the binding site for the inhibitors. A possible regulatory role for the concerted inhibition is suggested and the significance of this in terms of mitochondrial isocitrate oxidation is discussed.

CONTENTS

INTRODUCTION

NADP-linked isocitrate dehydrogenase (threo-D_S-isocitrate:NADP oxidoreductase (decarboxylating) E.C.1.1.1.42) was first discovered in the 1930's during work on the elucidation of the citric acid cycle[1,2,3]. At that time it was thought to be the enzyme involved in the conversion of isocitrate to α-ketoglutarate in the operation of this cycle. Today the function of this enzyme in metabolism and its relevance to the operation of the citric acid cycle is much disputed[4,5]. Further work is essential to elucidate the role of this enzyme in metabolism and its mechanism of action.

Several reviews on the subject of this enzyme have appeared in the literature[6,7]. The following represents a summary of some recent developments and of a number of general and specific topics related to this enzyme.

Occurrence

NADP-isocitrate dehydrogenase activity has been noted in tissues from various animals, plants and microorganisms. However, it has been obtained in purified form from relatively few sources, including baker's yeast[8], parsley root[9], *Thiobacillus novellus*[10] and *Escherichia freundii*[11]. The work cited here applies to the enzyme present in pig heart except where otherwise specified.

Subcellular Distribution

The enzyme isolated from pig heart appears to be of mitochondrial origin. From studies on isocitrate dehydrogenase, two immunologically and electrophoretically distinct isoenzymes have been identified in mammalian species — one from mitochondria and one from cytosol[12,13]. Early investigators of the purification and properties generally assumed that ICDH[14,15,16] was a homogeneous molecular species. It is perhaps fortuitous that pig heart, which contains an abundance of the mitochondrial isoenzyme and very little of the supernatant isoenzyme was chosen as enzyme source.

The cytoplasmic isoenzyme has only recently been isolated from pig liver and its properties studied[17]. A number of major differences between the two enzymes has already appeared from these initial studies (e.g. number of subunits, pH optimum, isoelectric point and molecular weight).

Further studies indicated that neither isoenzyme may be completely confined to either location, but that in certain tissues both isoenzymes may occur either in mitochondria or in cytosol[18,19]. The "mitochondrial" isoenzyme has also been detected in microsomal and nuclear fractions[19]. The fractionation techniques required for this work however make these results somewhat dubious.

The complexity of the localization is therefore undecided. There does appear to be definite compartmentalization and this may be of metabolic significance for the

different functions of the two enzymes.

Isolation

A number of procedures describing the isolation and purification of this enzyme from pig heart have been published [15,16]. Preparations with specific activities of 58 μmoles of $NADP^+$ reduced per minute per mg protein (58 μmoles/min/mg), the highest specific activities yet reported for this enzyme were obtained by Siebert and co-workers[14]. The highest specific activity reported by previous workers was 5.3 μmoles/min/mg[15]. Siebert et al developed two purification procedures. The more elaborate procedure produced enzyme with specific activities as high as 58 μmoles/min/mg, while a highly simplified procedure readily yielded enzyme ranging from 10.6 to 15 μmoles/min/mg. From ultracentrifugation studies on the purest preparations it appeared that 95-98% of the protein was present in the major peak corresponding to ICDH. On the other hand, electrophoretic analysis indicated that as much as 35-40% of proteins other than ICDH might be present in the preparation. However, the latter evidence may be of little significance due to the extreme lability of the enzyme under these analysis conditions.

More recently, Colman has obtained an ICDH preparation which appears homogeneous under exhaustive analysis[20,21]. In her method she used CM cellulose chromatography, a system

first used by Rose for purification of ICDH[22], and gel filtration on Sephadex G-150. However, the enzyme which she obtains has a specific activity of 29 μmoles/min/mg — only one-half the specific activity obtained by Siebert. The molecular weight and catalytic properties of the enzyme obtained in both cases are similar. The possibility that Colman's preparation is only 50% pure is unlikely on the basis of the characterization studies which she has carried out. The enzyme exhibits a single peak in sedimentation velocity experiments and a single band on both disc electrophoresis and cellulose acetate electrophoresis. These experiments, as well as sedimentation equilibrium studies, indicate that the enzyme is homogeneous. The reason for the discrepancy between her work and that of Siebert is not immediately apparent. In connection with this it may be noted that Rose, in repeating part of the procedure of Siebert, obtained appreciably lower specific activities at the various stages[22]. It may be mentioned that, as first suggested by Moyle[23], the absolute activity of ICDH is low compared to that of many enzymes, whether it be 29 or 58 μmoles/min/mg.

Enzyme Characteristics

A. Physical properties

ICDH, isolated from pig heart, has a molecular

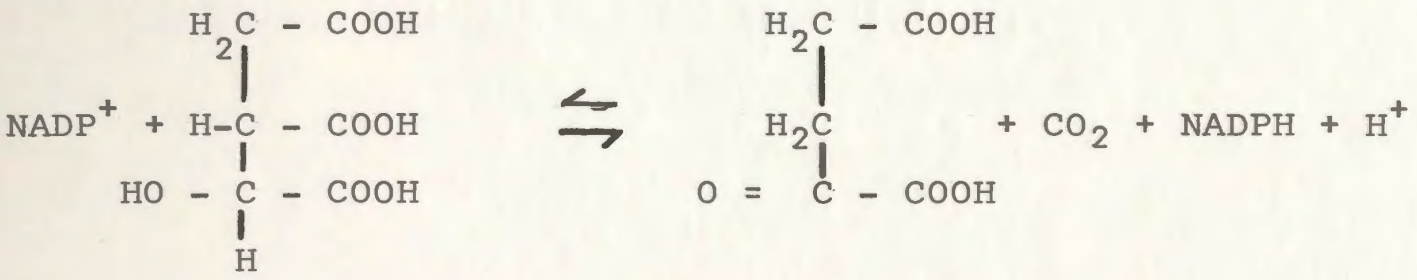
weight of approximately 58,000 [20]. This has been confirmed by a number of workers using a variety of techniques [14,15,21]. Wich such a molecular weight ICDH falls into the grouping of the smaller enzymes.

Conflicting reports have appeared on the structure of the enzyme. Colman contends that it is a monomer[21] while Magar and Robbins state that it appears to be composed of two identical subunits[24]. Colman has shown that her enzyme preparation is homogeneous, while Magar and Robbins stated only that their enzyme had a specific activity comparable to that of Colman. Their purification procedure has not yet been published. An amino acid analysis of the enzyme has been published by Colman[25].

Investigations of the enzyme's properties have been hampered by its instability in solutions of low ionic strength. Its isoionic point has been determined to be pH 4.0 [23], and its isoelectric point was originally estimated to be approximately pH 7.4 [14]. Determination of the enzyme's isoelectric point was complicated by the extreme lability of the protein which can however be stabilized by high salt concentrations or by glycerol. In an electrophoretic analysis of the protein, Colman noted that the enzyme showed a tendency to migrate toward the cathode, even at pH 9.0 [20]. This is a variance with earlier workers and indicates an isoelectric point above pH 9.0.

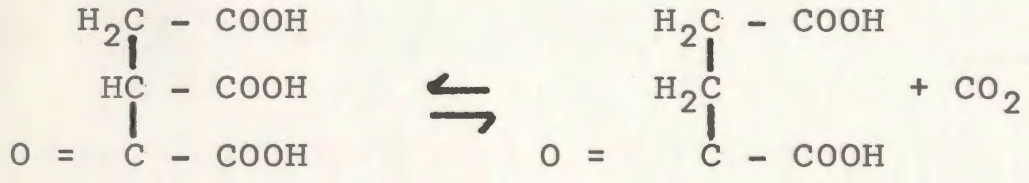
B. Catalytic Properties

From an enzymological point of view ICDH is of particular interest because of its multifunctional nature. Early investigations[26,27,28] with aqueous extracts from acetone powders of pig heart revealed that such preparations catalyzed the oxidative decarboxylation of isocitrate to α-ketoglutarate and the reversal (Eq. 1).



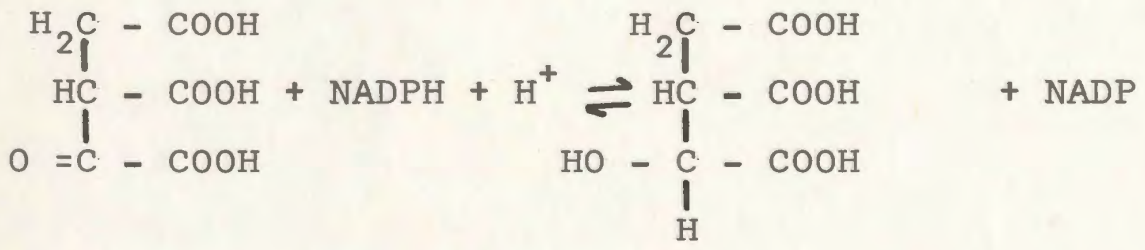
isocitrate α-ketoglutarate [1]

The decarboxylation of oxalosuccinate (Eq. 2)



oxalosuccinate α-ketoglutarate [2]

and the reduction of oxalosuccinate to isocitrate (Eq. 3)



oxalosuccinate isocitrate [3]

At that time it was uncertain whether or not a single enzyme catalyzed each of these reactions. Investigation of this problem with purified preparations has shown that the reactions represented in equations 1 - 3 are indeed inherent properties of isocitrate dehydrogenase[29]. The ratios of the activities were shown to be constant over an 84-fold purification of the enzyme[30].

The enzyme is absolutely specific for $NADP^+$ and is inactive with NAD^+ [3]. The dehydrogenase and decarboxylase and possibly also the reductase activity show a metal ion requirement with Mn^{++} being more effective than Mg^{++} [30,23 3]. The metal ion is not essential for the binding of substrate or co-enzyme, but may serve to enhance such binding[31,32]. The mechanism does not appear to require any specific order of binding of substrates and cofactors to the enzyme, i.e. the reaction follows random order kinetics[33,20].

Mechanism

A. Reaction Sequence -

The presence of oxalosuccinate decarboxylase (Eq. 2) and reductase (Eq. 3) activities in this enzyme suggests that the overall reaction (Eq. 1) involves sequentially the oxidation of isocitrate to oxalosuccinate (reversal of Eq. 3) followed by its decarboxylation to α-ketoglutarate (Eq. 2)[6]. Free oxalosuccinate is not thought to participate in the overall reaction and it is possible that the true

intermediate is an enzyme-bound form of oxalosuccinate in equilibrium with free oxalosuccinate[23,30]. The formation of oxalosuccinate from isocitrate (reversal of Eq. 3) or from α-ketoglutarate + CO_2 (reversal of Eq. 2) could not be demonstrated under a variety of conditions. This may indicate that the rate of reaction of the bound form of oxalosuccinate to either product (i.e. to isocitrate or to α-ketoglutarate and CO_2) is considerably greater than the equilibrium between free and bound oxalosuccinate[30], or that such an equilibrium strongly favours the formation of the oxalosuccinate enzyme complex[23].

From the varying sensitivities of these activities to protein modification, from solvent isotope effects and from other studies, it has been inferred that the dehydrogenation step in the oxidative decarboxylation of isocitric acid is rate-determining[20,32,34]. This was initially suggested by Moyle[23] in 1956 from her studies on the maximum rate of the various reactions and later reinforced by the work of Siebert et al[30]. Colman's extensive studies on the enzyme have led to a similar conclusion. Recently, in agreement with these workers use of heavy atom isotopes has clearly demonstrated that the decarboxylation step is not rate limiting but is much faster than the rate determining step[35]. The slow step in the reverse reaction is thought to be the carboxylation of α-ketoglutarate[30].

B. Active site -

It is apparent that two mechanistically distinct

reactions, dehydrogenation and decarboxylation, are carried out by a relatively small enzyme molecule. However, at the present time knowledge of its active site is still insufficient to enable the proposal of a detailed mechanism.

Information about the mechanism has emerged from the studies of Colman who has compared the response of the various catalytic activities to a number of different treatments[20, 32, 36,37, 38]. Most of the reagents used to modify the enzyme activity have been sulfhydryl binding agents. Sulfhydryl groups were implicated in the activity of ICDH by the work of Lotspeich and Peters[39], who found that o-iodosobenzoate, copper and mercury compounds, and diphenylchloroarsine inhibited the enzyme. Since only SH groups are thought to respond to these inhibitors the SH nature of ICDH was established.

Seibert et al showed that both dehydrogenation and decarboxylation were inhibited by the SH reagent pCMB[30]. Protection by substrates and cofactors against inactivation has lent weight to these observations, indicating that the observed effect is indeed due to reaction with the enzyme. These studies showed that modification of the SH groups of ICDH resulted in loss of enzyme activity. The significance of this in terms of the enzyme's structure was not established. While the possibility exists that these groups are directly involved in the catalytic activity of the enzyme, other possibilities also exist. Introduction of the reagent into the enzyme may

sterically or electrostatically hinder reactions proceeding at the active center or merely disrupt the structure of the enzyme.

As well, in ICDH the situation is somewhat more complex. In such a dual function enzyme the catalytic site may be viewed as a composite one in which certain amino acid residues are involved in one catalytic reaction, other amino acid residues are involved in a second reaction, and a third group of residues bridges the two regions of the active site, and is essential for both. From this point of view Colman has explored the effects of various reagents on the different enzyme activities and properties. With such an approach it may be possible to differentially modify the catalytic activities of the enzyme and thus link certain amino acid residues with different parts of the active site.

In studying the SH character of the enzyme, she has shown that it contains a total of 13 SH groups[32, 20]. Colman also noted that both the rate of SH modification and the extent of the reaction varied with the thiol reagent used. Thus DTNB reacted with five SH residues whereas NEM[36, 37] reacted with only two. However, in the presence of substrate and cofactor, only three residues react with DTNB and the enzyme activity is retained. This indicates that these three SH groups, while available for reaction with DTNB, are not involved in the active site. A maximum of two SH groups is therefore involved in the loss of activity. Work with NEM

implicates only one SH group directly in the catalytic activity. With DTNB and NEM Colman has found that all three enzyme activities (Eq. 1 and its reversal and Eq. 2) were effected to the same extent. At this point therefore SH groups cannot be directly related to any one particular activity.

Iodoacetate has been generally used as a sulfhydryl reagent. The SH nature of ICDH was initially disputed due to conflicting reports on the effectiveness of this substance as an inhibitor of ICDH [39, 3, 40]. Colman found that iodoacetate does indeed inhibit ICDH when incubated with the enzyme below pH 6.0 [20,25]. However, the inhibition is not due to binding of SH groups but to the alkylation of a single essential methionine residue. The binding was shown not to alter the physical characteristics of the enzyme. The reaction destroys the dehydrogenase activity more than the decarboxylase activity, thus indicating that the active sites for the two reactions may be at least partially separable. The loss of activity on alkylation of this residue indicates that the methionine residue is essential for activity. Since isocitrate protects against inactivation the reaction is thought to occur at the dehydrogenase active site.

Allostery

The activity of intracellular enzymes may be governed by a variety of factors such as changes in the rates of synthesis or destruction of the enzymes, or fluctuations in

substrate or product levels. However, certain key enzymes are regulated by a finer control process - allostery[41]. Whilst such allosteric enzymes frequently exhibit a variety of properties including anomalous kinetics and a subunited structure, their major feature is that they are subject to the influence of low molecular weight substances, termed effectors, which may or may not be structurally related to the substrate[42]. The binding of an effector molecule at one site (the allosteric site) is able to alter the properties of the separate catalytic site in the enzyme. Since allosteric effectors can either enhance or inhibit the catalytic activity, allostery has been postulated as one of the major processes of metabolic regulation in the cell.

In the case of ICDH, folic acid and mixtures of glyoxalate and oxaloacetate have been suggested as possible allosteric effectors although as yet the evidence is somewhat inconclusive.

A. Folic acid -

During a survey of the effect of physiological compounds on a number of nicotinamide nucleotide dehydrogenases, Magar and Homi[43] observed that folic acid inhibits the reduction of NADP by pig heart NADP-ICDH. In the presence of folate a plot of reaction velocity at variable isocitrate concentration yields a sigmoid curve — often a characteristic of allosteric effectors. In addition, the inhibition is neither competitive nor non-competitive with any of the substrates of ICDH. Their

preparation of ICDH was shown to be free of dihydrofolate reductase activity. On the basis of their results they concluded that folate could be inducing a conformational change in the protein and might therefore represent an allosteric effector of the enzyme. No further reports on this inhibition have appeared in the literature to date.

B. Glyoxalate and oxaloacetate -

The inhibition of NADP-ICDH by mixtures of glyoxalate and oxaloacetate was brought to light as a result of extensive studies by a number of workers, leading from the initial observation that glyoxalate inhibits the oxygen uptake of various respiring tissue suspensions[44, 45, 46, 47, 48]. Several studies indicated that the inhibition was due to a product formed by a nonenzymic condensation of glyoxalate and oxaloacetate — either oxalomalate (αhydroxy-β-oxalosuccinate)[47,49] or γhydroxy-α-ketoglutarate[50]. Both substances were shown to inhibit IDCH from a number of sources including pig heart[49]. However, in a further investigation of the inhibition, Shiio and Ozaki determined that while it was evident that oxalomalate was a potent inhibitor of ICDH, the condensation product could not be detected under the conditions of the enzyme assay[51].

In examining the nonenzymic condensation they showed that at low concentrations, such as those used to inhibit the enzyme, the condensation reaction, followed by determining the decrease in glyoxalate was not significant. At higher concentrations the condensation was almost complete and was stimulated

by the presence of 20 mM Mg^{++} or Mn^{++} ions in agreement with Ruffo *et al*[47]. Marr and Weber reached a similar conclusion because of the long period of time required to form the compound (2 - 3 hours) and the short duration of the enzymatic reaction (30 - 60 seconds)[52].

Shiio and Ozaki examined the kinetics of the inhibition. Inhibition by glyoxalate, glyoxalate plus oxaloacetate, or the condensation product was competitive with respect to isocitrate, while inhibition by oxaloacetate was of mixed type. Kinetic analysis has shown that when glyoxalate has bound to the enzyme, the subsequent binding to the enzyme of oxaloacetate is enhanced 60,000 fold. Similarly oxaloacetate enhances glyoxalate binding 60,000 times. Therefore it appears that the inhibition of ICDH activity, by the presence of oxaloacetate and glyoxalate in the assay system, is due to the interaction of these individual components with the enzyme molecule, without any prior condensation. The inhibition is of a concerted type, first described by Datta and Gest[53] for homoserine dehydrogenase.

On the basis of the available evidence, the significance of the concerted inhibition cannot be interpreted with certainty. It may possibly represent a form of feedback inhibition.

C. Implications of allostery in ICDH -

The possible allostery of ICDH is of interest from

several points of view. Although the current models for allosteric interactions are based on the presence of interacting subunits[41], if one defines an allosteric enzyme as one in which the binding of one small molecule at one site influences the properties of a distant site in the enzyme, there seems no a priori reason why non-subunited enzymes should not exhibit allostery. It should indeed be possible for such effects to be mediated within a single polypeptide chain. In this respect the occurrence of allostery in ICDH, a small protein which probably does not possess subunits, would indeed be significant. Furthermore, confirmation of the metabolic significance of these regulators, whether allosteric or otherwise, may shed some light on the in vivo role of ICDH. Part of the evidence discrediting NADP-ICDH from a functional role in the TCA cycle is in fact its lack of regulators[4]. Investigation of possible regulators may indicate a role for the enzyme either in the TCA cycle or in some other metabolic sequence.

Interactions of Ionizing Radiations with Enzymes

It is of particular interest to irradiate an enzyme possessing the properties of ICDH. This is true whether viewing such a study as a tool for further investigation of the properties of this enzyme or as a means of increasing our understanding of radiobiology at the molecular level.

A. Direct and Indirect Action -

Radiobiologists have established the existence of two types of action which lead to loss of biological activity — direct and indirect. If the absorption of radiation occurs in the molecule in which the damage appears then this is the direct action of radiation, while with indirect action the absorption of the radiation energy and the response to this energy occur in different molecules[54]. The radiosensitivities of molecules to these two different types of action is markedly different since the causative agents are different. In indirect studies in aqueous solutions such species as $OH^{\cdot}$, $H^{\cdot}$, the hydrated electron (e^{-}_{aq}) and H_2O_2 interact with the biological molecules[54].

B. Target Molecules *in vivo* -

From a variety of studies DNA has been generally acknowledged as the critical *in vivo* target for ionizing radiations[55,56]. Despite this, *in vitro* radiation sensitivity of both DNA and enzymes is of the same order of magnitude and yet less than the sensitivity of living cells. The difference lies in the fact that *in vivo*, replication amplifies any damage to an individual DNA molecule. Thus DNA is a key radiation target because of its function as a template. It is now apparent that the cell is able to modify the effects of high energy radiation, partly via an enzyme mediated repair system[57,58]. Therefore, radiation damage to DNA repair enzymes

with concomitant damage to DNA, would be very serious indeed. From this point of view damage to other proteins with vital functions would be of consequence. For example, it has been suggested that the mitotic spindle proteins contain sulfhydryl groups which might render them susceptible to radiation damage. In fact, hypotheses have been advanced suggesting that sulfhydryl groups of various cellular components, including enzymes, might be radiation targets[56,59]. While the evidence for this is not as conclusive as the DNA hypothesis, it certainly indicates that SH groups may be one of the modifying factors in the radiosensitivity of a cell.

C. Effects of Irradiation of Enzymes *in vitro* -

Since it is important to examine the possibility of radiation damage to vital proteins, the interaction of radiation with enzymes has been considered. Okada and Fletcher have suggested that *in vivo* there may be an amplification of the action of irradiation on one or more parts of a series of enzyme reactions in which one system serves as the substrate for the next[60]. Under such conditions an almost undetectable effect of radiation on a single enzyme could lead to a large change in the final products of the reaction chain. Correlations of the *in vitro* inactivation with chemical and physical alterations in the molecule have been carried out for a number of enzymes. By studying the effects of radiation on these purified enzyme preparations, one removes the complicating factor of the cell's ability to protect against and

modify the effects of radiation damage. From such studies two general causes of the loss of biological activity of enzymes have emerged: (i) conformational changes in the enzyme, and (ii) alteration of specific amino acid residues in the active site.

(1) Changes in molecular properties and catalytic activity - Certain amino acid residues have been found to be considerably more radiosensitive than others. With the indirect effect cysteine, tryptophan, cystine and methionine have been shown to be among the most sensitive of the amino acids[61,62]. However, the sensitivity of a residue is modified by its presence in a protein molecule and is indicative of its environment. With the S-peptide of RNAse, methionine, phenylalanine, histidine and arginine were more sensitive than the other residues in the protein[62]. Mee and Adelstein were able to show however that x-ray inactivation can be linked to modification of the essential methionine residue with very little destruction of the other amino acids[63]. Damage to SH groups has been implicated in the loss of activity of papain [64, 65, 66], phosphoglyceraldehyde dehydrogenase[67], alcohol dehydrogenase[68] and several other enzymes[69]. With glutamate dehydrogenase, an extensive study has shown that inactivation is the result of a variety of contributing factors[70,71]. Irradiation resulted in modification of amino acid residues

and this led to modification in the tertiary structure with resulting loss of maximum activity.

In enzymes which exhibit a number of different measurable activities, radiation has been shown to differentially affect some of these activities, indicating that inactivation events may indeed be complex. When some irradiated enzymes are assayed with substrates of different types the extent of apparent inactivation is found to be dependent on the type of substrate. X-irradiation differentially affects the esterase and proteinase activities of chymotrypsin[72] and trypsin[73]. With other enzymes kinetic examination shows that the effect of radiation on substrate binding ability is different from that on catalytic activity[74,75].

(2) Changes in regulatory properties -

Studies on the effects of irradiation on the biological activities of enzymes have now been extended to include examination of the effects on the allosteric or regulatory properties. From these studies Sanner and Pihl postulated that the allosteric sites of enzymes may be more radiosensitive than the catalytic sites[76,77]. This hypothesis, if validated, could add a new dimension to the study of radiobiological effects.

Damjanovich et al showed that inactivation of phosphorylase b involved largely an effect on the allosteric sites with loss of ability to bind the essential activator and consequent loss of ability to bind substrate[77]. As well, aspartate transcarbamylase[76,78], fructose-1,6-diphosphatase[79], glutathione

peroxidase[80] and threonine-sensitive aspartokinase-homoserine dehydrogenase[81] have all shown some allosteric or regulatory functions which are of greater radiosensitivity than the normal catalytic activity. This provides a mechanism for a more subtle form of radiation damage in which the cell may still function, but activities are no longer under control.

D. Irradiation and ICDH -

There have been no previous studies carried out on the irradiation of ICDH in vitro. The radiation sensitivity of protein SH groups is parallelled by their reactivity towards chemical agents[82]. ICDH has been shown to contain chemically reactive SH groups which might respond to irradiation. As well the enzyme possesses a methionine residue which has been implicated in the enzyme activity and this might likewise be affected. In this manner x-rays might represent another reagent capable of specifically modifying the active site. As well, the enzyme is multifunctional and the possibility exists that x-rays might differentiate between these activities as in the case of chymotrypsin. Finally, the possible allostery of this molecule provides a system to examine the hypothesis of Sanner and Pihl while also affording a closer look at these regulators and their possible binding sites.

The following reports an extension of the work on the enzymology of ICDH. The use of sulfhydryl agents to impair both the catalytic and the possible regulatory properties has been examined. There have been no previous attempts to examine the

response of the regulatory properties of ICDH to enzyme inhibitors. The effect of x-rays on these activities has similarly been studied and compared with the effects of certain functional group specific chemical reagents in an attempt to correlate molecular damage with loss of activity.

MATERIALS AND METHODS

Materials

Pig heart $NADP^+$-linked isocitrate dehydrogenase, (Type IV) was supplied by Sigma or by Boehringer-Mannheim as a solution in 50% glycerol and was further purified by the method of Colman[20] (see Results section). Glutathione reductase, trypsin and alkaline phosphatase were also obtained from Sigma. Tritiated-iodoacetate was purchased from New England Nuclear. Hyamine hydroxide was obtained from Packard. Ampholine carrier ampholytes were obtained from LKB Producter, Sweden. All other biochemicals were supplied by Sigma. Other reagents used were of the analytical grade of purity. Oxalosuccinic acid, triethyl ester, was hydrolyzed prior to use as described by Ochoa[83]. Linoleic acid hydroperoxide was prepared as described by Little and O'Brien[84] and stored as a 0.5 mM solution in ethanol at -20°.

Methods

1. Enzyme assays -

Isocitrate dehydrogenase activity was measured at 30° by modification of the method of Ochoa[85]. The assay system contained 0.025 M Tris-HCl buffer, pH 7.4, with 2×10^{-4} M, 4×10^{-3} M and 6×10^{-3}M NADP, DL isocitrate and manganese sulfate respectively in a total volume of 3.0 ml.

Activity was assessed spectrophotometrically using either a Unicam SP500 or a Beckman DB-G in conjunction with a Beckman Log/Linear recorder. Initial velocities were determined by measuring the rate of increase in absorbance at 340 nm due to formation of NADPH.

Specific activity is defined in terms of this assay as micromoles of NADP reduced per minute per mg of protein. Protein was estimated spectrophotometrically at 280 nm ($E_{280}^{1\%} = 9.10$) [20] and also by the method of Lowry[86] with bovine serum albumin as standard.

Reductive carboxylase activity was also assessed spectrophotometrically at 340 nm and 30° according to the method of Colman[20]. The assay contained 0.04 M Tris-HCl buffer, pH 7.4, with 2×10^{-4} M, 2×10^{-2}M, 4×10^{-2} M and 2×10^{-3} M, NADPH, α-ketoglutarate, potassium bicarbonate and manganese sulfate respectively, in a total volume of 3.0 ml. The potassium bicarbonate stock solution was saturated with carbon dioxide prior to use.

Oxalosuccinate decarboxylase activity was measured spectrophotometrically at 240 nm and 22° by the method of Grafflin and Ochoa[16]. The assay contained 1.34×10^{-1} M, 1.67×10^{-3}M and 2.3×10^{-4} M potassium chloride, oxalosuccinate and manganese sulfate respectively in 0.2 M sodium acetate buffer, pH 5.6, in a total volume of 1 ml.

Reduction of oxaloacetate was measured following the

procedure developed by Illingworth and Tipton[87] for the NADP-ICDH from pig liver cytoplasm. The assay contained 2×10^{-3} M oxaloacetate, 2.5×10^{-2} M magnesium chloride and 0.125 M NADPH in 0.1 M potassium phosphate buffer, pH 6.0 at 30°. The reaction was monitored by following NADPH oxidation at 340 nm, or oxaloacetate disappearance at 283 nm. At the pH of the assay system a nonenzymic decomposition of the nucleotide occurs. This control rate was corrected for in all assays of OAA reductase carried out at 340 nm. Similarly assays at 283 nm were corrected for the non enzymic decomposition of oxaloacetate.

2. Supplementary assays -

tate dehydrogenase (EC 1.1.1.27). The activity of the enzyme was determined using the method of Kornberg[88]. The assay contained in a total volume of 3.0 ml, 3.4×10^{-2} M potassium phosphate buffer, pH 7.4, 3.3×10^{-4} M sodium pyruvate and 6.6×10^{-5} M NADH.

Glutathione reductase (EC 1.6.4.2). The enzyme was measured spectrophotometrically at 340 nm according to the method of Racker[89].

Trypsin (EC 3.4.4.4). The assay was conducted spectrophotometrically at 253 nm by the method of Schwert and Takenaka[90] using N-benzoyl-L-arginine ethyl ester (BAEE) as substrate.

Alkaline phosphatase (EC 3.1.3.1). Activity was measured by monitoring the release of p-nitrophenylate anion at 410 nm, using a modification of the method of Lowry et al[91]. The assay system contained 5 x 10^{-2}M glycine-NaOH buffer, pH 10.0, with 8 x 10^{-3} M magnesium chloride, 4.6 x 10^{-4} M p-nitrophenyl phosphate and 50 µg enzyme in a total volume of 3 ml. Malic dehydrogenase (EC 1.1.1.37). Using the method of Ochoa[92] the decrease in absorbance at 340 nm due to oxidation of NADH was measured with oxaloacetate as substrate. Malic dehydrogenase activity was also assayed in the opposite direction by following the rate of reduction of NAD at 340 nm. The latter assay system contained 0.1 M glycine-NaOH buffer, pH 10.0, with 1.6 x 10^{-3} M malate and 2 x 10^{-3} M NAD.

3. Enzyme purification -

A number of methods of purification of ICDH were investigated and these will be discussed in the Results section.

4. Kinetic parameters -

In measuring the individual Michaelis constants each substrate was varied in turn with the other substrates being kept at the concentrations specified in the above assay procedures.

5. Enzyme modifiers -

The modifiers glyoxalate, oxaloacetate and folate, were made up in deionized water and adjusted to pH 7.4 with

NaOH. Solutions of glyoxalate and oxaloacetate were freshly prepared before use. Unless otherwise specified, modifiers and enzyme were preincubated for two minutes in the assay system and the reaction then started by addition of NADP(H).

6. Determination of sulfhydryl content -

Sulfhydryl assays were carried out by the method of Ellman[93]. Available thiol was assessed by direct spectrophotometric measurement of the 2-nitro-5-mercaptobenzoate released upon incubation of the native enzyme with 5,5'dithiobis (2-nitrobenzoic acid) for 15 minutes, at pH 7.7. For measuring total sulfhydryl content the enzyme was first denatured in 30% (w/v) guanidine hydrochloride.

7. Methionine measurement -

The essential methionine residue of the enzyme was measured by following the binding of H^3-labelled iodoacetate at pH 5.5 and 30°. Under these conditions only one methionine and no cysteine residues react[20]. The reaction mixture contained 2×10^{-7} M enzyme, 5.2×10^{-4} M H^3-iodoacetate and 0.05 M sodium acetate buffer, pH 5.5. After 45 minutes incubation the protein was precipitated by addition of perchloric acid (0.2% (w/v) final concentration). The solution was then centrifuged and the precipitate neutralized with ammonium hydroxide, dried and dissolved in hyamine solution. The

scintillation medium (0.49% (w/v) PPO and 0.01% (w/v) POPOP in toluene) was then added and after the mixture had remained for 12 hours at 2°, the sample was counted in an Intertechnique SL30 scintillation spectrometer. The uptake of label was proportional to the amount of protein.

8. Irradiation conditions -

The enzyme was usually irradiated in 50 mM phosphate buffer, pH 6.8 and 1% glycerol. When determining the G-value for enzyme inactivation no glycerol was present in the solution. Prior to irradiation the enzyme was passed down a Sephadex G-25 column which had been equilibrated with 0.05M phosphate buffer, pH 6.8. The enzyme was irradiated in glass vials in equilibrium with air at 0° with a Philips MG100 x-ray machine. The irradiation parameters were 100 kv, 10 ma with 1 mm Be and 0.1 mm Al filtration. The dose rate as measured by Fricke dosimetry was 2.0 kR/min.

In studying the effect of pH on the radiation response of the enzyme the latter was diluted in 0.1 M phosphate or tris buffer of varying pH and then irradiated.

In the protection experiments the various substrates and cofactors were incubated in the enzyme solution during irradiation.

9. Studies with chemical inactivators -

Inactivation studies on the enzyme were carried out

using a variety of reagents known to bind specifically to sulfhydryl or to methionine residues of proteins. The enzyme was incubated with the thiol reagents DTNB and NEM and the methionine reagent iodoacetate under conditions analogous to those used by Colman[20,32,36]. Incubations with pCMB and H_2O_2 were at 0° and at concentrations specified in the Results. Linoleic acid hydroperoxide incubation was carried out at 22°.

10. Isoelectric focusing -

Focusing was carried out at 2° according to the method of Vesterberg and Svensson[94] using a focusing column of 110 ml capacity and accessory equipment purchased from LKB Producter, Sweden. Runs were continued from 24 to 72 hours depending on the ampholine used.

RESULTS

Enzyme Purification

Pig heart NADP-linked isocitrate dehydrogenase as obtained commercially varied in specific activity from 2.8 to 10 μmolar units per mg protein assayed at 37°. Since ICDH preparations of specific activities up to 58 μmolar units per mg protein assayed at 25° have been reported[14], it was desirable for this set of experiments that the commercial enzyme be further purified at least to homogeneity.

1. Examination of Published Purification Procedures -

A number of purification procedures have been published. Several of these were examined but in no case was ICDH obtained completely free of other enzyme activities.

Illingworth and Tipton have recently purified NADP-ICDH from pig liver cytoplasm[87]. Their method which involves elution of the enzyme from a carboxy-methyl cellulose column, using 0.2 mM sodium isocitrate in 10 mM sodium succinate buffer, pH 6.0, was examined. However, the enzyme from heart mitochondria appears to have a much greater affinity for the cellulose column and could not be eluted even with 0.6 mM isocitrate.

The method of Magar and Homi[43], using an ionic strength gradient on CM Sephadex, yielded a final product of variable specific activity. Furthermore, the enzyme was unstable to lengthy storage in the 0.08 M phosphate buffer, pH 6.8,

in which it was eluted.

The method originated by Colman was found to give the greatest and most constant purification[20]. It had the further advantages that the published outline was sufficiently detailed to allow repetition, the techniques practicable and the yields good. Colman has determined that the enzyme which she obtains in this manner is homogeneous.

2. Purification by the Method of Colman -

The enzyme was purified initially by chromatography on CM-cellulose (Sigma) with a column measuring 2.5 x 45 cm. The flow rate was 60 ml per hour and all operations were carried out at 4°. Prior to packing the cellulose was regenerated by successive washings with 0.1 N HCl and 0.5 N KOH. After a final acid washing the cellulose was washed with water, packed and equilibrated with the starting buffer of 0.018 M triethanolamine chloride, pH 7.0, containing 0.9 mM EDTA and 10% glycerol. After elution of an inactive protein peak with the starting buffer, the eluting buffer was changed to 0.1 M triethanolamine chloride, pH 7.7, containing 1 mM EDTA, 0.3 M Na_2SO_4, and 10% glycerol and a second protein peak with ICDH activity was obtained. The peak tubes, when pooled, exhibited a specific activity of 11. The enzyme was concentrated by ultrafiltration using a membrane with a retention minimum of 30,000 molecular weight (Diaflo PM-30 membrane).

The enzyme was further purified by gel filtration on Sephadex G-150 using a column measuring 2.5 x 45 cm. Two protein peaks, the second of which contained enzymic activity were eluted using 0.1 M triethanolamine chloride, pH 7.7, containing 0.1 mM EDTA, 10% glycerol and 0.3 M Na_2SO_4. The flow rate was 4 ml per hour. The purified enzyme was again concentrated by ultrafiltration, centrifuged to remove denatured protein and stored at 2°. This procedure produced an enzyme with a specific activity of 29 µmoles NADP reduced per minute per mg protein at 25°. At 30° the rates are approximately 1.40 times as rapid. A summary of the purification procedure is given in Table I. Although this procedure yielded an enzyme with specific activity similar to that obtained by Colman, it was later discovered that this enzyme preparation is not completely pure in that other enzyme activities are detectable. However, enzyme prepared in this manner was used to carry out the experiments reported here except where otherwise specified.

3. Separation of MDH and IDCH -

NADP-ICDH is a multifunctional enzyme catalyzing dehydrogenation of isocitrate, decarboxylation of oxalosuccinate and reduction of α-ketoglutarate. It has been established that all three activities are inherent properties of the enzyme[29]. These activities could be readily demonstrated in the above preparation of purified enzyme by using the assay conditions described under Methods.

TABLE I

PURIFICATION OF NADP-ISOCITRATE DEHYDROGENASE

Treatment	Specific Activity [a]	Yield (%)
Commercial enzyme (Sigma)	5	100
CM cellulose chromatography	11	77
G-150 chromatography	29	50

[a] Assays were generally conducted at 30°. Specific activities reported here are corrected to give the appropriate specific activity at 25°.

NADP-ICDH from pig heart cytoplasm has recently been purified and an attempt made to compare its properties to those of the mitochondrial enzyme[87]. In their studies of the cytoplasmic enzyme, Illingworth and Tipton[17] discovered yet another catalytic activity — the NADPH dependent reduction of oxaloacetate. Using their assay procedure the mitochondrial enzyme preparation was examined and also showed this activity.

However, malate dehydrogenase also has NADPH-OAA oxidoreductase activity[92] and, consequently, the ICDH preparations were examined for the presence of MDH. Using the assay system outlined in Methods, different batches of purified enzyme were shown to contain significant quantities of MDH. However, since MDH is such an active enzyme, the actual contamination by malate dehydrogenase on a protein weight basis may be less than 5% (Appendix I). Commercial enzyme from both Sigma and Boehringer showed comparable MDH activity. The possibility still exists, however, that MDH was contributing some or all of the NADPH-OAA oxidoreductase activity. In order to clarify this point attempts were made to prepare ICDH free from malate dehydrogenase.

Initially slight modifications of Colman's procedure were attempted. The distribution of malate dehydrogenase was followed throughout the purification. Fig. 1 shows the distribution of MDH on the CM cellulose column. The protein peak eluted with the 0.018 M buffer contains malate dehydrogenase activity and NADPH-OAA oxidoreductase activity but no

Fig. 1. CM cellulose chromatography of ICDH

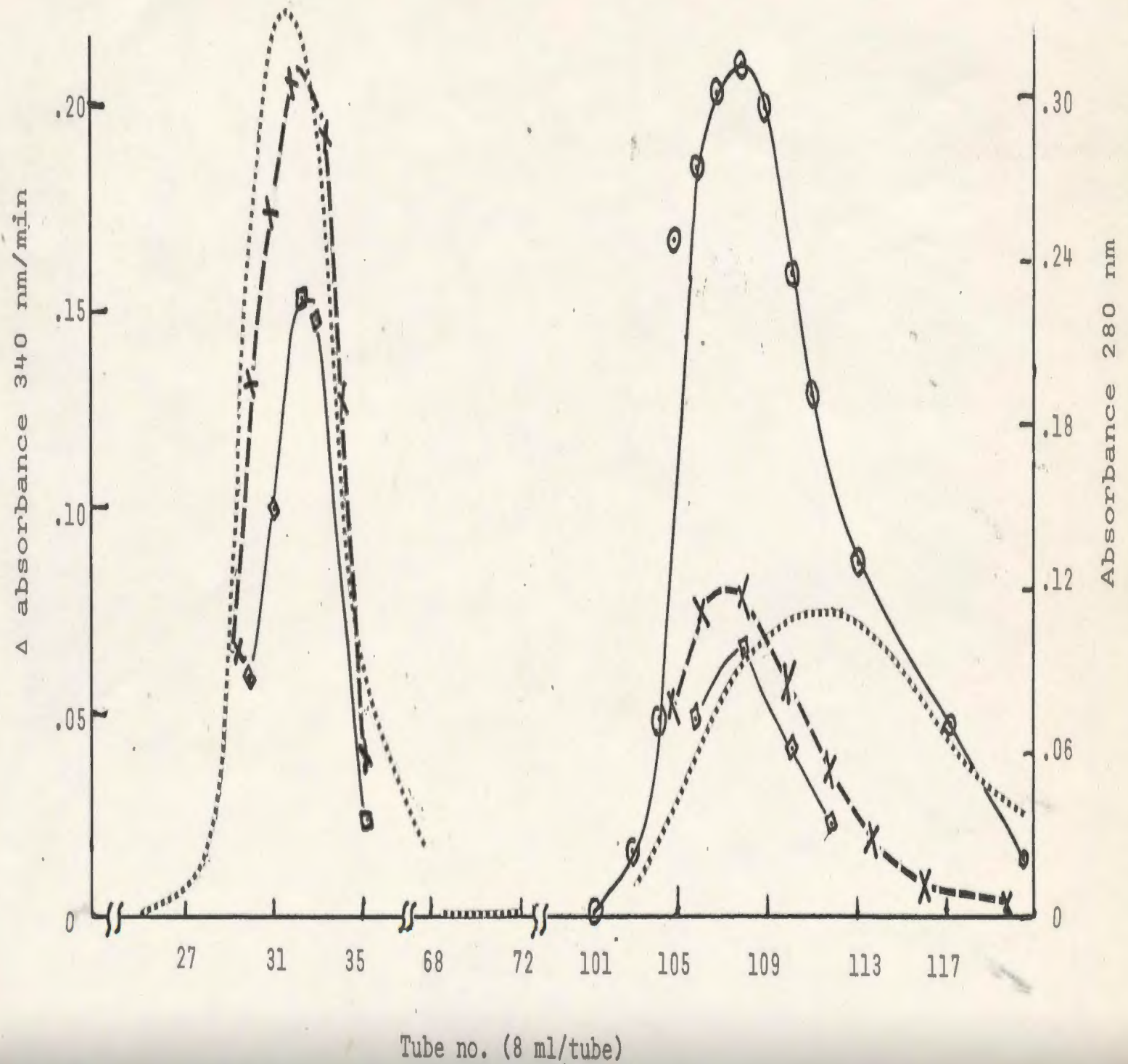


Fig. 1. CM cellulose chromatography of ICDH.

Purification was conducted on a column 2.5 x 40 cm at 4°. With tubes 1 - 68 the eluting buffer was 0.018 M triethanolamine chloride, pH 7.0, containing 0.9 mM EDTA and 10% glycerol and with the remainder was 0.1 M triethanolamine chloride, pH 7.7, containing o.3 M Na_2SO_4, 1 mM EDTA and 10% glycerol. Activities were assessed spectrophotometrically at 340 nm, as described in Methods. Reported activities are for 0.1 ml ICDH (O), 0.5 ml OAA reductase (□), and 0.05 MDH (X).

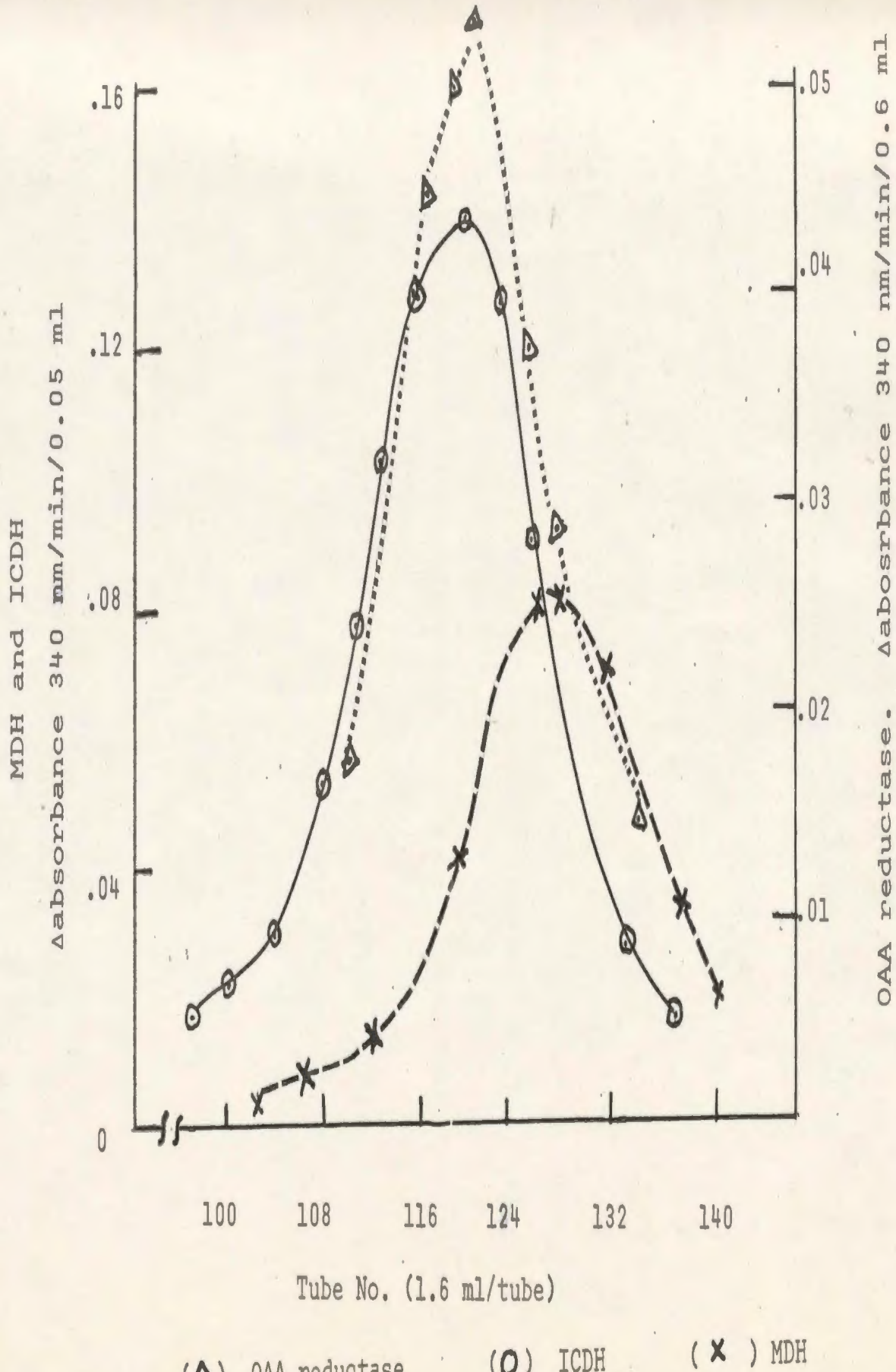
ICDH. On the other hand, the protein peak eluted with the 0.1 M buffer contained ICDH, MDH and NADPH-OAA oxidoreductase activity.

It was of some interest to determine whether or not the commercial enzyme contained lactate dehydrogenase since this was a possible contaminant. Use of the assay system described under Methods confirmed the presence of the enzyme. It was eluted from the CM column with MDH by the 0.018 M buffer. (Specific activity of the peak tube was 17.8 μM/min/mg.) No LDH activity was detected in the protein peak containing the ICDH activity.

In attempting to separate ICDH and MDH, the G-150 column was increased in size to 2.5 x 90 cm. The results of a fractionation of this column are shown in Fig. 2. While the peak tubes for MDH and ICDH do not coindide they are not sufficiently well separated to allow isolation of ICDH free from MDH. It is however interesting to observe that the distribution of the NADPH-OAA oxidoreductase activity corresponds not to the malate dehydrogenase peak but to the ICDH peak. Although this is hardly conclusive it does suggest that the activity may well be a property of ICDH.

Fractionation of the commercial enzyme without prior CM cellulose chromatography was attempted using 3 consecutive G-150 columns, each of 2.5 x 35 cm. Such a system is more efficient than a single long column of 105 cm. While this set

Fig. 2. Gel filtration on Sephadex G-150



up was shown to improve the separation, it does not yield MDH free ICDH (Fig. 3). Direct use of the commercial enzyme in this was unsatisfactory due to the large amount of malate dehydrogenase present. The insert in Fig. 3 shows that the major OAA-NADPH peak corresponds to the MDH peak. However, even in the presence of such high MDH activity a shoulder of the OAA-NADPH activity is seen to correspond to the ICDH activity. The measured OAA reductase activity may be broken down into two components and curves calculated giving the separate contributions of MDH and ICDH to the overall activity. (See Fig. 3 for calculated curves and an explanation of the calculation.) On this basis, MDH accounts for less than 40% of the OAA reductase activity in the region of the ICDH peak.

Since a satisfactory separation on the basis of molecular weight could not be obtained, separation methods based on other parameters were investigated. The use of a combination pH and ionic strength gradient on CMœllulose was tried but did not effect a separation.

Isoelectric focusing, a method acclaimed for its ability to resolve components of very similar nature, was then examined. Although several attempts were made to purify the enzyme by a number of variations of this technique no MDH free ICDH was obtained.

High recoveries of ICDH were obtained by using

Fig. 3. Gel filtration on Sephadex G150 using 3 columns in series.

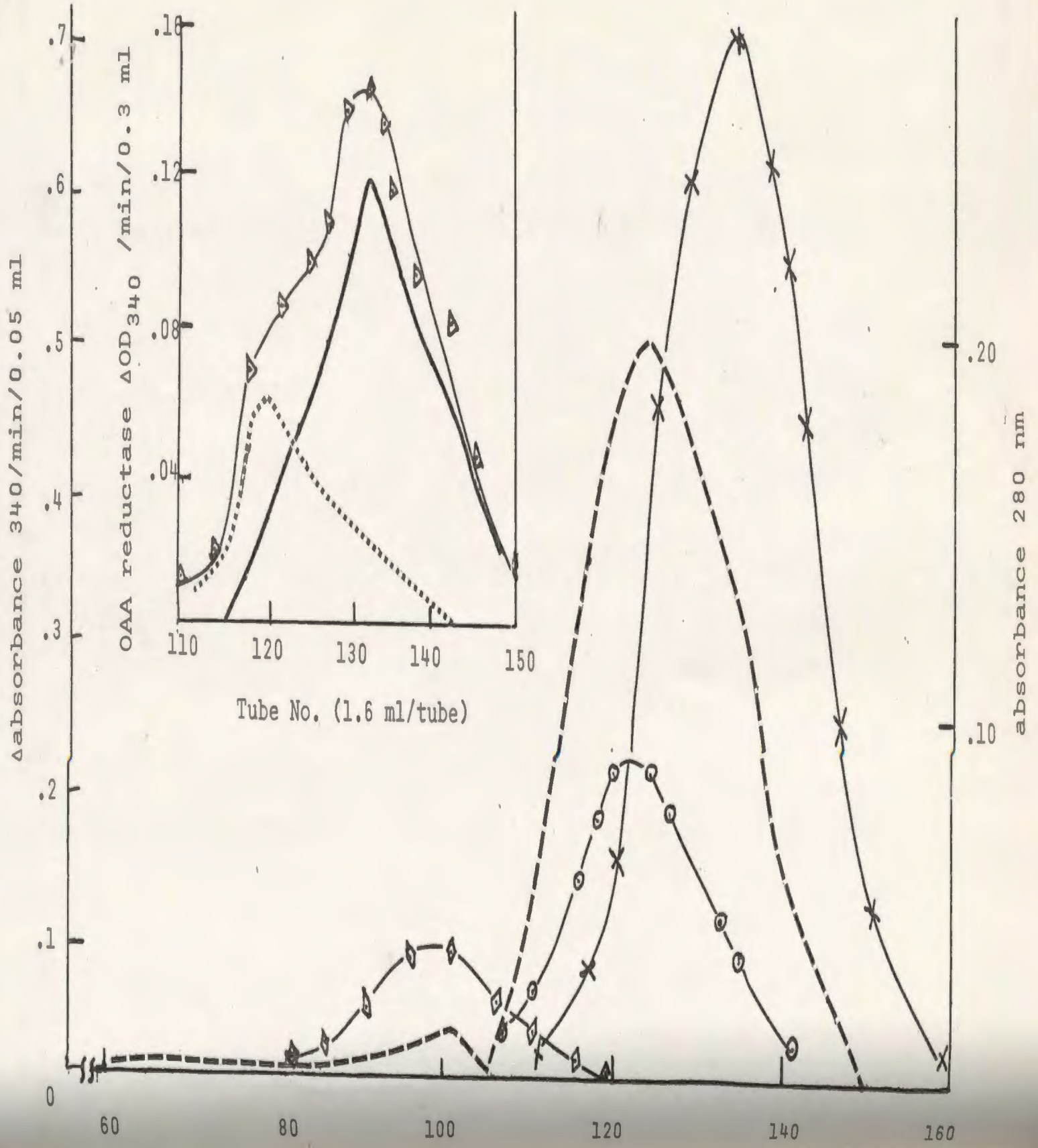


Fig. 3. Gel filtration on Sephadex G150 using 3 columns in series.

(X) MDH, (O) ICDH, (◊) LDH, (- -) protein

The insert in Fig. 3 shows the observed (Δ) OAA reductase eluted from the column and gives the calculated contributions of ICDH (···) and MDH (—) to the overall activity. In calculating these separate contributions one may assume that in tubes 145 to 150 where the ICDH activity in negligible, all the OAA activity is due to MDH. Therefore, MDH accepts NADPH at 2.2%, the rate at which it accepts NADH (i.e. ΔOD_{MDH}/0.3 ml/min = 1.8, OD_{OAA} reductase / 0.3 ml/min = 0.04; therefore the relative activity = $\frac{.04}{.3}$ x 100 = 2.2%. This should be constant throughout and the OAA activity of the other tubes should therefore be 2.2% of the MDH activity. Subtracting this value from the observed OAA activity one obtains the contribution of ICDH to the overall activity.

ampholine to give a pH range of 3 to 10, but resolution was poor. The peak ICDH tube always focused between pH 8.3 and pH 8.7. Two MDH peaks were obtained focusing respectively at pH 5.6 and pH 9.3. Collecting the peak ICDH tubes from such a column and refocusing to give a narrower pH range resulted in loss of activity.

These results suggest an isoelectric point of between pH 8.3 and pH 8.7. This value is a variance with the isoelectric point determined by Siebert et al[14]. Their value was however determined under conditions which may cause denaturation and therefore invalidate the result. An isoelectric point above pH 9.0 was suggested by Colman who observed that in an electrophoresis experiment conducted at pH 9.0 the enzyme still exhibited a net positive charge[20]. The somewhat lower value obtained here may be a result of differences in conformation due to the very different environments to which the enzyme is subjected in the two methods.

Using ampholine designed to give a narrower pH range - pH 6.0 to pH 8.0 - both the commercial enzyme and a partially purified sample from CM cellulose were unstable under the focusing conditions. The MDH was much more stable and readily focused (Fig. 4). Although the recovery of ICDH is small the peak is somewhat removed from the MDH peak. In migrating beyond pH 8.0, the ICDH may be moving outside the effective buffering range of this particular ampholine and is therefore

Fig. 4. Electrofocusing of commercial preparation of ICDH.

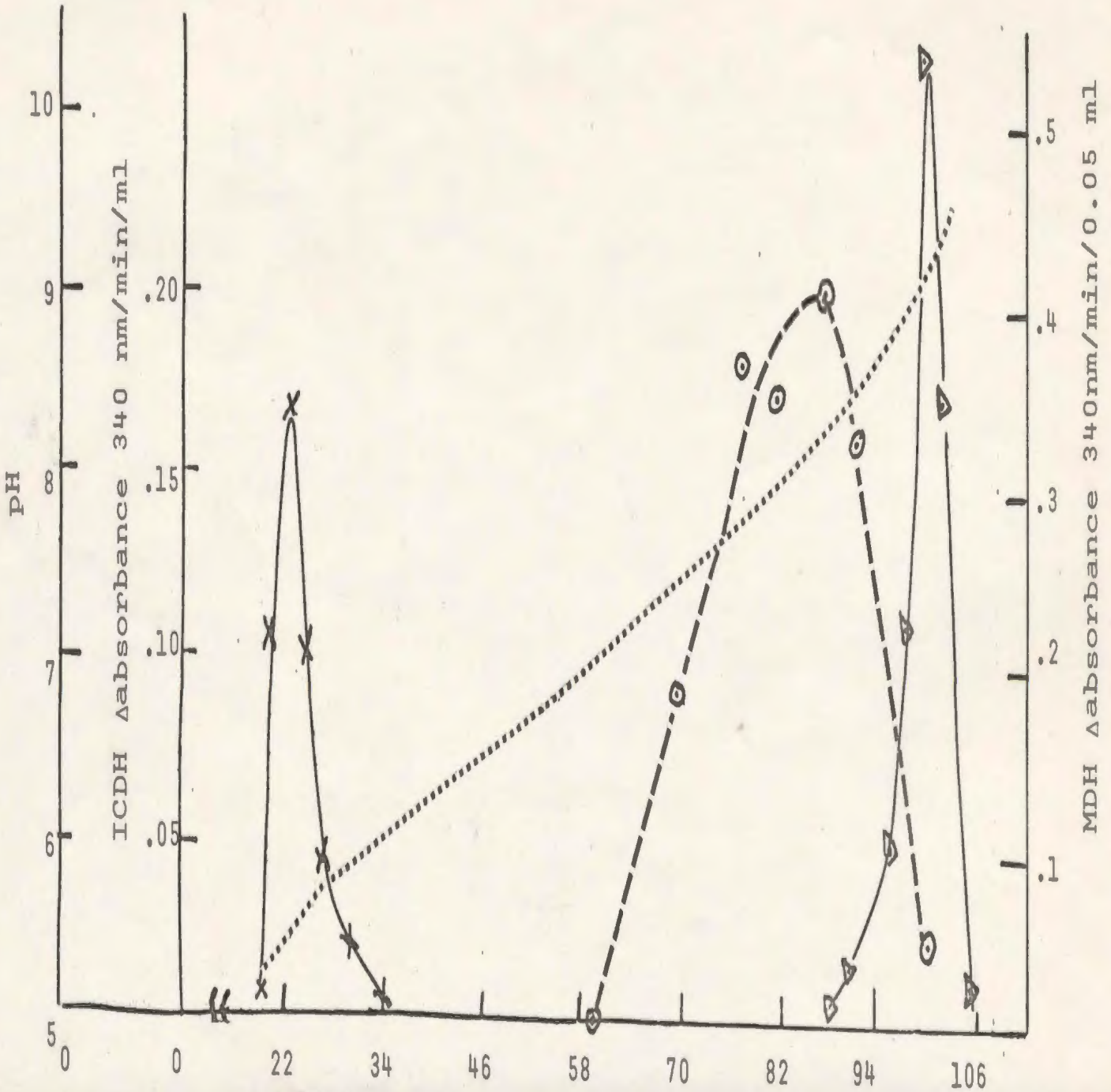


Fig. 4. Electrofocusing of commercial preparation of ICDH.

Focusing was carried out as described in Methods, using 2.0 ml commercial enzyme (10 mg/ml, specific activity 8 μmoles/min/mg) with 4 ml carrier ampholytes (range pH 6.0 to pH 8.0).

(·····) pH

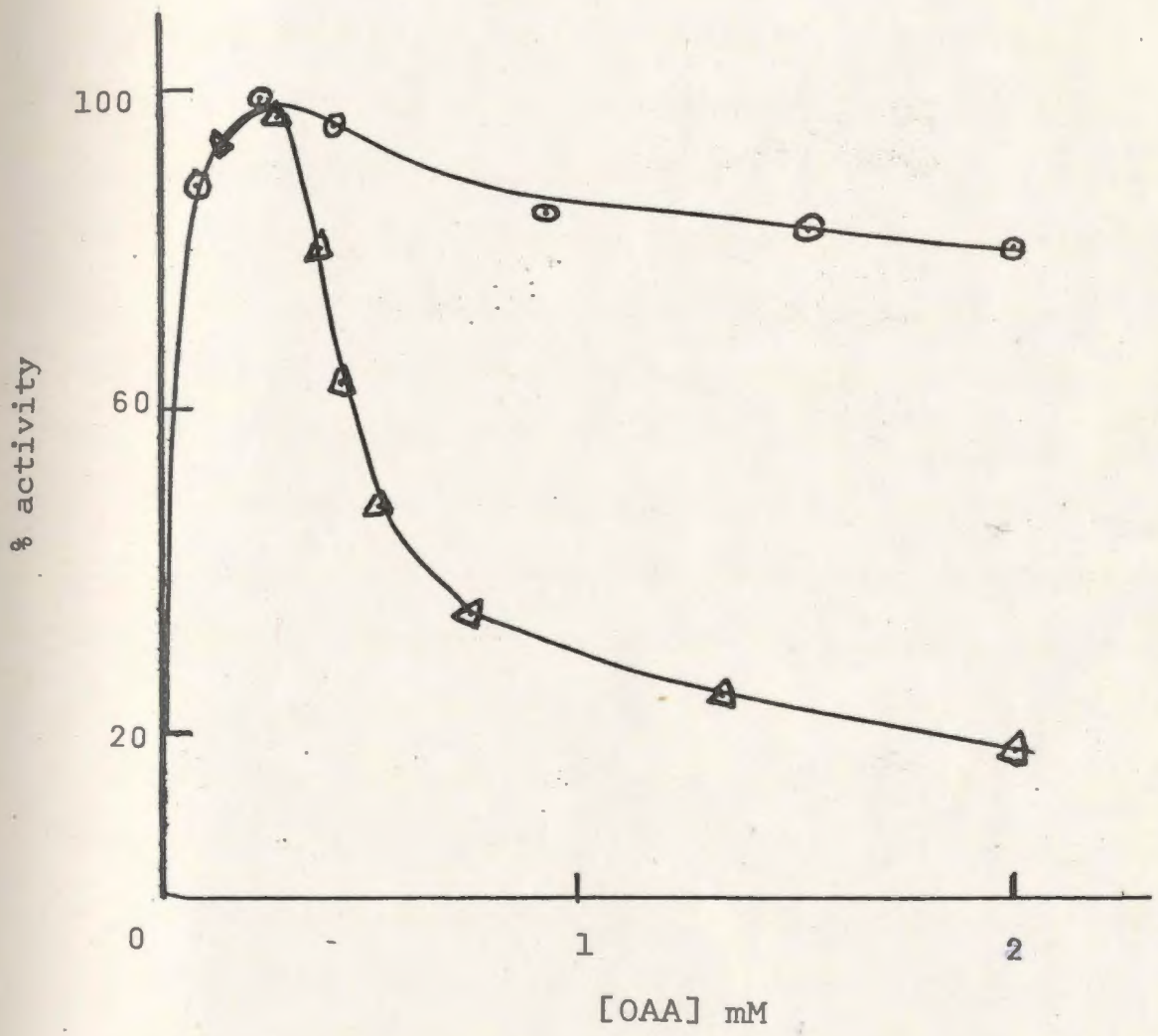
(o) ICDH

(X , Δ) MDH

being denatured. Only the cathodal species of MDH is detected if the enzyme is first passed through the CM column. Therefore, as had been suggested by CM cellulose chromatography, the isoelectric focusing pointed out that there were at least two forms of MDH in the commercial preparation - one of which is readily separable from the ICDH. The separation of MDH and ICDH was made extremely difficult by two factors: (1) the high lability of ICDH especially in solutions of low ionic strength or in the absence of glycerol and (2) the remarkably similar physical properties of the two enzymes. An examination of the properties of the malate dehydrogenases showed that both forms appear to use NADPH as a cofactor but at a very low rate compared to NADH. As well they appear to have similar molecular weights of less than 60,000 since they are both eluted immediately after ICDH on the G-150 column but they have distinct isoelectric points. The effect of substrate concentration on the two MDH's is shown in Fig. 5. One form is markedly inhibited by high substrate concentration while the other form, which separates readily from ICDH, is not.

Taken as a whole, the present work suggests that ICDH exhibits NADPH-OAA oxidoreductase activity. However, in the absence of a completely MDH free sample of ICDH, no definite statements can be made.

Fig. 5. The effect of [OAA] on the activity of the two forms of MDH.



The two forms of MDH were separated by electrofocusing. (O) is the anodal species and (Δ) the cathodal species. Assays were conducted using aliquots from tubes 24 and 102 respectively from Fig. 4.

The Multifunctional Nature of ICDH

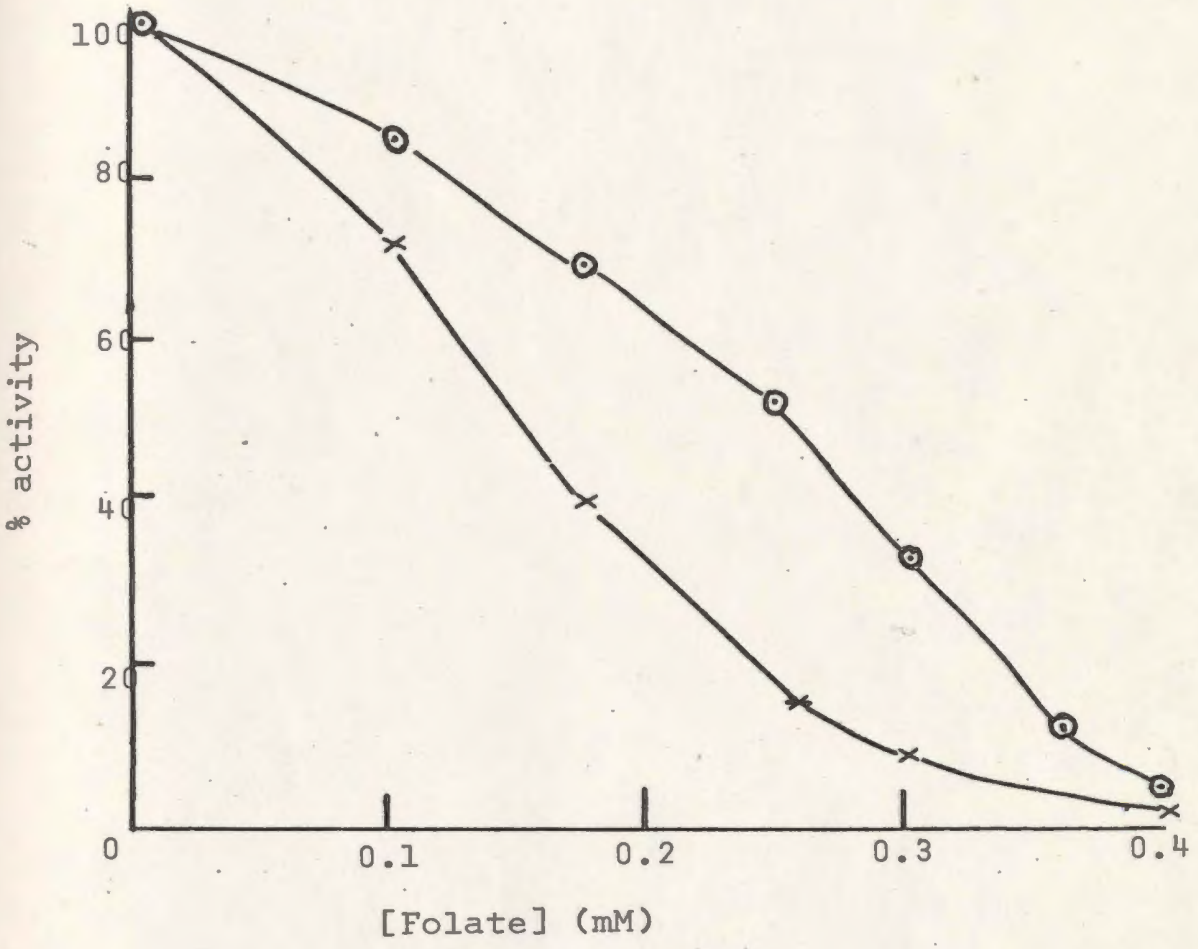
1. The Regulatory Properties of the Enzyme -

The $NADP^+$-ICDH is not subject to control by the modifiers which affect the NAD-ICDH, i.e. AMP, ADP, and citrate. However, inhibition by folate[43] and by the combination of glyoxalate and oxaloacetate[49] has been observed and suggested to be allosteric in nature.

(a) Folic acid - Folic Acid has been shown by previous workers to affect the isocitrate dehydrogenase activity of the enzyme. In examining its effect on the other enzyme activities folate was found to inhibit the reductive carboxylase activity of the enzyme more strongly than the dehydrogenase activity (Fig. 6). 0.15 mM folate inhibits the back reaction 56% while the forward reaction is only inhibited 26%.

(b) Glyoxalate and oxaloacetate - Concerted inhibition by glyoxalate and oxaloacetate has been demonstrated for NADP-ICDH from a number of bacterial and mammalian sources, including pig heart. However, for the pig heart enzyme the inhibition has been demonstrated only in crude preparations (specific activity 1.1 μmoles/min/mg), and therefore it was necessary to determine if this was a property of the purified enzyme. Table II shows that over a 6-fold purification of the enzyme there was no significant change in the ability of the glyoxalate and oxaloacetate to act in a concerted inhibition. Inclusion of NADPH in the assay system offered some protection against this inhibition.

Fig. 6. Folate inhibition of dehydrogenation and reductive carboxylation.



(O) dehydrogenation

(X) reductive carboxylation

TABLE II

CONCERTED INHIBITION OF ICDH BY GLYOXALATE AND OXALOACETATE

Additions to Assay System	Inhibition of dehydrogenase (%) Commercial Enzyme (specific activity 5)	Purified Enzyme (specific activity 29)
None	0	0
0.08 mM glyoxalate + 0.08 mM oxaloacetate	69	67
0.16 mM glyoxalate + 0.16 mM OAA	90	87
0.24 mM glyoxalate + 0.24 mM OAA	95	94
0.08 mM glyoxalate + 0.08 mM OAA + 0.08 mM NADPH	-	38
0.16 mM glyoxalate + 0.16 mM OAA + 0.08 mM NADPH	-	57

Enzyme and inhibitor were preincubated at 30° and the reaction started by the addition of NADP. In the protection experiments enzyme and NADPH were first preincubated for 1 min, the inhibitors then added and incubation continued for a further 2 min before starting the reaction by addition of NADP.

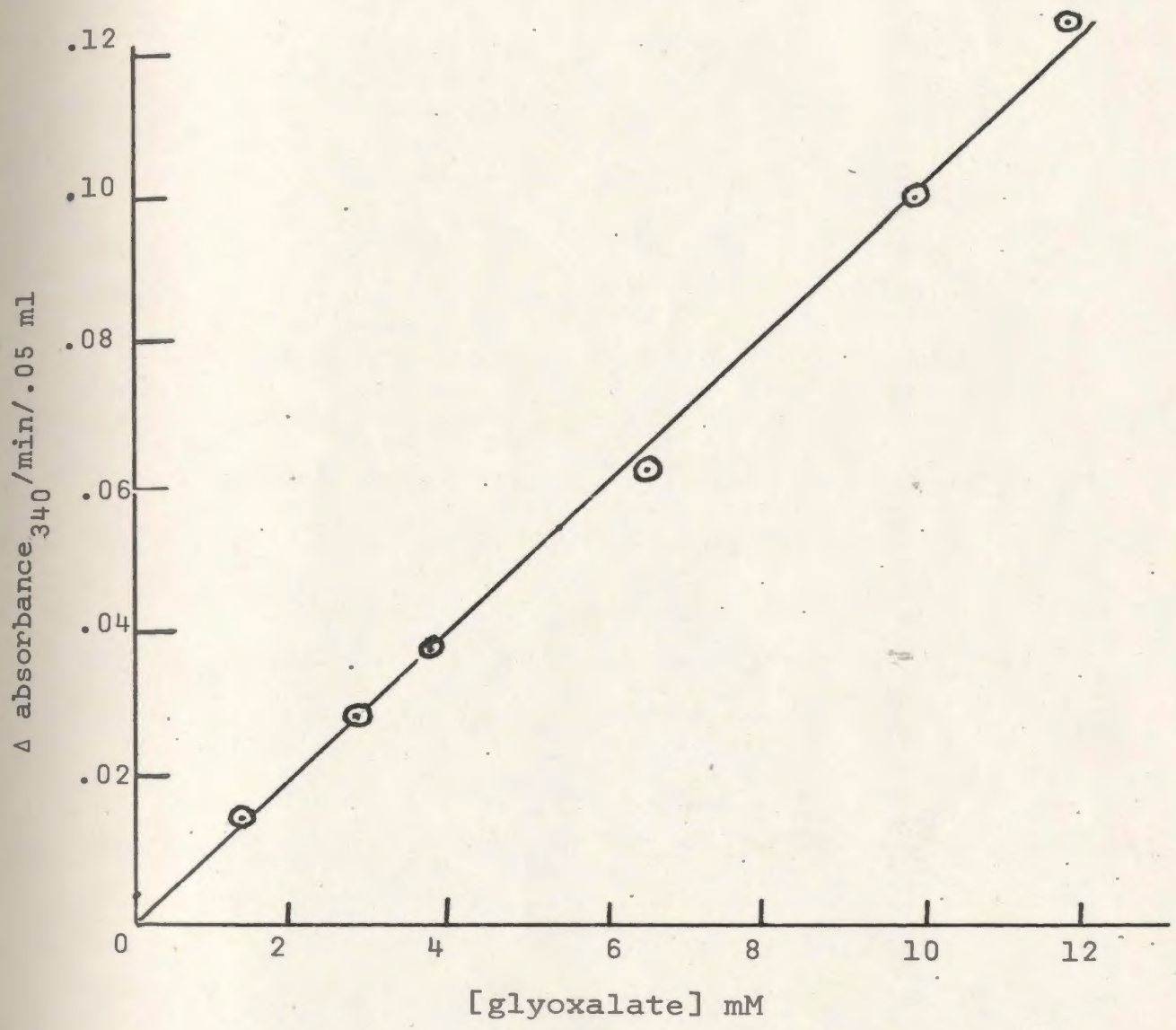
Since the purified enzyme contained some malate dehydrogenase which could utilize the oxaloacetate as its substrate, the effect of glyoxalate on MDH was examined. Over the concentration range used to inhibit ICDH, glyoxalate had no apparent effect on MDH activity.

The possibility of the presence of other contaminants which somehow mediated the inhibitory effect was considered. Using glyoxalate as substrate commercial enzyme was demonstrated to oxidize NADH but not NADPH. Fig. 7 shows the effect of glyoxalate concentration on the rate of NADH oxidation. Lactate dehydrogenase, which will accept glyoxalate as substrate[95,96] was also shown to be present in this preparation. LDH and the glyoxalate-NADH activity eluted together from the CM cellulose column with .018 M triethanolamine chloride buffer. Neither activity was present in the ICDH peak eluted from the CM column using the 0.1 M triethanolamine chloride buffer. It is therefore probable that both activities are due to lactate dehydrogenase. Removal of these activities did not alter the concerted inhibition.

Illingworth and Tipton reported that OAA reductase activity of cytoplasmic ICDH was inhibited by glyoxalate[17]. The OAA reductase activity of the pig heart mitochondrial enzyme did not appear to be inhibited by 2 mM glyoxalate.

Glyoxalate and oxaloacetate were found to inhibit reductive carboxylation as well as dehydrogenation. However, in contrast to the folate effect, glyoxalate and oxaloacetate

Fig. 7. Glyoxalate-NADH reductase activity of commercial preparation of ICDH.



Activity was assessed spectrophotometrically at 30° by following the oxidation of NADH at 340 nm. The concentration of glyoxalate was varied in a system containing 2×10^{-4} M NADH and .05 M potassium phosphate buffer, pH 7.4.

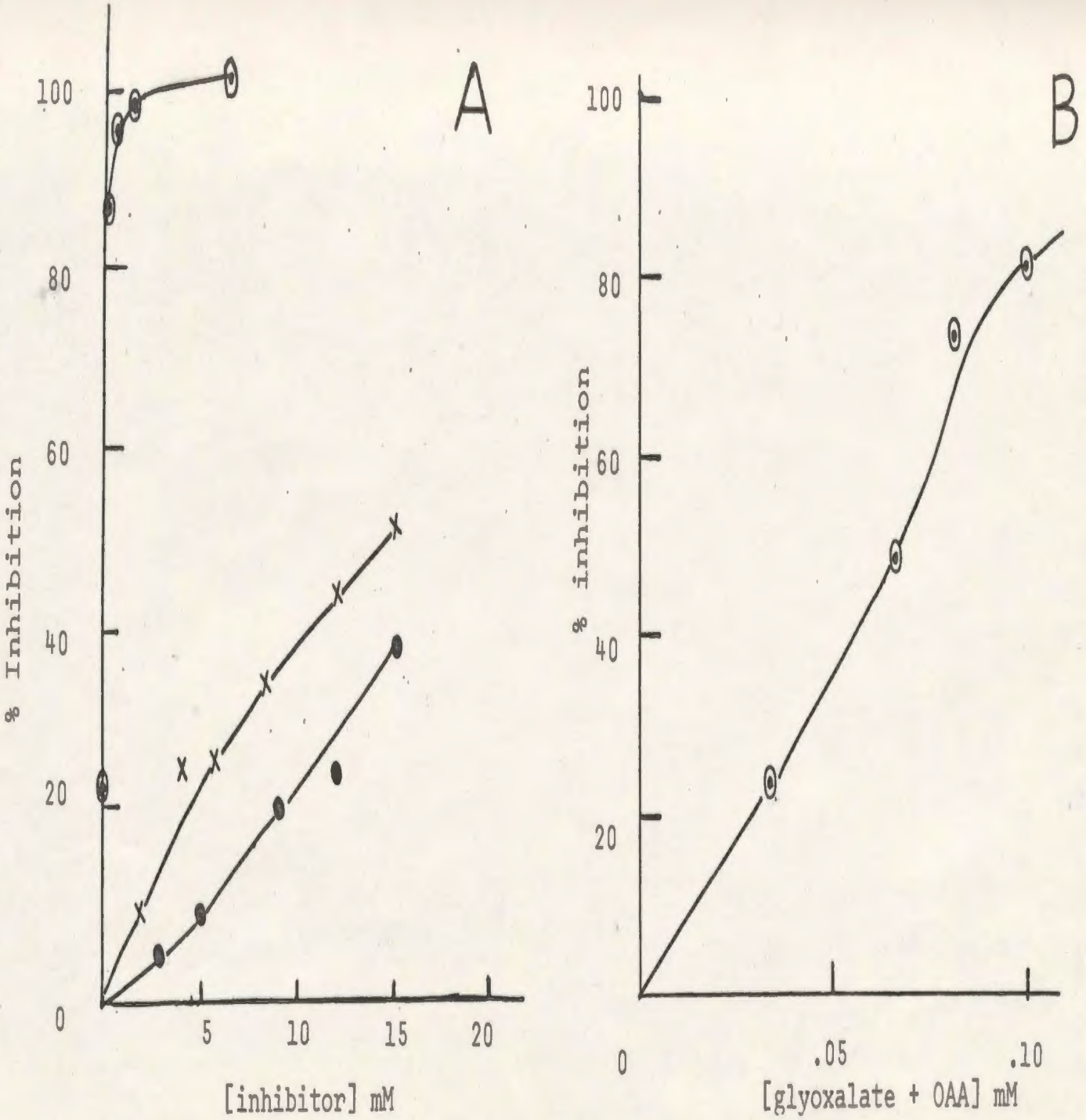
both separately and together, have a greater inhibitory effect on dehydrogenation than on reductive carboxylation. Fig. 8 shows their effect on the isocitrate dehydrogenase activity. Glyoxalate and oxaloacetate are individually able to inhibit ICDH. Oxaloacetate appears slightly more inhibitory. Inclusion of both substances together in the assay mixture at concentrations of less than 0.2 mM each, produced 100% inhibition. Such concentrations separately produce less than 5% inhibition.

Fig. 9 shows the sigmoidal curve of the inhibitory effect of glyoxalate and oxaloacetate on the reductive carboxylation. Such a curve is often taken as being indicative of allosteric effectors. An interesting feature of this inhibition is its apparent maximum at about 60% inhibition. The inhibition of the dehydrogenase reaction on the other hand goes to 100%. High concentrations of glyoxalate and oxaloacetate are required to inhibit the back reaction separately. Inclusion of 4 mM glyoxalate or oxaloacetate in the reaction mixture does not inhibit enzyme activity.

2. Response of the Enzymic Functions to X-irradiation -

As pointed out in the Introduction, irradiation of isocitrate dehydrogenase may yield useful information on the amino acid residues involved in the catalytic and regulatory activities. That X-rays can be used successfully to yield such information has been demonstrated in the case of fructose-1,6-diphosphatase[79]. A study was therefore undertaken of the

Fig. 8. Inhibition of ICDH by glyoxalate and oxaloacetate.



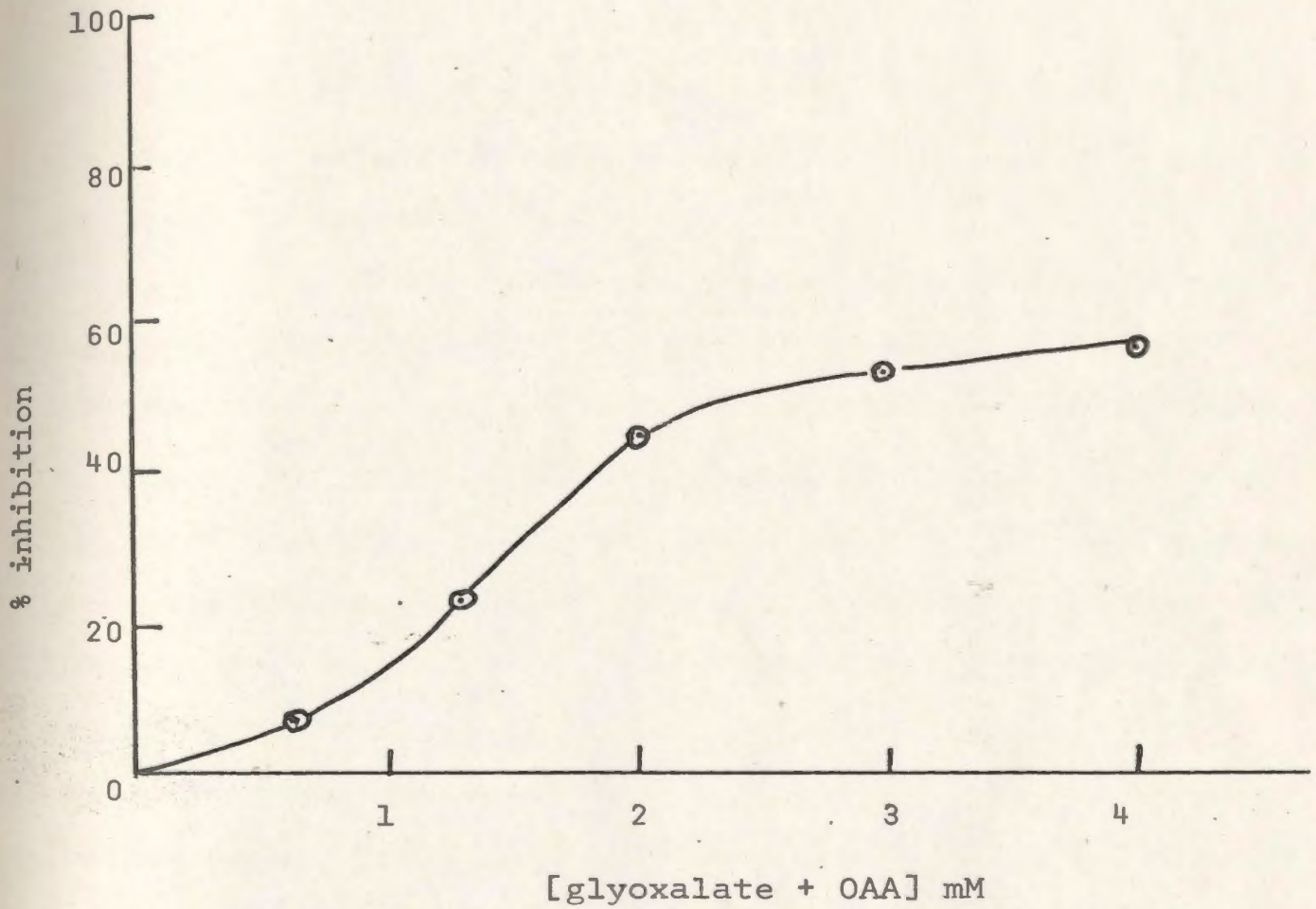
A. Concerted and separate inhibitions

B. Concerted inhibition.

(X) OAA, (O) concerted inhibition, (●) glyoxalate

In the concerted inhibition equimolar concentrations of glyoxalate and oxaloacetate were used.

Fig. 9. Concerted inhibition of reductive carboxylation by glyoxalate and oxaloacetate



Equimolar concentrations of glyoxalate and oxaloacetate were used.

effects of X-rays on the various enzymic properties of ICDH and an attempt was made to correlate these changes with modifications of specific amino acid residues.

Effect of X-rays on catalytic properties -

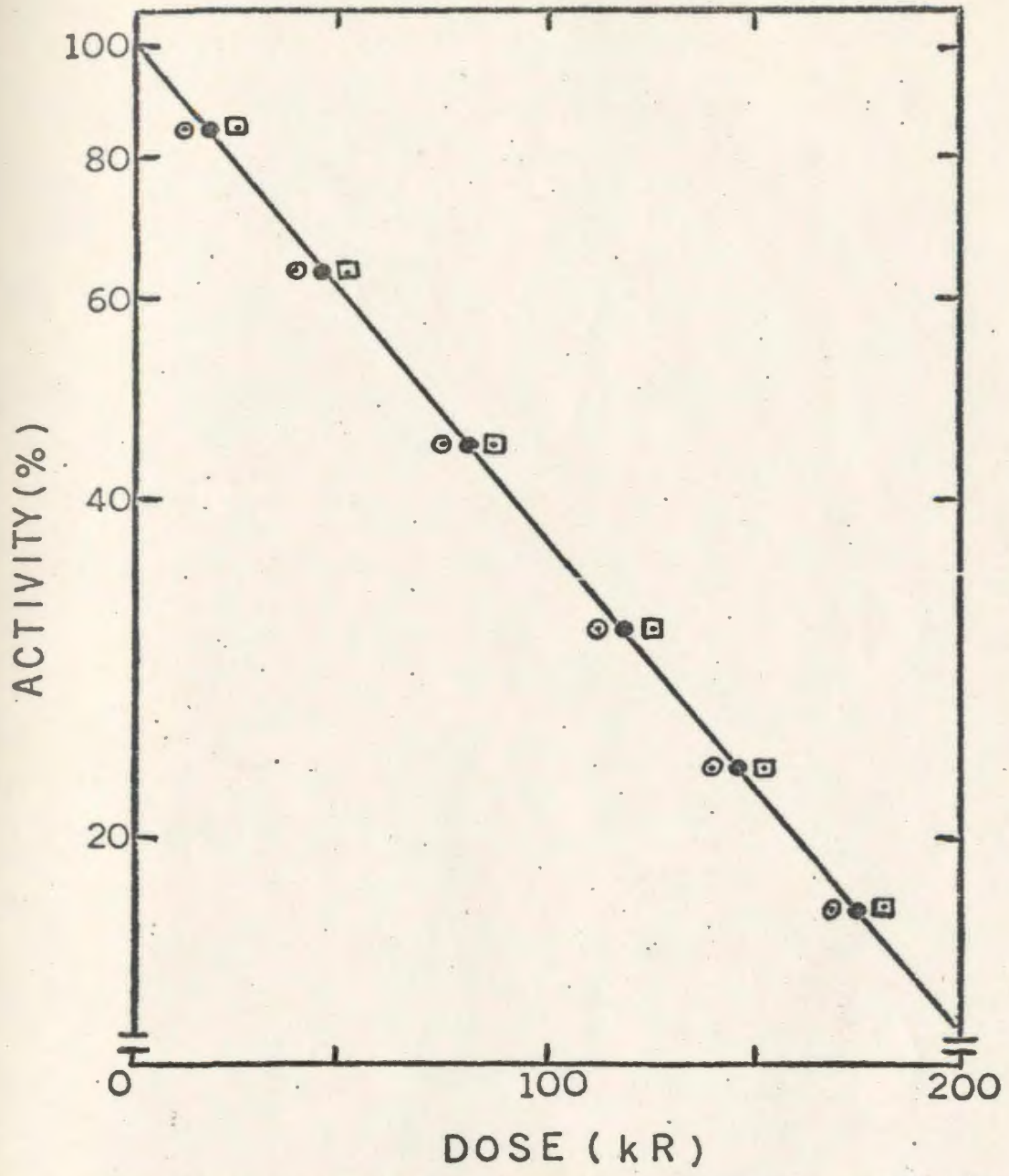
X-irradiation with increasing doses led to a progressive simultaneous decrease in all three catalytic activities (Fig. 10). Thus, the three catalytic functions have identical radiosensitivities. As is normal with X-ray inactivation of enzymes, the catalytic activities decreased as exponential functions of the dose[97].

In order to compare the radiosensitivity of this enzyme with that of other enzymes, the inactivation yield and G-value were calculated. G-values (the number of enzyme molecules inactivated per 100 eV absorbed by the solution) are used to quantitate the radiosensitivities of molecules in order to make comparisons meaningful. The inactivation yield may be obtained from a plot of D_{37} dose (the dose required to reduce the particular enzyme solution to 37% residual activity) against enzyme concentration (Fig. 11)[98]. From this plot a G-value of 0.5 was calculated, suggesting that ICDH is quite a radiosensitive enzyme. G-values for other enzymes range from 0.01 for aspartate transcarbamylase[76] to 1.8 for papain[65].

Effect of X-rays on regulatory properties -

The response of the enzyme to folate and to glyoxalate-oxaloacetate was examined after various doses of X-rays. Irradiated enzyme was just as sensitive as native

Fig. 10. X-ray inactivation of isocitrate dehydrogenase.



The enzyme (0.2 mg/ml) was irradiated under conditions given in Methods. The figure shows loss of the three enzymic activities: (O) oxidative decarboxylation of isocitrate, (●) reductive carboxylation of α-keto-glutarate, and (□) decarboxylation of oxalosuccinate.

Fig. 11. Response of ICDH to X-irradiation as a function of enzyme concentration.

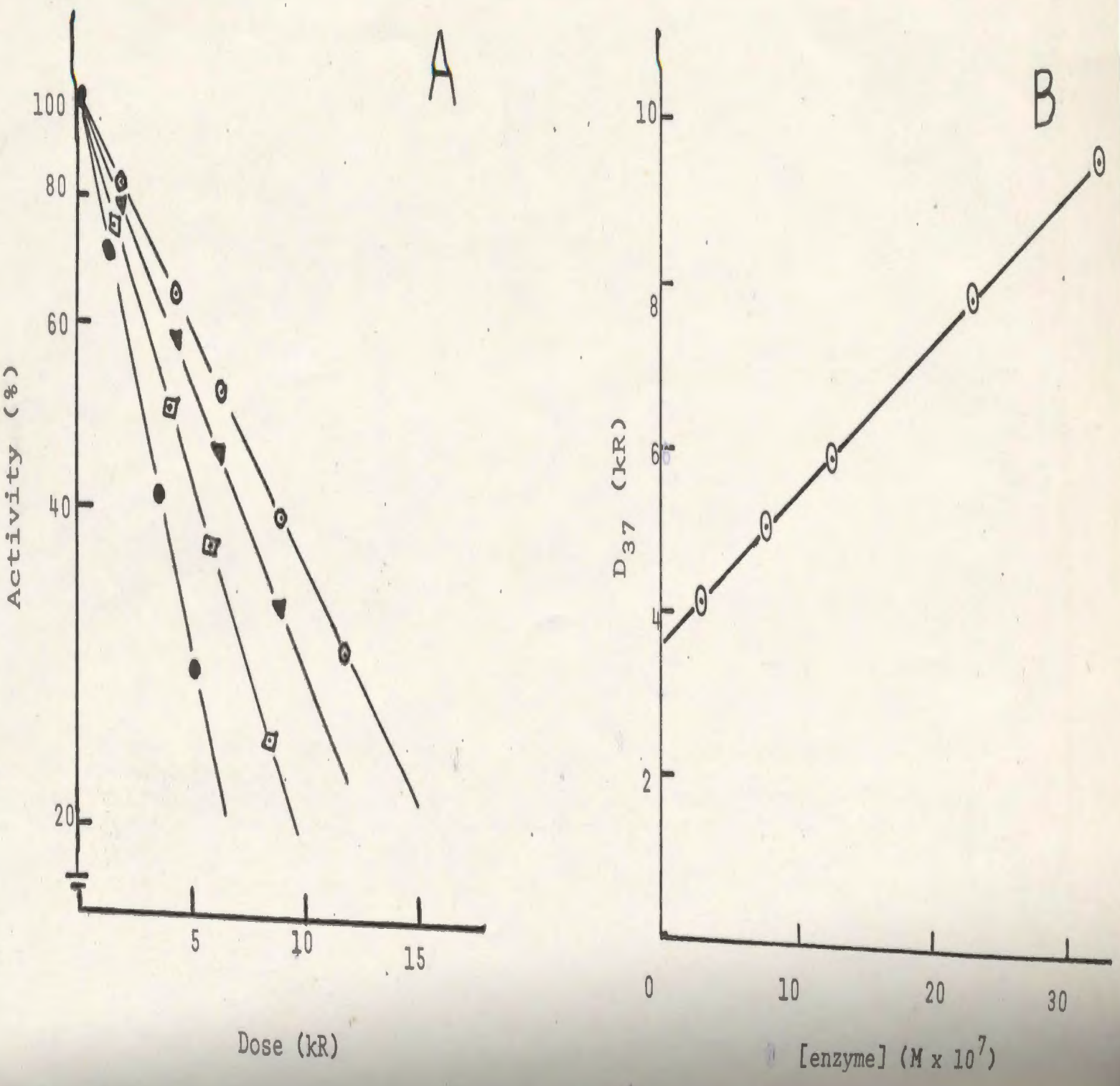


Fig. 11. Response of ICDH to X-irradiation as a function of enzyme concentration.

A. Dose response curves at various enzyme concentrations

(O) 3.4×10^{-6} M, (▲) 2.3×10^{-6} M, (◇) 1.3×10^{-6} M, and (●) 2.6×10^{-7} M.

B. The D_{37} dose as a function of enzyme concentration. The calculation of the G-value from this data is explained in Appendix II.

enzyme to these inhibitors. Enzyme irradiated to 50% residual activity displayed the same response to various concentrations of folate as native enzyme (Table III). Even quite high doses of X-rays which were sufficient to destroy over 90% of the catalytic activity caused no diminution in the response of the remaining enzyme activity to the inhibitors (Table IV).

Kinetic Parameters of Irradiated Enzyme -

As pointed out above, irradiation decreased the V_{max} value for the enzyme. However, the values of the Km for the substrate and cofactor are also modified. The greatest effect is on the Km for isocitrate which at 50% inactivation of the enzyme is almost doubled, from 2.6 μmoles to 4.3 μmoles, as determined from the double reciprocal plot of Fig. 12. A smaller increase in Km for manganese ions is observed with no significant effect on the Km for $NADP^+$, (Table V). The changes in the Km's are not large enough to account for the decreased activity measured at the high substrate concentrations of the standard assay. Therefore, weaker substrate binding by an otherwise active molecule cannot be advanced as the reason for the observed loss of activity.

Effect of X-rays on the pH dependence of the enzyme -

Native and irradiated enzyme were assayed at a variety of pH's over the range of 4.5 to 9.0 using sodium acetate, potassium phosphate, and tris-HCl buffers, Fig. 13 shows that the curves obtained for native enzyme and enzyme irradiated to 24% residual activity are superimposable within experimental

TABLE III

EFFECTS OF X-RAYS ON THE RESPONSE OF ICDH TO VARIOUS CONCENTRATIONS OF FOLATE

[Folate] mM	Activity of Native Enzyme (%)	Activity of Irradiated Enzyme[a]
0	100	100
0.04	96	96
0.10	87	86
0.15	78	77
0.20	65	62
0.25	54	55
0.30	35	35
0.40	5	6

[a] Enzyme (0.2 mg/ml) was irradiated to 50% residual activity. Activity is presented as a normalized percentage.

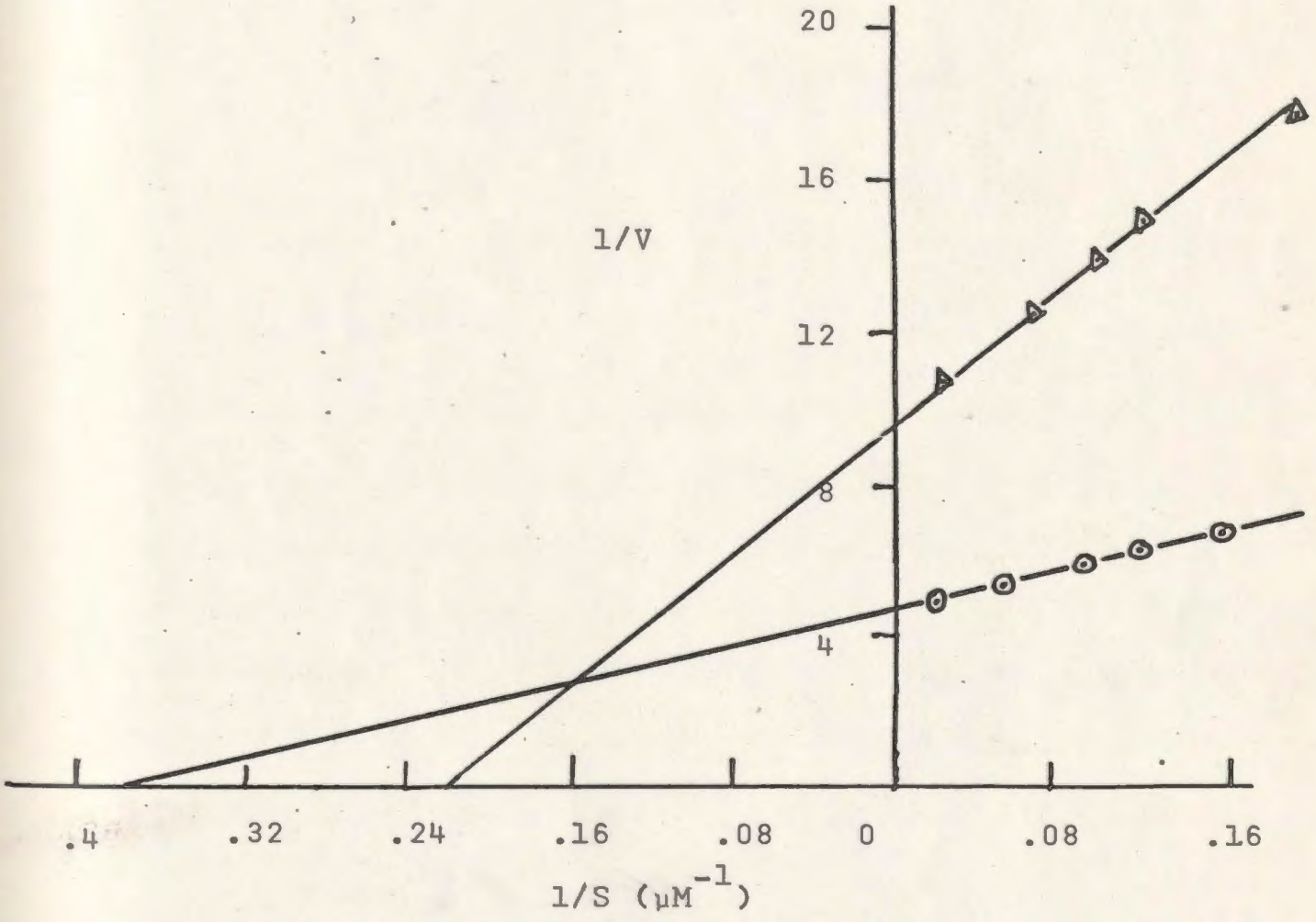
TABLE IV

THE RELATIVE RADIOSENSITIVITIES OF THE CATALYTIC AND ALLOSTERIC FUNCTIONS

Residual Catalytic Activity (%)	Inhibition by 0.4 mM folate (%)	Inhibition by 0.17 mM glyoxalate + 0.17 mM OAA (%)
100	58	77
10	60	78

Enzyme (0.2 mg/ml) was irradiated to 10% residual activity (180 rads) under the conditions described in Methods. Enzyme and inhibitors were preincubated at 30° and the reaction started by addition of NADP.

Fig. 12. Effect of X-rays on the Km for isocitrate.



S [isocitrate], o native enzyme, Δ enzyme irradiated to 47% residual activity

Assays were conducted using an SP800 spectrophotometer with a 0 - 0.1A scale expansion accessory and external chart recorder.

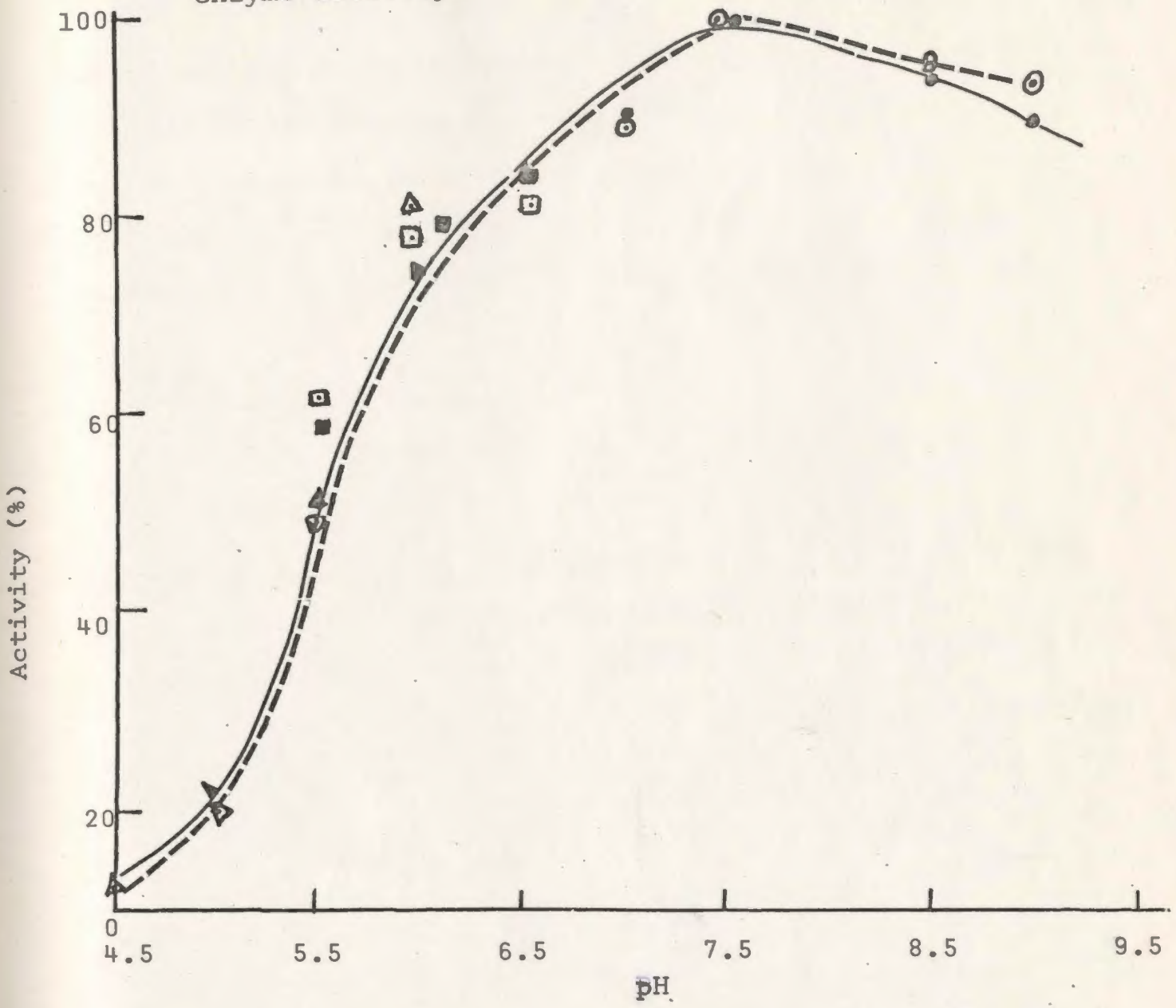
TABLE V

EFFECT OF X-IRRADIATION ON KINETIC PARAMETERS

Residual Enzymic Activity (%)	K_m (μM)		
	D-isocitrate	$NADP^+$	Mn^{+2}
100	2.6	4.15	0.7
50	4.3	4.0	1.2

Enzyme (0.2 mg/ml) was irradiated to 50% residual activity (90 kR). The Km's are calculated from Lineweaver-Burk plots for native and irradiated enzyme. When determining Km values each substrate was varied in turn with the other substrates remaining at the concentrations used in the normal assay procedure. Assays were carried out using a SP800 spectrophotometer with a 0 - 0.1A scale expansion accessory and external chart recorder.

Fig. 13. Effect of X-irradiation on the pH dependence of the enzyme activity.



Enzyme was irradiated in .05M tris-HCl pH 7.4 and the activity of the enzyme assessed at a number of pH's before and after irradiation. (—)Native enzyme assayed in Tris-HCl (●), phosphate (■) and acetate (▲). (– –) Enzyme irradiated to 25% residual activity assayed in Tris HCl (○), phosphate (□), and acetate (△).

error. Irradiation therefore does not alter the pH response of the enzyme and the observed inactivation cannot be attributed to a large shift in the pH dependence of the activity in the irradiated enzyme. These results suggest that the carboxylate anion to which the pH dependence has been attributed[20] has not been modified by the X-rays, either directly or indirectly, by significant conformational changes in the enzyme.

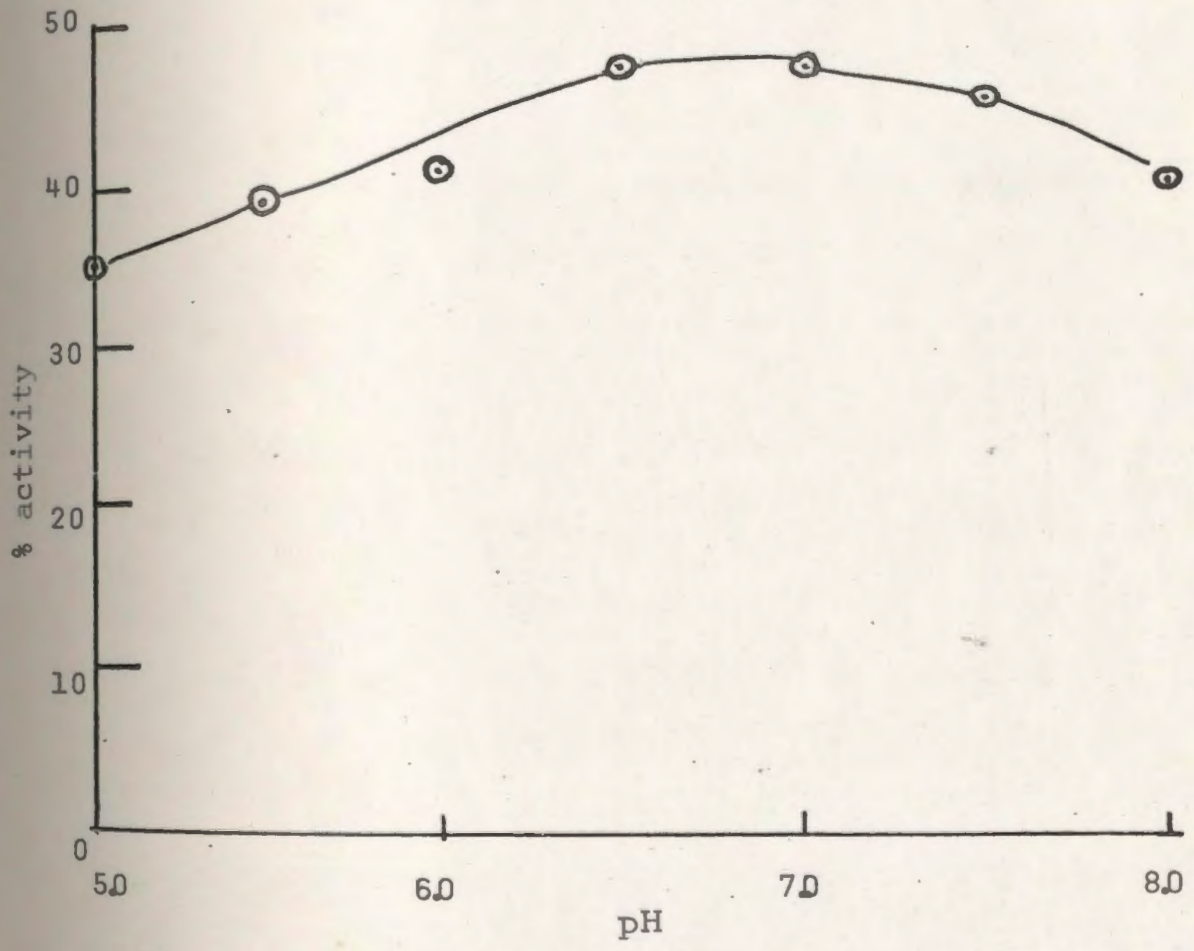
Effect of pH on radiosensitivity -

The relative radiosensitivity of the enzyme to irradiation in solutions of different pH was also considered. Enzyme was irradiated in potassium phosphate buffer, pH 5 to pH 8. Aliquots were removed and all assays conducted as described under Methods using tris-HCl buffer, pH 7.4. Fig. 14 shows that the enzyme was only slightly more sensitive at pH 5 and at pH 8 than at the other pH's. This greater sensitivity may possibly arise from conformational changes which render certain amino acid residues more sensitive to the x-rays.

Protection Experiments -

Chemical reagents which bind reversibly to amino acid residues of proteins have been known to protect these groups during irradiation[82]. Since substrates analogously bind to amino acid residues in the active center there exists the

Fig. 14. Effect of pH on radiosensitivity.



Enzyme was irradiated in 0.1 M phosphate buffer of varying pH; aliquots were then removed and assayed in Tris-HCl, pH 7.4. Activity is expressed as a percentage in terms of the activity of the native enzyme diluted in the respective buffers for equivalent periods of time.

possibility that they may offer some protection against inactivation. This has been shown to be so with fructose-1,6-diphosphatase[79]. Colman has shown that combinations of isocitrate and manganese ions or NADPH and manganese ions protect against inactivation by sulfhydryl reagents[32,36]. As shown in Table VI such mixtures did not protect against X-ray inactivation. At first glance it appears that there is significant protection. However, it may be seen that this is equivalent to that obtained using manganese sulfate alone and is therefore possibly an effect of radical scavenging by the manganese sulfate. Isocitrate or NADPH alone have no significant effect.

Site of X-ray Damage -

Since it was clear that this enzyme was extremely sensitive to irradiation further experiments were carried out in an attempt to localize the site of radiation damage. Previous workers have shown that on treatment of ICDH with chemical reagents, total inactivation results from modification of a single methionine residue[20] or between 2 to 5 sulfhydryl groups depending on the exact reagent used[32,36,99].

(i) Role of methionine -

The role of methionine in the X-ray inactivation was first examined. Colman has determined that the enzyme contains approximately 10 methionine residues[25]. However, the single essential methionine is significantly more reactive than the others and it is the only amino acid to bind iodoacetate at pH 5.5. Cysteine residues do not react under these conditions[20].

TABLE VI

EFFECT OF SUBSTRATE AND COFACTORS ON X-RAY INACTIVATION OF ICDH

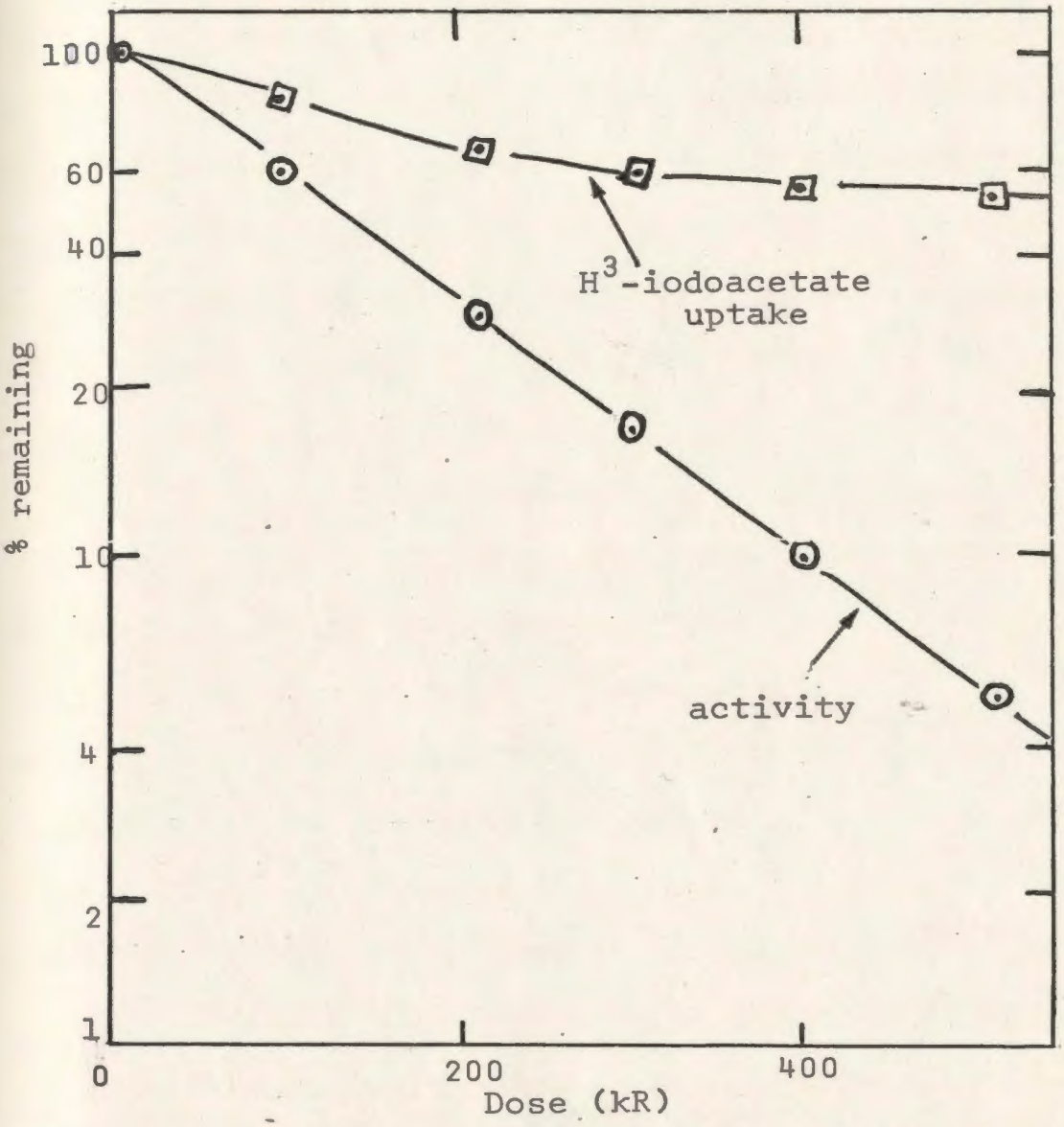
Protective substance	Residual Activity after 45 kRads (%)	Residual Activity after 150 kRads (%)
None	65	25
5×10^{-4} M isocitrate	67	26
1.26×10^{-4} M $MnSO_4$	78	45
1.26×10^{-4} M $MnSO_4$ + 5×10^{-4} M isocitrate	76	41
3×10^{-4} M $MnSO_4$ + 5×10^{-4} M isocitrate	80	50
2×10^{-4} M NADPH	63	24
2×10^{-4} M NADPH + 3×10^{-4} M $MnSO_4$	77	45

The enzyme (0.2 mg/ml) was irradiated under the conditions given in Methods.

Destruction of the essential methionine would therefore completely prevent the binding of radioactivity to the enzyme in the presence of H^3-labelled iodoacetate. Fig. 15 shows the effect of irradiation on the binding of radioactivity and on the enzyme activity. It is apparent that marked x-ray inactivation can occur with relatively little diminution of the enzyme's capacity to bind labelled iodoacetate. 60% of the reactive methionine remains intact when approximately 90% of the catalytic activity of the enzyme is destroyed. Activity is being lost much more rapidly than the single essential methionine is being destroyed. This would suggest that methionine destruction is not a significant mechanism in the X-ray inactivation. However, this evidence is not conclusive since X-irradiation might cause slight denaturation of the enzyme, thereby exposing other methionine or other amino acids for reaction with iodoacetate.

Further evidence against the involvement of methionine however may be obtained from an examination of the relative inactivation of dehydrogenase and decarboxylase activities. As has been demonstrated by Colman[20] and is confirmed by these results, modification of the essential methionine by binding of iodoacetate to the native enzyme causes a greater loss of dehydrogenase activity than of decarboxylase activity. X-rays have an equal effect on the two activities. On this basis, methionine appears to contribute very little to the x-ray inactivation.

Fig. 15. Effects of X-rays on the uptake of ^{3}H-iodoacetate.



The enzyme 0.2 mg/ml was irradiated under the conditions given in Methods. After various doses the enzyme was adjusted to pH 5.5, incubated with ^{3}H-iodoacetate and counted as described in Methods. The radioactive uptake is expressed as a percentage of the unirradiated control. (□) ^{3}H-iodoacetate uptake (O) activity.

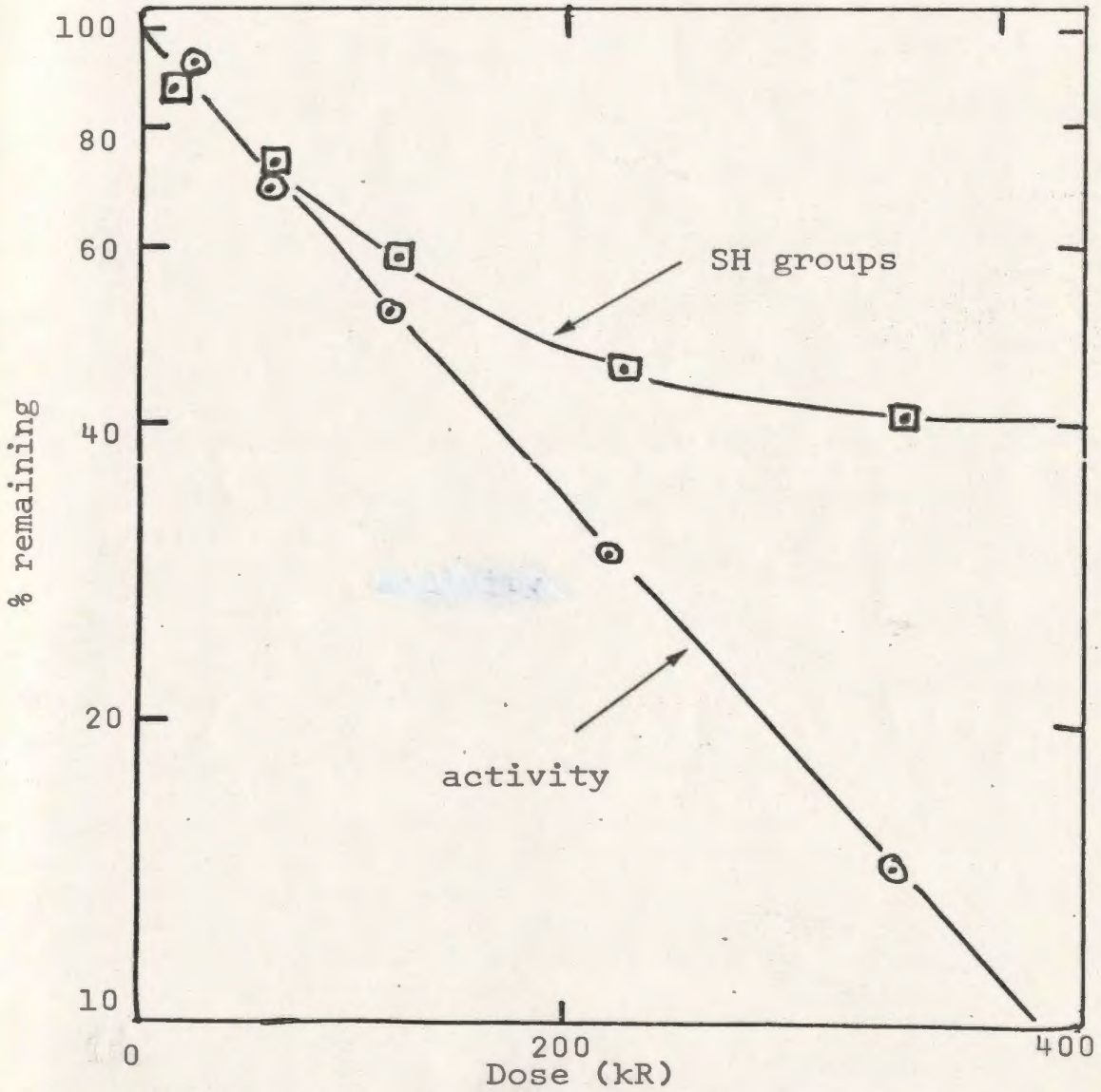
(ii) Role of sulfhydryl residues

The major mechanism of X-ray inactivation of sulfhydryl enzymes seems to be cysteine destruction[82]. As previous workers have implicated thiol groups in the catalytic activity of this enzyme[32,39,30,36] the sulfhydryl content was measured before and after exposure of the enzyme to X-rays. Fig. 16 shows a significant decrease in sulfhydryl content following irradiation. It can be calculated from the data in the figure that complete inactivation is accompanied by the loss of approximately 7-8 sulfhydryl groups. The enzyme contains a total of 13 thiol groups[32].

Only one of these has been implicated in catalysis by Colman. Of the thirteen, eight are buried within the enzyme and five, including the essential one, are available for reaction with DTNB in the undenatured enzyme. It is reasonable that the most reactive sulfhydryls, including those essential for activity, would be preferentially affected by X-rays. Thus, cysteine destruction may well play an important role in the mechanism of X-ray inactivation of the enzyme.

From Fig. 16 it is apparent that sulfhydryl destruction, unlike catalytic inactivation, is not an exponential function of dose. This is presumably because the sulfhydryl groups of the enzyme are of varying reactivities and that the most reactive are modified preferentially. The G-value for destruction of the first four sulfhydryl groups is approximately 1.6 to 2.0 suggesting that they are amongst the most radiosensitive of enzyme sulfhydryl groups[82]. Sanner and Pihl[82] have pointed

Fig. 16. Loss of SH groups upon irradiation.



Enzyme (0.2 mg/ml) was irradiated as described in Methods. After various doses, the SH content of the enzyme and the activity were measured as described in Methods and expressed as a percentage of the unirradiated control.

(□) SH groups (O) activity

out that the most radiosensitive enzyme sulfhydryl groups are the most chemically reactive ones and will react with the weak electrophile cystamine. The present enzyme is no exception since incubation for 1 hr with 10 mM cystamine at pH 7.7 caused 68% inactivation of the native enzyme.

(iii) Protection and reactivation by thiols -

Attempts were made to protect the enzyme from X-ray inactivation and to reverse the inactivation. With sulfhydryl enzymes, blocking the sulfhydryl groups with pCMB before irradiation and then removal of the reagent just before assay usually affords considerable radioprotection of the enzymes[82]. With isocitrate dehydrogenase however, binding of the mercurial caused an inactivation which could not be reversed unless excess small thiol was added almost immediately. Therefore, attempts to protect the enzyme against irradiation damage by incubation with mercurials were unsatifactory since the mercurial could not be removed without marked loss of enzyme activity. Similarly protection experiments with DTNB were unsatisfactory since the enzyme could not be readily reactivated.

With certain enzymes it has been found that X-irradiation damage could be repaired by treating the enzymes with excess small thiol after exposure[69,79,82]. In attempts to reactivate ICDH after irradiation samples were incubated with reduced glutathione or cysteine. However, unlike certain other sulfhydryl enzymes, X-ray inactivated ICDH was not even

partially reactivated by exposure to excess small thiol. The reason for this is discussed later.

3. Response of the Enzymic Functions to Certain Site Specific Reagents -

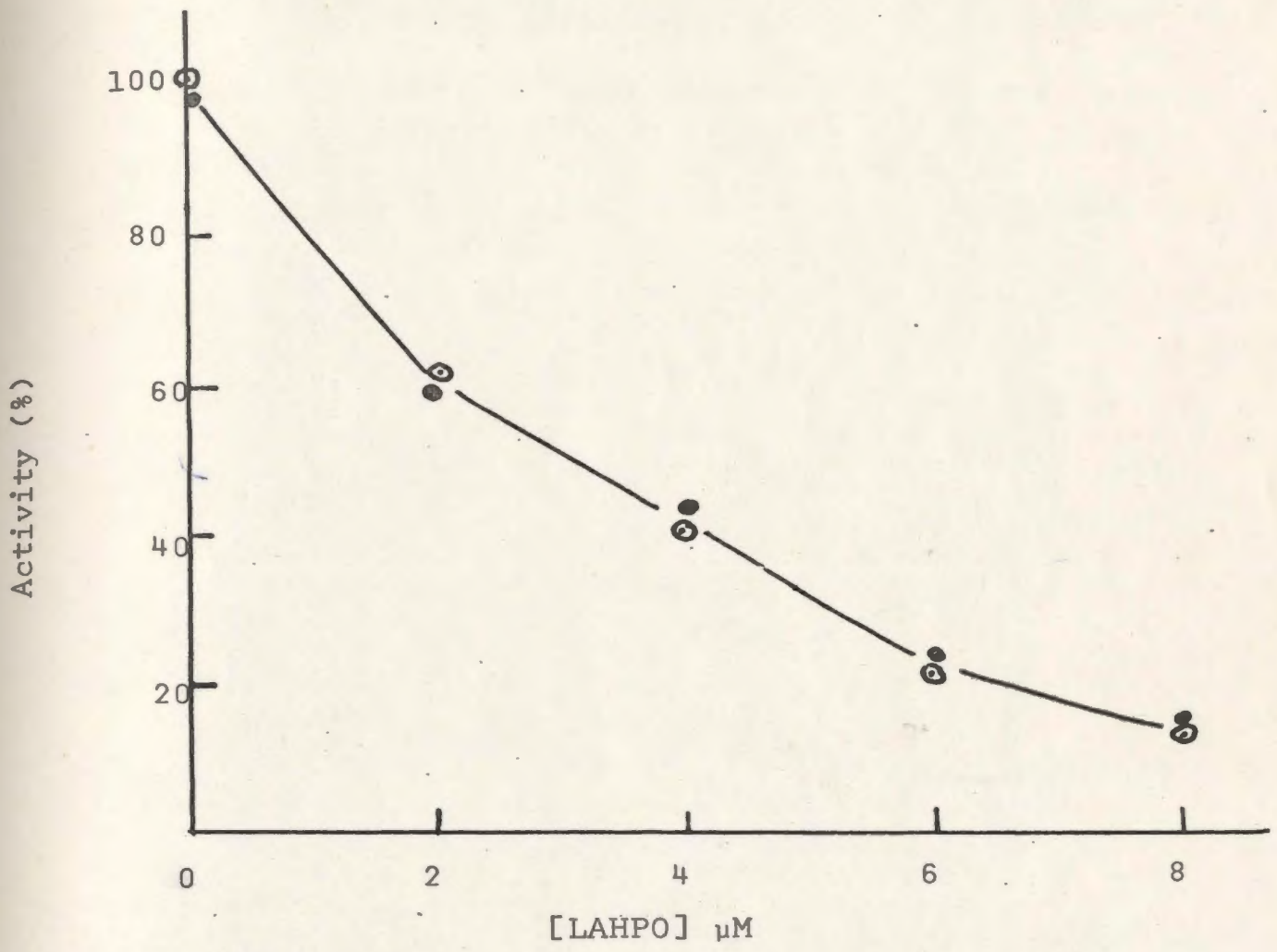
To investigate further the possible role of SH groups in the catalytic and regulatory functions of the enzyme, and in the X-ray response of ICDH, the effects of several chemical reagents on the various enzymic functions of ICDH were studied. The reagents chosen, pCMB, LAHPO, iodoacetate, DTNB, H_2O_2 and NEM are believed to modify SH or methionine residues in the enzyme.

(a) Effects of chemical reagents on catalytic properties -

The effects of NEM, DTNB, iodoacetate and LAHPO on the catalytic activities have been previously documented. NEM[36] and DTNB[32] bind to sulfhydryl groups and affect all three catalytic activities to the same extent. LAHPO[99] has been shown to affect dehydrogenase and decarboxylase activities to the same extent. Investigation of the reductive carboxylase showed that this was also similarly affected (Fig. 17). Iodoacetate[20] binds not to cysteine residues but to the single essential methionine residue. In so doing it disrupts the dehydrogenation more than the decarboxylation. In this its effect differs from that of the other reagents.

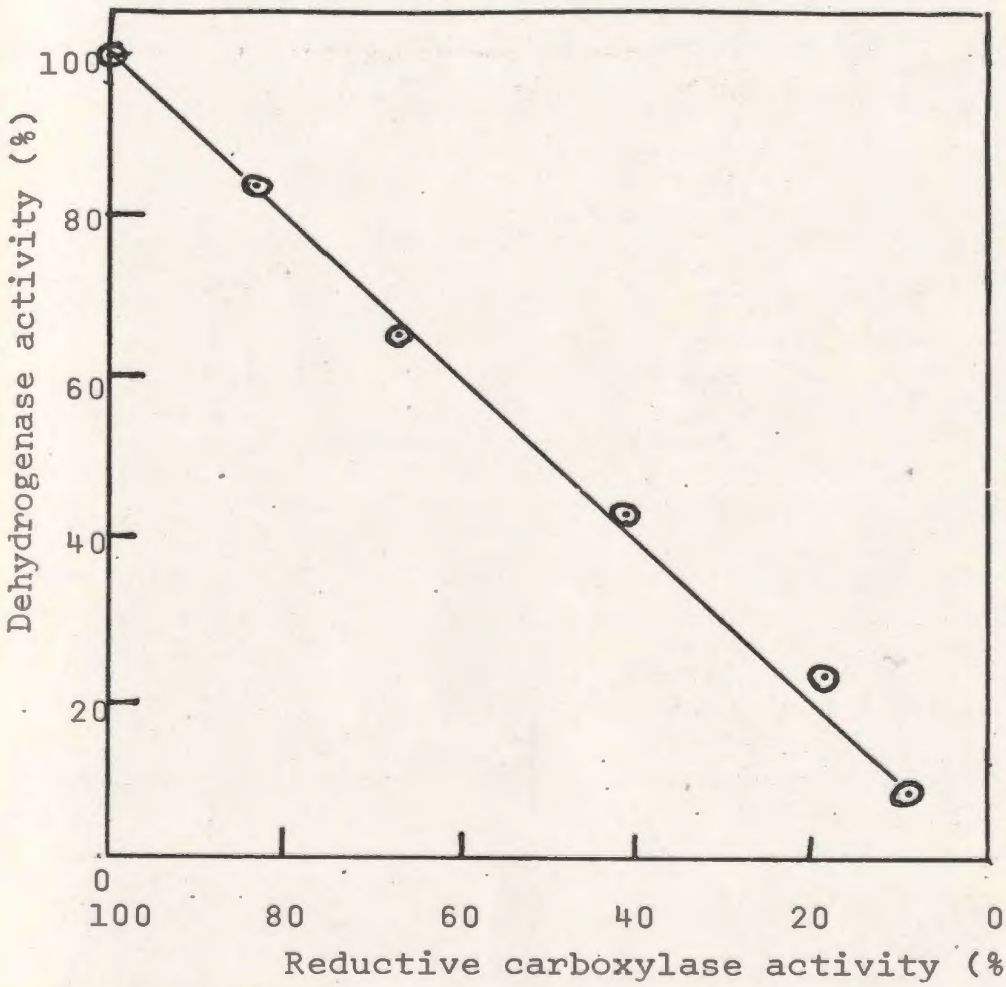
pCMB is known to inactivate ICDH by binding to its sulfhydryl residues[30]. Like X-rays it destroys all three catalytic activities at the same rate. Fig. 18 shows the effect

Fig. 17. Inactivation of dehydrogenation and reductive carboxylation by LAHPO.



ICDH (0.3 mg/ml) was incubated with LAHPO at 22° in 0.1 M Tris-HCl buffer, pH 8.5, for 4 min. Aliquots were removed, the reaction effectively stopped by dilution 60-fold and enzyme assayed for dehydrogenase (O) and reductive carboxylase (●) activities.

Fig. 18. Loss of dehydrogenase and reductive carboxylase activities by treatment with p-CMB.



ICDH (0.5 mg/ml) was incubated with varying concentrations of p-CMB up to 25 μM in 0.1 M triethanolamine chloride, pH 7.7, 0.3 M Na_2SO_4, and 10% glycerol at 0° for 20 minute intervals. Inactivation was effectively stopped by 30-fold dilution of an aliquot from the preincubation mixture and enzyme assayed for dehydrogenase and reductive carboxylase activities.

on dehydrogenation and reductive carboxylation: Green et al showed that decarboxylation is also similarly affected[99]. At variance with these results are the earlier studies of Siebert et al which had indicated that the dehydrogenation might be more sensitive than the decarboxylation[30]. However, Siebert and coworkers commented that with the extreme sensitivity of the enzyme to the reagent, the degree of inhibition will vary with enzyme concentration, and therefore, their use of different protein concentrations in the pCMB incubation systems for the two different activities is not a satisfactory basis for a quantitative comparison.

Hydrogen peroxide can also be used to inactivate the enzyme. It destroys all three catalytic activities at the same rate (Fig. 19). A comparison of H_2O_2 , cystamine and LAHPO shows that LAHPO is approximately 1700 times more effective than H_2O_2 in agreement with relative effectiveness in cruder preparations[99], while cystamine is approximately 10 times more effective (Figs. 17 and 19). The relative effectiveness is calculated from the concentration of reagent required to give 50% inactivation under similar incubation conditions.

(b) A comparison of the effects of various inactivators on sulfhydryl content -

The number of sulfhydryl groups modified by these reagents in destroying the isocitrate dehydrogenase activity was investigated. The reagents varied markedly in the number of thiol groups which they modified in order to completely

Fig. 19. Inactivation of ICDH by H_2O_2 and cystamine.

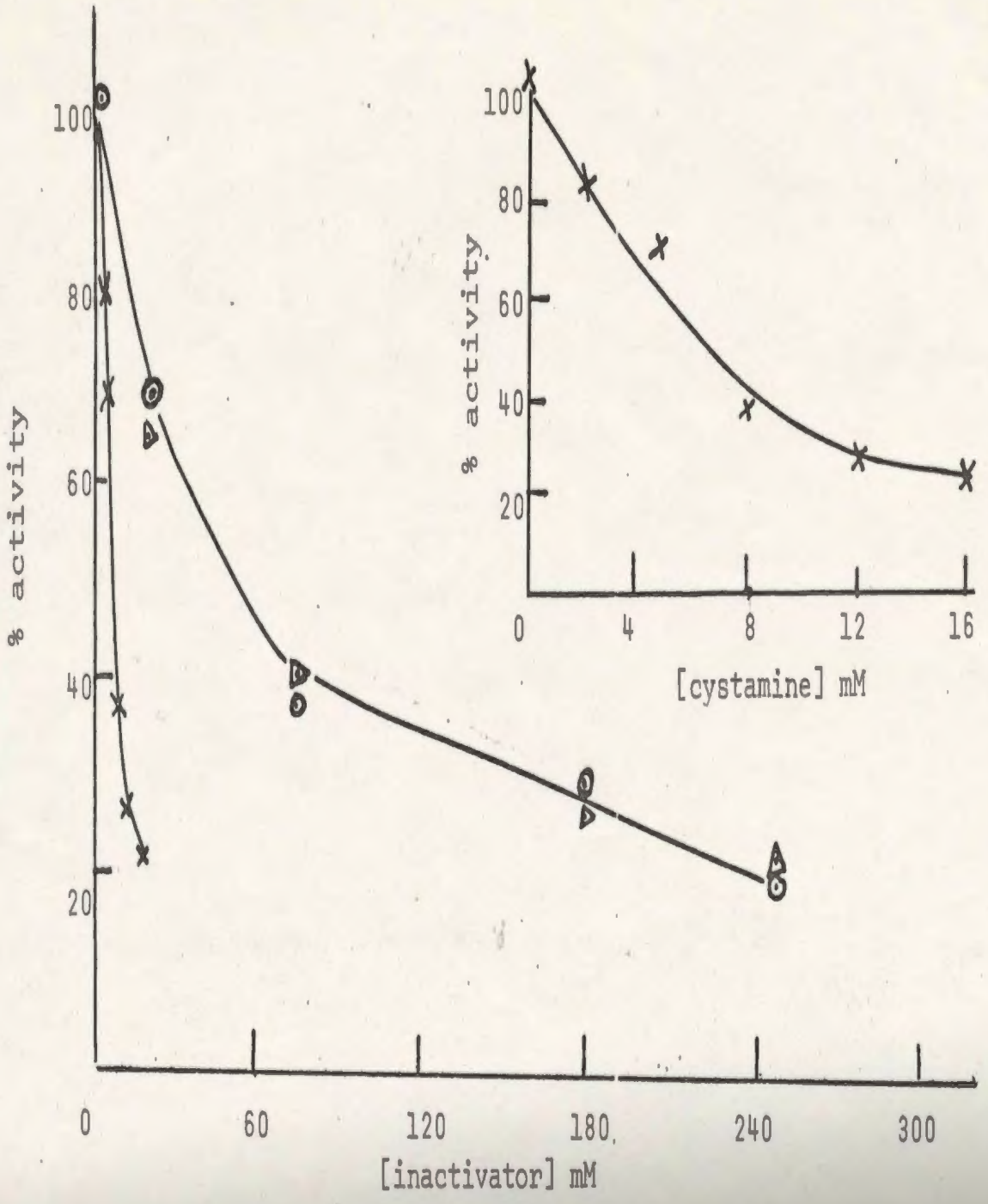


Fig. 19. Inactivation of ICDH by H_2O_2 and cystamine.

ICDH (0.18 mg/ml) was incubated with inactivator at 0° for 20 min. The reactions were effectively stopped by dilution (20-fold) and enzyme activity measured. (X) Residual dehydrogenase activity after incubation with cystamine in 0.1 M triethanolamine chloride, pH 7.7, containing 0.3 M Na_2SO_4 and 10% glycerol. (Δ) Dehydrogenase and (O) reductive carboxylase activities after incubation with H_2O_2 in 0.25 M Tris-HCl, pH 7.4.

Insert: Inactivation by cystamine replotted on an expanded axis.

inactivate the enzyme (Table VII). This is again shown in Fig. 20 where we have a plot of residual activity against number of SH groups modified by NEM, pCMB and X-rays. NEM is the most selective reagent and binds to only two SH groups for complete inactivation[36]. pCMB and X-rays were rather less selective and reacted with the essential sulfhydryl groups as well as with reactive non-essential SH groups.

(c) Effects of chemical reagents on allosteric properties -

Folate Response - Like X-rays, none of the sulfhydryl reagents tested had any effect on the folate inhibition of the enzyme. Iodoacetate was also without effect. The results, both with chemical reagents and X-rays, indicate that folate probably does not bind to sulfhydryl groups to cause enzyme inhibition.

Glyoxalate-Oxaloacetate Response - The effects of the various inactivating agents on the enzyme's response to glyoxalate and oxaloacetate proved to be rather complex - both in their effects on the concerted inhibition and on the inhibition due to the substances separately.

Inactivation of the enzyme by LAHPO or H_2O_2 did not alter the response to glyoxalate and oxaloacetate. Iodoacetate similarly was without effect. In inactivating the enzyme pCMB, DTNB and NEM decrease the enzyme's response to glyoxalate and oxaloacetate (Fig. 21). The inactivation of the regulatory property by these three reagents is markedly

TABLE VII

SULFHYDRYL MODIFICATION AND LOSS OF CATALYTIC ACTIVITY

Reagent	Number of SH groups modified for 100% inactivation
X-rays [a]	8
p-CMB [a]	6
DTNB [b]	5
NEM [c]	2
LAHPO [d]	3

[a] determined from Fig. 20 by extrapolation to zero enzyme activity

[b] taken from reference 32

[c] taken from reference 36

[d] taken from reference 99

Fig. 20. Loss of SH groups during inactivation of ICDH by various reagents.

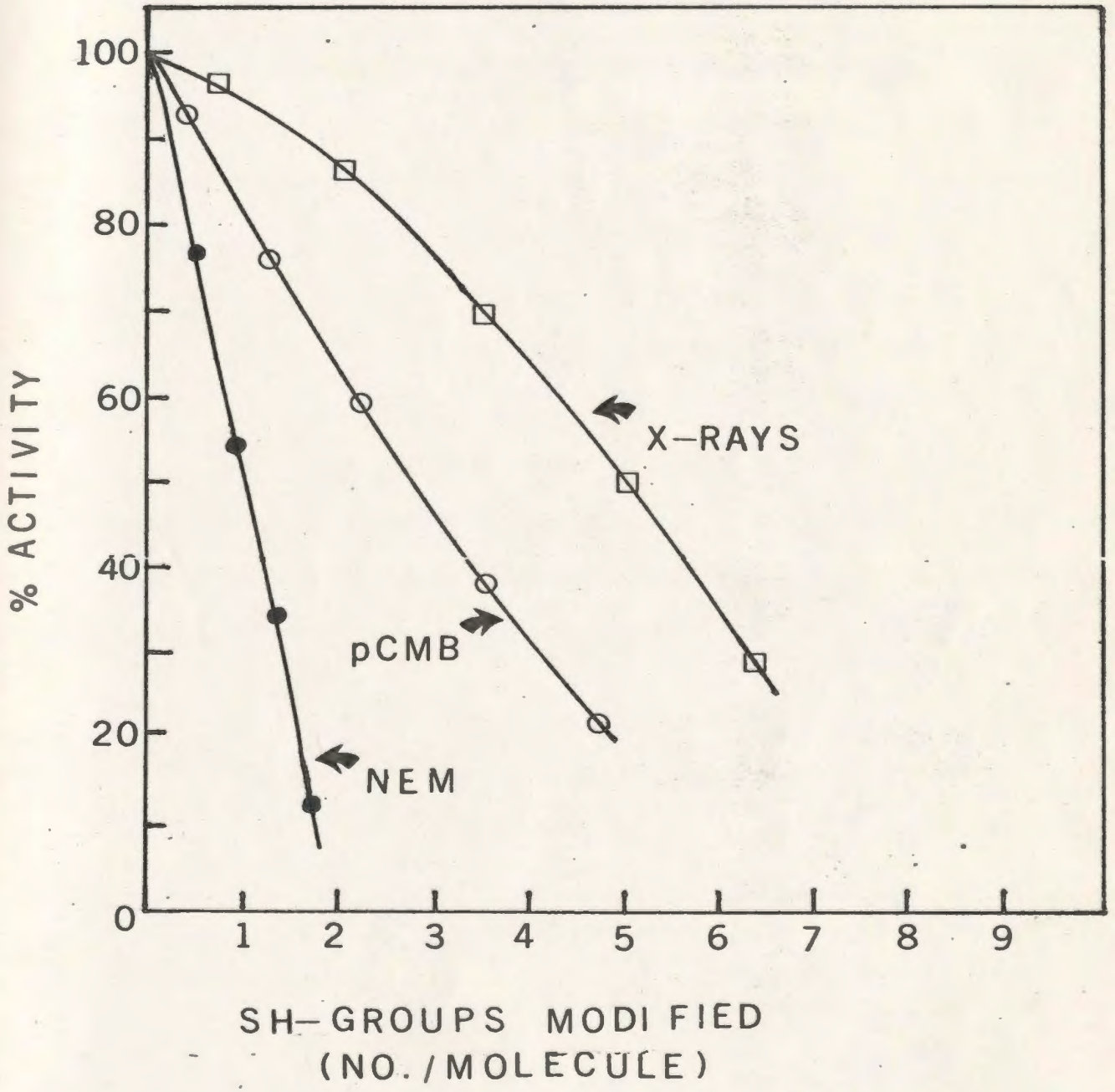


Fig. 20. Loss of SH groups during inactivation of ICDH by various reagents.

ICDH was subjected to the following treatments: (□) X-irradiation under the conditions described in Methods, (○) incubation with varying concentrations of pCMB up to 25 μM for 20 min at 0° in 0.05 M Tris-HCl, pH 7.4, (●) incubation with varying concentrations of NEM up to 80 μM for 20 min at 30° in 0.1 M triethanolamine chloride, pH 7.7, 0.3 M sodium sulfate and 10% glycerol.

In all cases enzyme was 0.5 mg/ml. With pCMB and NEM aliquots were removed and the reaction effectively stopped by dilution (60-fold). Enzyme was assayed for dehydrogenase activity and sulfhydryl content as described in Methods.

Fig. 21. Effect of SH reagents on the concerted inhibition by glyoxalate and oxaloacetate.

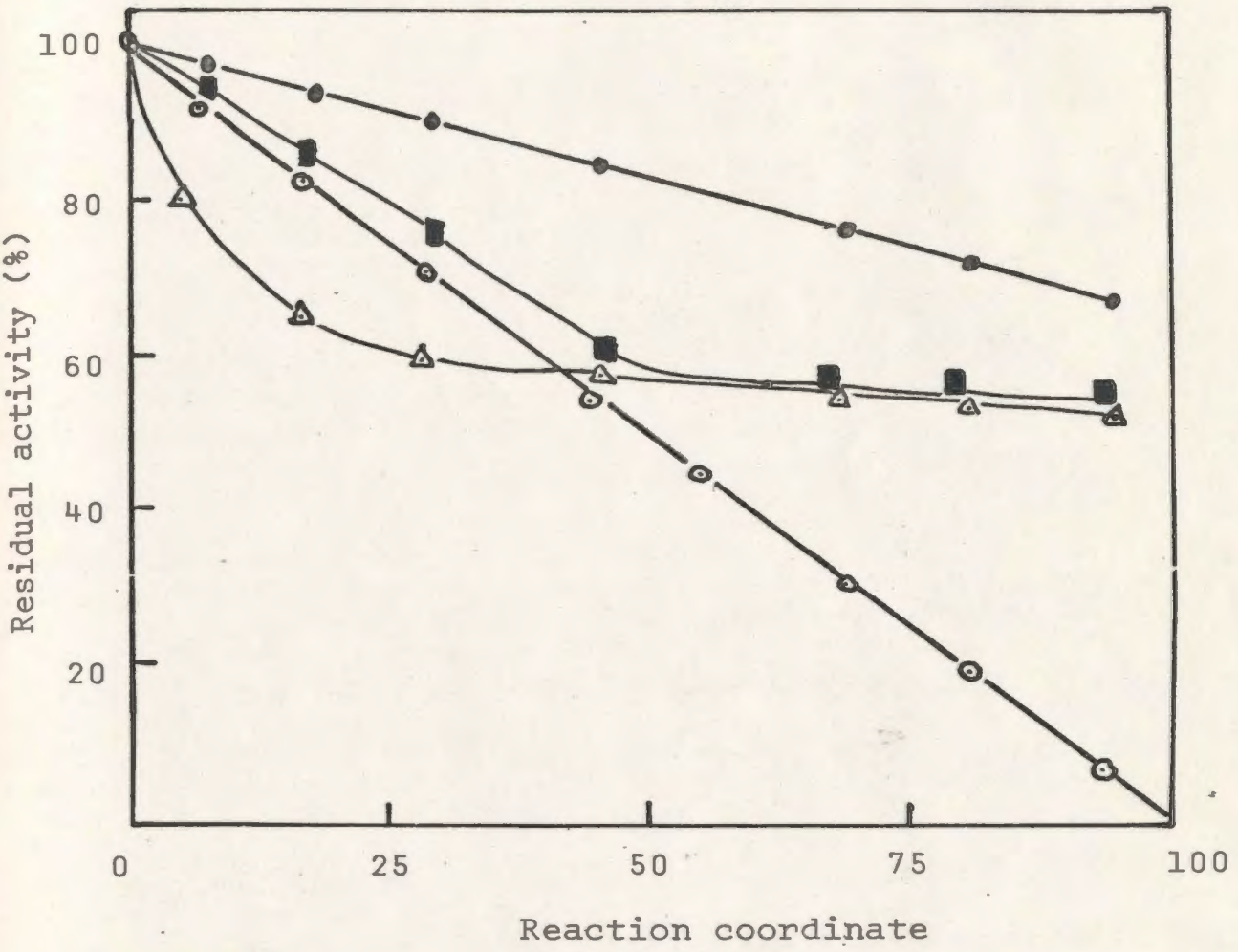


Fig. 21. Effect of SH reagents on the concerted inhibition by glyoxalate and oxaloacetate.

The reaction coordinate is an arbitrarily defined normalization giving a linear progress of the reaction independent of the reagent used. The residual regulatory response determined throughout the progress of the reaction was calculated as the degree of inhibition expressed as a percentage of the inhibition of the native enzyme. The reaction coordinate for the regulatory response corresponds to that for the residual catalytic activity at which the stated residual regulatory activity is observed.

The purified enzyme (0.5 mg protein/ml) was treated as follows: Incubated at 30° for various times up to 40 min with NEM (20-60 μM) or DTNB (40-80 μM) in 0.1 M triethanolamine chloride (pH 7.7), 0.3 M sodium sulfate, and 10% glycerol. Incubated at 0° for 20 min with various coneentrations of pCMB up to 25 μM in 0.05 M Tris-HCl, pH 7.4. The reactions were effectively stopped by 60-fold dilution.

Enzyme and inhibitors were preincubated for 2 min with the assay system, the reaction started by addition of NADP. Response of the concerted inhibition to (●) DTNB, (■) pCMB, and (△) NEM. Response of catalytic activity (▲).

different. With DTNB the loss of catalytic and regulatory activities bears a linear relationship to one another. However, the regulatory activity is affected to a much lesser extent. When 90% of the catalytic activity has been destroyed 65% of the regulatory activity remains intact. With NEM the regulatory activity is affected initially to a greater extent than the catalytic activity. However, with both NEM and pCMB, there appears to be a levelling off effect such that over 50% of the allosteric activity remains intact. The inactivation patterns were similar for commercial and pruified enzyme.

After having obtained an alteration in the concerted inhibition, it was desirable to investigate the inhibitions by glyoxalate and oxaloacetate separately. Only pCMB and DTNB were examined. Figs. 22 and 23 show the effects of these reagents on the separate inhibitions by oxaloacetate and glyoxalate. Both reagents had a stronger effect on the inhibition by glyoxalate than on that by oxaloacetate. The effect on the glyoxalate inhibition is at first much greater than that on the catalytic activity. However, in the case of DTNB (Fig. 22) it rapidly levels off at 66%. 96% of the oxaloacetate inhibitory capacity remains intact even when less than 10% of the catalytic activity is left after treatment with DTNB. With pCMB (Fig. 23) there is a more extensive destruction of the glyoxalate inhibition. At 10% residual catalytic activity 50% of the glyoxalate

Fig. 22. Effect of DTNB on the concerted inhibition and on the separate inhibitions by OAA and by glyoxalate.

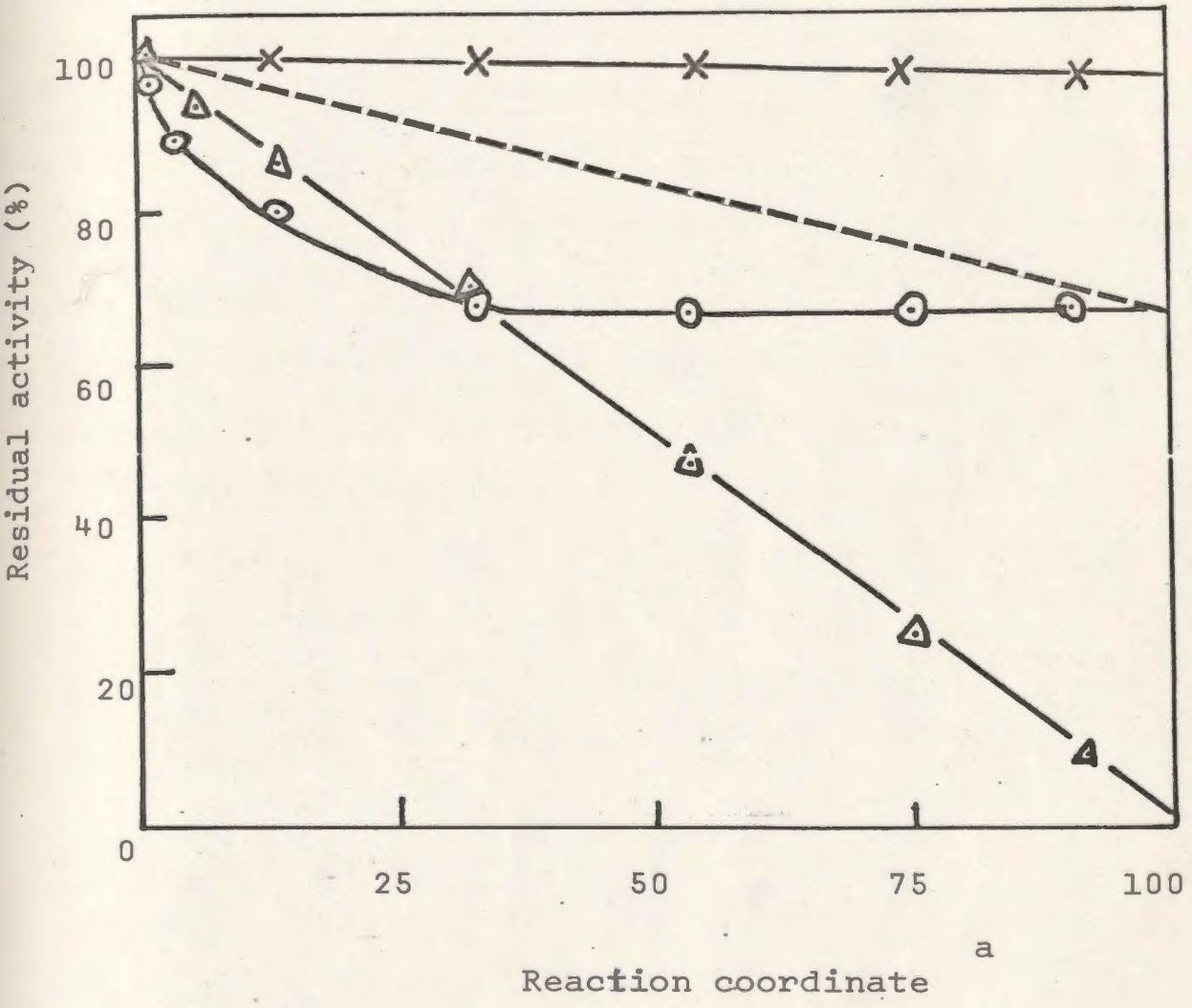


Fig. 22. Effect of DTNB on the concerted inhibition and on the separate inhibitions by OAA and by glyoxalate.

As with the concerted inhibition the separate inhibitors were preincubated with enzyme and the reaction started by addition of NADP. The response of the regulatory activity to DTNB is as shown here, using either 8 mM or 20 mM of glyoxalate or of OAA for the separate inhibitions. The response of the concerted inhibition is as shown using either 0.08 mM glyoxalate + 0.08 mM OAA or 0.16 mM glyoxalate + 0.16 mM OAA. Incubation system is as described in Fig. 21. Response of the regulatory activities to DTNB: (X) inhibition by OAA, (O)inhibition by glyoxalate, (--) concerted inhibition. Response of catalytic activity (Δ).

[a] Reaction coordinate is as defined in Fig. 21.

Fig. 23. Effect of pCMB on the concerted inhibition and on the separate inhibitions by OAA and glyoxalate.

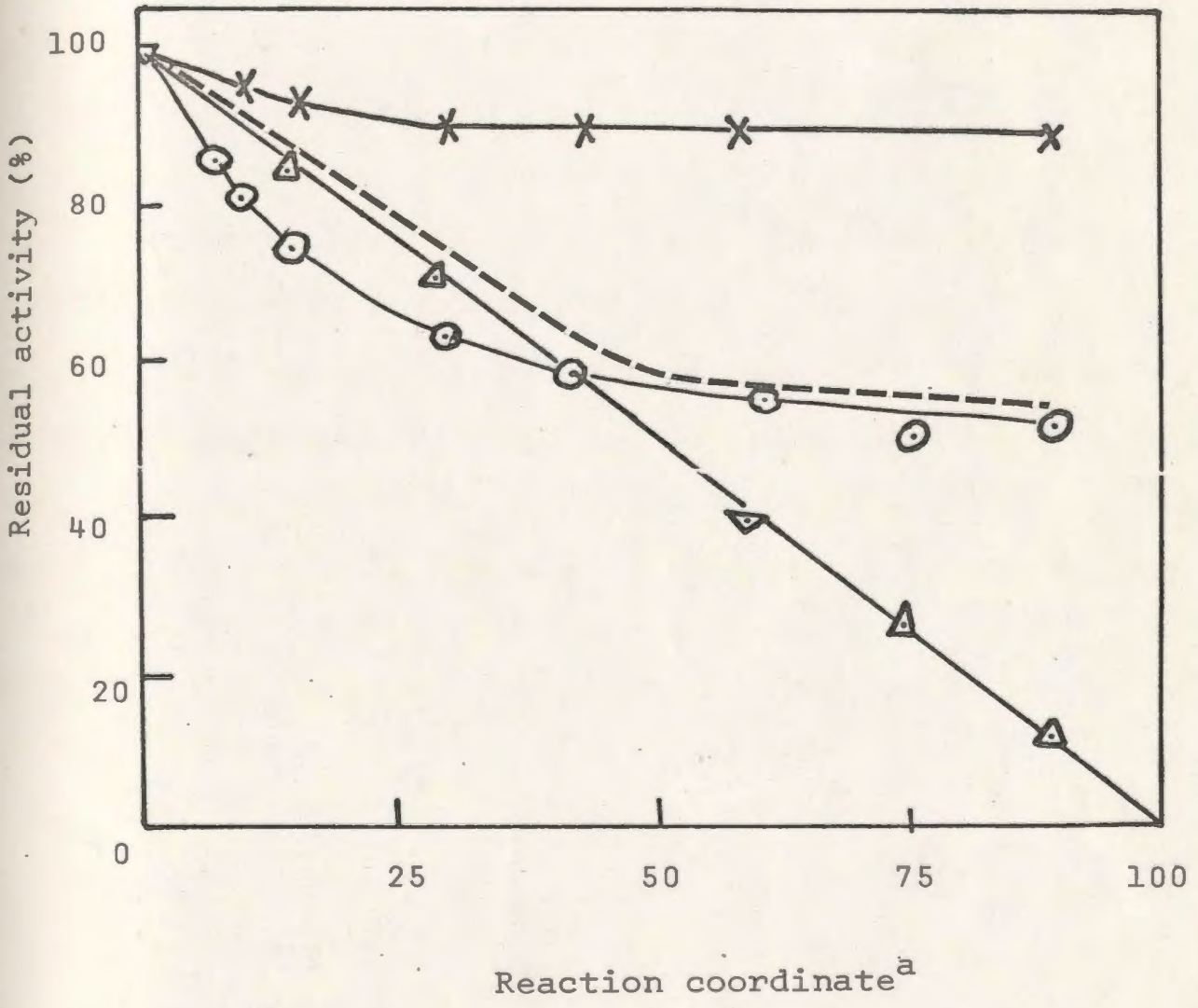


Fig. 23. Effect of pCMB on the concerted inhibition and on the separate inhibition by OAA and glyoxalate.

As with the concerted inhibition the separate inhibitors were preincubated with enzyme and the reaction started by addition of NADP. The response of the regulatory activity to pCMB is as shown here using either 8 mM or 20 mM of glyoxalate or of OAA for the separate inhibitions. The response of the concerted inhibition is as shown using either 0.08 mM glyoxalate + 0.08 mM OAA, or 0.16 mM glyoxalate + 0.16 mM OAA. Incubation described in Fig. 21. Response of the regulatory activities to pCMB: (×) inhibition by OAA, (o) inhibition by glyoxalate, (– –) concerted inhibition. Response of catalytic activity (Δ).

[a] Reaction coordinate defined in Fig. 21.

inhibition has been destroyed. pCMB also appears to affect the OAA inhibition more than DTNB. However, even in this case, the OAA inhibition is only slightly diminished. The data previously given (Fig. 21) for these reagents on the concerted inhibition is replotted here in Figs. 22 and 23 for comparison purposes.

The overall results of the comparison of the effects of X-rays and chemical reagents on ICDH are summarized in Table VIII. With the exception of iodoacetate all the reagents are believed to inactivate the enzyme by sulfhydryl modification. X-rays, LAHPO and H_2O_2 appear to have similar effects. All three reagents are unable to modify the regulatory properties while affecting the three catalytic activities to the same extent. Iodoacetate was also ineffective in modifying the regulatory properties. However, it was the only reagent capable of differentially modifying the catalytic activities. The other reagents, pCMB, DTNB and NEM all react specifically with sulfhydryl groups. While they do not distinguish between the three catalytic activities, all of them affect the concerted inhibition by glyoxalate and oxaloacetate. None of the reagents affected the folate inhibition.

4. The Nature of Folate Inhibition of the Enzyme -

Folate could be readily demonstrated as an inhibitor of ICDH. The curves exhibited a tendency to sigmoidicity (see Fig. 6) and the extent of the inhibition by various concentrations of folate was in agreement with the results of Magar and

TABLE VIII

EFFECT OF X-RAYS AND SEVERAL CHEMICAL REAGENTS ON THE VARIOUS ENZYMIC FUNCTIONS

	Catalytic activity (%) with the substrates			Allosteric activity (%)[a]	
	Isocitrate	Oxalosuccinate	α-Ketoglutarate	Response to folate	Response to oxaloacetate + glyoxalate
None	100	100	100	100	100
X-rays (130 kR)	50	50	50	100	100
Iodoacetate[b] (5 mM)	50	67	67	100	96
p-Chloromercuribenzoate[c] (10 μM)	50	50	50	100	57
N-Ethylmaleimide[d] (50 μM)	50	50	50	100	58
5,5'-dithiobis (2-nitrobenzoate)[e] (80 μM)	50	50	50	100	82
Linoleic acid hydroperoxide[f] (7 μM)	50	50	50	100	100
H_2O_2[g] (350 mM)	50	50	50	100	100

[a] The allosteric activity was calculated as the degree of inhibition by 0.4 mM folate or 0.17 mM glyoxalate + 0.17 mM oxaloacetate, expressed in percent of the inhibition of the native enzyme.

NOTE: Enzyme (0.5 mg/ml) was treated with the above reagents under conditions where 50% loss of dehydrogenase activity occurred. The reaction conditions were:

[b] 40 min at 30° in 0.2 M sodium acetate (pH 5.5)

[c] 15 min at 0° in 0.1 M triethanolamine Cl (pH 7.7), 0.3 M sodium sulfate, and 10% glycerol

[d,e] 22 min at 30° in 0.1 M triethanolamine Cl (pH 7.7), 0.3 M sodium sulfate, and 10% glycerol

[f] 4 min at 22° in 0.1 M Tris-HCl (pH 8.5)

[g] 20 min at 0° in 0.05 M Tris-HCl (pH 7.4)

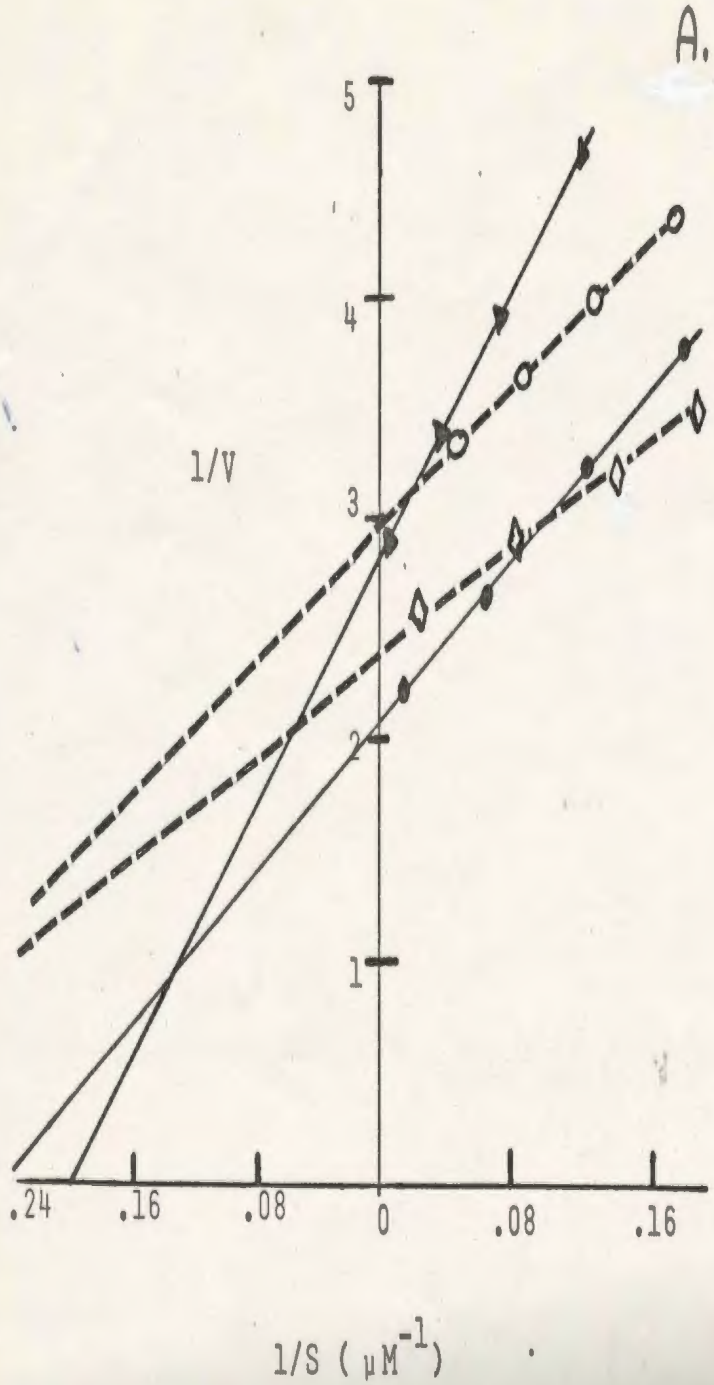
Homi. Also in agreement with their results, the inhibition was neither competitive nor non-competitive with the substrates of ICDH (Fig. 24A). However, since all attempts to modify the folate inhibition were without effect, the nature of this inhibition was more closely examined.

The effect of folate on the other components present in the commercial enzyme was initially examined. It was shown to be an effective inhibitor of the OAA reductase activity. Double reciprocal plots indicated that this inhibition was neither competitive nor strictly non-competitive with the substrate oxaloacetate (Fig. 24B). Folate was also found to inhibit both lactate and malate dehydrogenases to extents comparable to that of isocitrate dehydrogenase (Table IX).

In view of this the effect of folate on an enzyme not occurring in the commercial preparation was examined. GSSG reductase was chosen as a suitable candidate on this basis. Also it employed a somewhat similar assay system and required a pyridine nucleotide cofactor. As with the other activities, folate was a potent inhibitor (Table IX).

Since all of these assays required a pyridine nucleotide cofactor, the possibility existed that folate might some how be interferring with the binding of the cofactor. Fig. 25 shows that NADPH appears to alter the folate absorption spectrum indicating some possible interaction. The peak and the shoulder at 208 nm and 219 nm respectively are replaced by a single symmetrical peak at 217 nm in the presence of NADPH. NADH caused

Fig. 24. Effect of folate on the kinetic parameters of ICDH and NADPH-OAA oxidoreductase.



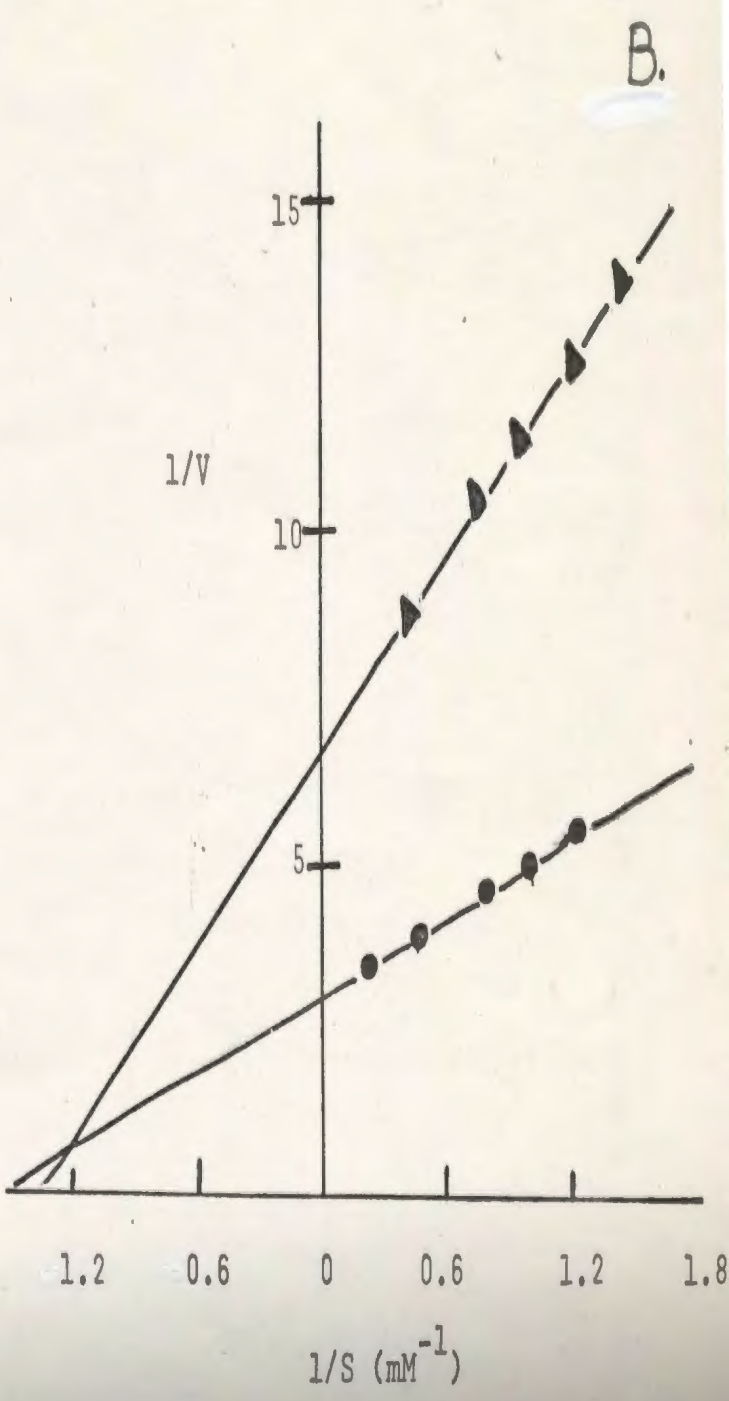


Fig. 24. Effect of folate on the kinetic parameters of ICDH and NADPH-OAA oxidoreductase.

A. Double reciprocal plots at variable (—) NADP and (--) isocitrate in the presence and absence of folate. Assays conducted using a SP800 spectrophotometer with a 0 - 0.1A scale expansion accessory and external chart recorder.

- ● NADP
- ▲ NADP + 0.21 mM folate
- ◊ Isocitrate
- o Isocitrate + 0.21 mM folate

B. Double reciprocal plots at variable OAA in the (▲) presence of 0.122 mM folate and in the absence (●) of folate. The enzyme sample used in part B was a commercial preparation of specific activity 8 μmoles/min/mg.

TABLE IX

FOLATE INHIBITION OF ENZYMIC ACTIVITIES

Enzyme	Substrate	Cofactor	pH	% Inhibition by 0.2 mM Folate
ICDH	Isocitrate	NADP	7.4	17
MDH	Oxaloacetate	NADH	7.4	20
LDH	Pyruvate	NADH	7.6	21
GSSG reductase	GSSG	NADPH	7.4	23
Trypsin	BAEE	-	8.0	97
Alkaline phosphatase	p-nitrophenylphosphate	-	10.0	2
MDH	Malate	NAD	10.0	5

Enzymes were assayed as described in Methods. Enzyme and inhibitor were preincubated in the assay system and the reaction started by the addition of cofactor except in the case of trypsin and alkaline phosphatase where reaction was started by addition of substrate.

Fig. 25. Effect of pyridine nucleotides on the absorption spectrum of folic acid.

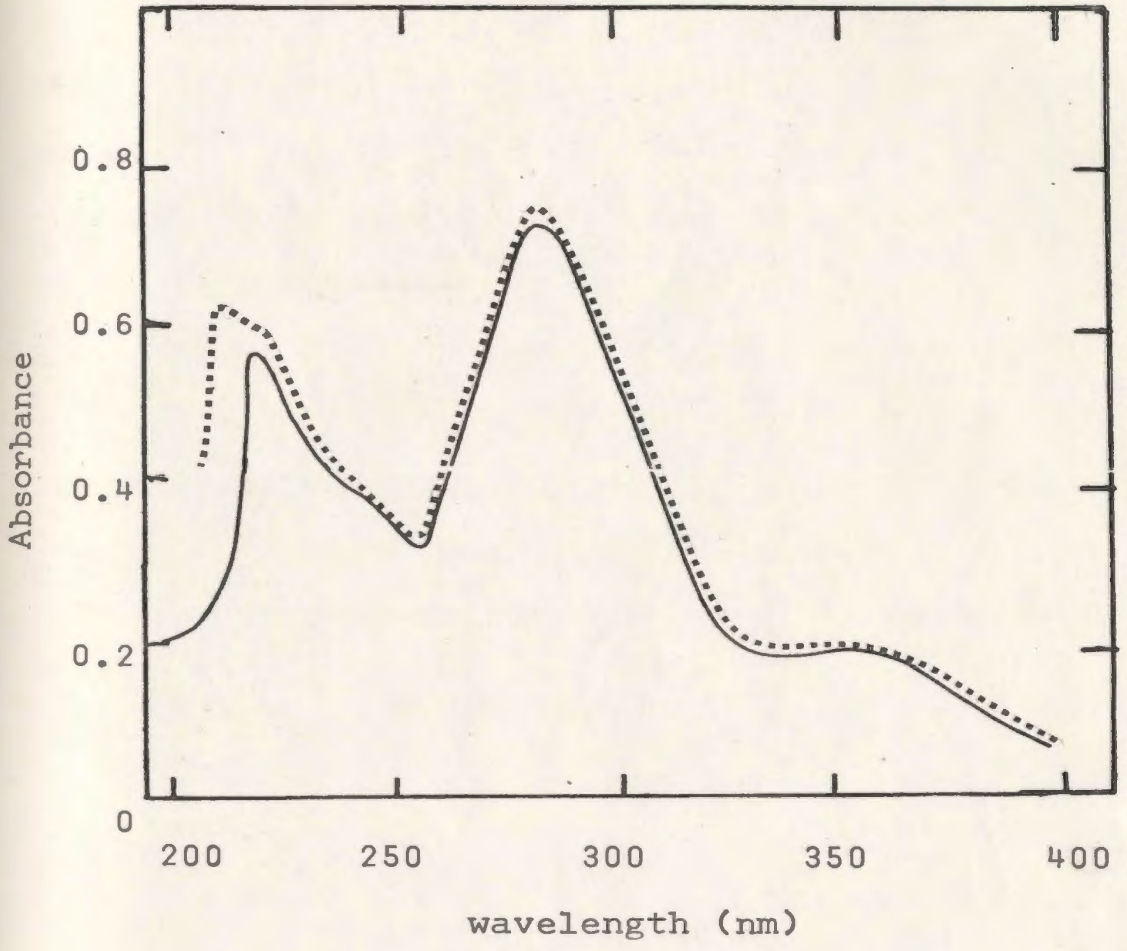


Fig. 25. Effect of pyridine nucleotides on the absorption spectrum of folic acid.

The spectra were determined in 0.1 M potassium phosphate buffer, pH 7.1, using a Unicam SP1800 double beam spectrophotometer. The folate spectrum was determined against a blank of 0.1 M phosphate buffer. That for folate in the presence of NADPH was determined against an equivalent concentration of NADPH in 0.1 M phosphate. Reference and sample cells containing NADPH were zeroed relative to one another before addition of folate; on addition of folate to the sample an equivalent volume of phosphate was added to the reference. Spectra obtained in this way were reproducible.

.... folic acid (25 μM)

—— folic acid in the presence of 30 μM NADPH

a similar spectral change. This may be one part of the effect but is perhaps not the only factor.

The effect on two other assay systems was also considered. Using the assay for trypsin described under Methods, folate was found to inhibit this system more strongly than any of the other systems which had been examined (Table IX). This assay is conducted at 253 nm and does not employ a pyridine nucleotide cofactor. The effect of folate on alkaline phosphatase was also examined by monitoring the formation of the p nitrophenylate anion at 410 nm. Under the conditions of the assay (wavelength 410 nm and pH 10.0), the folate absorption is very much decreased. In concentrations required to inhibit the other enzymes, folate did not significantly affect the alkaline phosphatase assay.

In view of this malate dehydrogenase was assayed in the forward direction using malate as substrate. Although this assay is carried out at 340 nm, the pH of the system is 10, and the folate absorption is greatly reduced. As shown in Table IX the folate inhibition is much less than that on the reverse reaction.

Folate appears to have a broad specificity as an inhibitor. It is therefore possible that at least part of the effect of folate is due to a nonspecific binding of this substance to proteins. In the two cases where folate inhibition was relieved the assays were carried out at pH 10.0. The high pH may decrease

interactions between either enzyme and folate or coenzyme and folate. The results suggest that folate is certainly not specific enough to be a unique metabolic inhibitor of any physiological significance with regard to ICDH.

DISCUSSION

The literature contains an abundance of data indicating that the relatively small protein ICDH possesses the ability to catalyze some quite complex reactions. This is borne out by the present work which further supports the contention that the enzyme may be regulated by mixtures of glyoxalate and oxaloacetate and expands on the involvement of sulfhydryl groups in the enzymic functions.

Regulatory properties

The mechanism by which folic acid produces its apparent inhibition has not been thoroughly investigated here, but contrary to the suggestion of previous workers[43], evidence has been adduced to indicate that it is not acting as an effector molecule which specifically binds to ICDH. While the present studies have not refuted the actual observations, indeed, they are completely reproducible, alternate explanations of these observations are suggested. Using spectrophotometric assays, folate was found to be a potent inhibitor of several enzymes of unrelated functions and with dissimilar assay systems. Magar and Homi stated that they first detected the folate inhibition during a survey of the effects of physiological compounds on a number of nucleotide-linked dehydrogenases. However, they did not specify whether or not the effect of folate on these other enzymes was actually investigated and, if so, whether or not it proved inhibitory.

In agreement with the present results (Table IX) Vogel et al[100] noted that folate does inhibit a variety of enzymes including lactate and malate dehydrogenases. Vogel and coworkers did not determine the exact mechanism of inhibition but suggested a complex inhibition with multiple folic acid molecules participating in the reaction[101]. Since the inhibition appears so non-specific, the binding may be via weak ionic, hydrophobic or hydrogen bonds. However, folic acid is capable of forming charge transfer complexes with addition occurring across the 5,6 double bond of the pteridine ring[102]. In this way is could bind to certain exposed amino acid residues, e.g. tryptophan. Folate is believed to complex with plasma proteins possibly via such a mechanism[103].

With the enzymes requiring a pyridine nucleotide cofactor formation of a charge transfer complex between folate and coenzyme may possibly represent a secondary mechanism of inhibition. Difference spectra (Fig. 25) show that NADPH or NADH, in the absence of enzyme or substrate, modifies the folate absorption. In such an interaction either the folate or the pyridine nucleotide might act as the nucleophile since the nucleotides are also capable of forming complexes — addition occurring across the 5,6 double bond of the nicotinamide ring[104,105]. Inhibition of DPNase by p aminobenzoic acid was attributed by Guardiola et al to the interaction of NAD and p aminobenzoic acid to form an addition compound with altered spectral characteristics[106].

Analogously, folic acid, i.e. N-[4 [(2-amino-4-hydroxy-6-pteridinyl)methyl] amino benzoyl] glutamic acid, might act as a nucleophile and add to the nucleotide via its p aminobenzoic acid moiety.

With MDH it is possible that folate does affect the forward and reverse reactions differently. Differential inhibition does occur with alcohol dehydrogenase but for two distinctive reactions — dehydrogenation and isomerization[101]. Therefore, a similar situation is possible but not as likely for the forward and reverse direction of a one step reaction. The lack of effect on alkaline phosphatase may indicate no suitable exposed residues. However, with both alkaline phosphatase and malate dehydrogenase, the decreased inhibition may be a pH effect in contrast to the suggestion of Vogel that the binding is pH independent[101].

Folic acid is a highly reactive species and while these effects are sufficient to cause inhibition, other factors may also be operating.

Evidence is presented however that ICDH does possess specific regulatory properties. Concerted inhibition by glyoxalate and oxaloacetate was observed in purified pig heart NADP-ICDH as has been observed for the purified enzyme from numerous bacterial sources.

Modification of the inhibition by sulfhydryl reagents suggests that glyoxalate and oxaloacetate exert their effect by binding to ICDH and not to any of the substrates. The protection

by NADPH (Table II) may be interpreted as further support of this, although it could also result from interaction of inhibitors and coenzyme. From the first point of view, however, the protective effect of NADPH might be due to a blocking of the approach of glyoxalate and oxaloacetate to their respective binding sites, either by the presence of NADPH at or near these binding sites, or because of conformational changes in ICDH on binding NADPH. Conformational changes in ICDH on binding NADPH have been suggested by Rose[22].

With regard to the active site of the enzyme the different extents of the concerted inhibition, i.e. 100% for dehydrogenation and 60% for reductive carboxylation, and the quite different concentrations required to reach these maxima again suggest that the regions of the active site required for the different enzyme activities are at least partially distinct.

Involvement of SH Groups in Catalytic and Regulatory Properties -

1. Catalytic activities -

In agreement with previous workers, the present results show that sulfhydryl modification leads to loss of catalytic activity.

Rapid inactivation occurred with micromolar concentrations of LAHPO (Fig. 17) or pCMB (Fig. 18) pointing to the high reactivity of the sulfhydryl groups of ICDH. Inactivation resulted from incubation with the relatively weak electrophile

cystamine — a reagent capable of modifying only highly reactive SH groups (Fig. 19)[82].

Since ICDH appears to contain only one catalytically essential SH group together with almost equally reactive non-essential sulfhydryls[36] it might be expected that the number of SH groups modified for complete inactivation would depend on the modifier used. The rather unreactive N-ethylmaleimide blocks only two sulfhydryls whereas the highly reactive pCMB blocks approximately six for total inactivation (Fig. 20). X-ray inactivation in aqueous enzyme solutions at pH's around neutrality seems mainly due to the hydroxyl radical. This is a reactive species capable of modifying various amino acid residues. However, it has been pointed out that SH groups of high chemical reactivity are usually the most radiosensitive. Total inactivation of the enzyme by X-rays involves the modification of seven to eight sulfhydryls, indicating that the highly reactive hydroxyl radical is even less able than the mercurial to distinguish between the essential sulfhydryl and the reactive non-essential ones. The high G-value for SH destruction (1.6 - 2) correlates well with the high chemical reactivity of these groups in ICDH. In fact, these values are among the highest so far obtained for protein sulfhydryl groups comparing well with the value of 1.8 for the essential sulfhydryl group of papain[82].

Taking a closer look at the X-ray inactivation, such a reactive species as the hydroxyl radical should of course be

capable of modifying most amino acid residues and causing general conformational changes. However, as pointed out in the Results, SH modification appears sufficient to account for the observed loss of catalytic activity.

In agreement with this, the lack of effect on the pH profile (Fig. 13) and the small changes in the kinetic parameters (Table V) suggest that no extensive conformational changes have occurred. The observed change in the Km for isocitrate indicates that modified enzyme molecules are still capable of catalytic activity, depending of course on the extent of modification, and that the observed residual activity does not represent only the population of unmodified enzyme molecules. The observed change in Km may reflect a small conformational change in the enzyme or may be due to modification of a particular SH group. Although no evidence has been advanced for the second possibility, a suitable candidate would be Colman's "Group B sulfhydryl" which binds NEM to alter the Km for isocitrate without causing loss of catalytic activity[36,37].

Fig. 15 indicates that the essential methionine residue of ICDH is not especially radiosensitive. While it is possible that this methionine is being destroyed and simultaneously replaced by another methionine, made available to iodoacetate by denaturation of the enzyme, the smooth curve obtained suggests that this is unlikely.

Aside from methionine and cysteine, destruction of other residues was not measured. However, since a sulfhydryl group — chemically reactive and radiosensitive — is required

for catalysis, it seems quite reasonable that it would be destroyed readily by X-rays during the extensive destruction of the enzyme sulfhydryl groups. Destruction of this group would yield an inactive enzyme.

The various inactivators used here may be compared with regard to the attacking species and the residues which they introduce into ICDH. X-ray inactivation could not be reversed by the addition of an excess of small thiol such as cysteine or GSH. This suggests that the essential sulfhydryl had been oxidized to the more stable sulfonic or sulfinic acids rather than to the easily reduced sulfenic or disulfide forms. Thus two or three oxygen atoms are added to the SH groups upon irradiation. Although X-rays lack selectivity they have the advantage of causing less steric hindrance than the SH reagents which introduce much larger residues. An observed effect with X-rays is therefore more likely to be directly related to modification of the SH groups.

LAHPO and H_2O_2, which are also thought to inactivate ICDH by sulfhydryl oxidation to a product which cannot be reversed by small thiols[99], were similar to X-rays in their effects on the enzymic properties (Table VIII). However, in the absence of a catalyst, with LAHPO and also H_2O_2, the attacking species is the peroxy group. It is a less reactive species than the hydroxyl radical and modifies only a small number of SH groups for complete inactivation (Table VII). Only NEM is more selective than LAHPO. The present results are in agreement with

those of Green et al[99] who noted that LAHPO was 1700 times more effective than H_2O_2 and suggested that the high reactivity is due to binding of the fatty acid moiety to the ICDH molecule so that the peroxy group is in a particularly favorable position to effect SH oxidation.

Substrate and cofactor did not protect against X-ray inactivation of ICDH. Colman found that isocitrate was just as good a protector as isocitrate plus manganese in the case of inactivation by iodoacetate[20]. To protect against SH reagents however, both isocitrate and manganese are required for efficient protection[32,36]. With X-rays neither combination appears particularly effective. Isocitrate alone does not protect: apparent protection by manganese alone is observed. Since manganese ions alone do not protect against SH reagents in general[32,36] and against LAHPO in particular,[99] it is not likely that they would protect against X-rays. It therefore seems reasonable to attribute the effect to radical scavenging which Mn^{++} is definitely capable of doing. It appears that the binding of substrate and cofactor blocks the approach of the SH reagents but with X-rays the small hydroxyl radical can approach the sensitive sulfur even when the substrate is bound to the enzyme. While LAHPO is thought to introduce the same change in ICDH as X-rays, protection is observed since the attacking species, the peroxy group of LAHPO, is attached to a sizeable fatty acid residue whose entry could readily be blocked by substrate and cofactor.

A number of chemical reagents capable of reversibly binding SH groups have been shown to protect these groups during X-irradiation[82]. With ICDH it has been demonstrated that pre-treatment of the enzyme with pCMB protects against inactivation caused by short term incubation (2 min) with LAHPO[99]. However, the reversibility of pCMB binding decreases with time and is effectively zero after the normal time required to irradiate the enzyme. Radioprotection studies with pCMB were therefore not feasible. Similarly difficulties were encountered using DTNB, where 72 hr incubations with mercaptoethanol are required for reversal of SH blocking[32]. Since secondary changes in the irradiated enzyme might occur during this time, radioprotection by DTNB was not investigated.

No sulfhydryl reagent examined was able to differentially affect the various catalytic activities even though a variety of reagents modifying from 2 to 8 SH groups for complete inactivation has been tried. This data is in agreement with that of Colman[32,36] and at variance with earlier results of Siebert who suggested differential inactivation by pCMB[30]. The problems encountered by Siebert do not arise in the present experiments using a single incubation system from which aliquots were removed and assayed for the various activities. Only iodoacetate produced a differential effect. It has not been determined that the single methionine bound is necessary for catalysis and possibly iodoacetate exerts its effect by sterically blocking approach of substrate or cofactor to an adjacent SH group. The presence of such a group

was suggested by the apparent decrease in the number of sulfhydryl groups by one as measured by DTNB under nondenaturing conditions after incubation with iodoacetate[32]. Colman has suggested an overlap of the reactive sites for the catalytic activities. It thus appears that the essential SH groups is in the region of total overlap whereas the methionine residue is closer to the dehydrogenase area of the active site.

More information on the differentiation of the catalytic sites might be obtained if reagents specific for other amino acid residues were to be employed.

2. Regulatory properties -

Significant differences were observed in the effects of various treatments on the regulatory properties of the enzyme. Since folate is probably not of regulatory significance it will not be discussed here. A summary of the effects of the various reagents on the concerted inhibition by glyoxalate and oxaloacetate is given in Table VIII. X-rays, like the peroxides and iodoacetate, do not diminish the concerted inhibition. The other reagents which introduce relatively large residues onto the SH groups in the molecule cause differential loss of catalytic and regulatory activity. These results suggest that the catalytic and regulatory sites are not identical.

Since X-rays and peroxides probably introduce the same modifications into the enzyme it is reasonable that they should have similar effects on the regulatory properties (viz. no effect).

With iodoacetate however, the site of attack is quite different. Colman has suggested that iodoacetate affects dehydrogenation in an all-or-none fashion so that residual dehydrogenase activity is entirely due to unmodified enzyme molecules[20]. Hence the reagent would not be expected to affect the response of the dehydrogenase activity to modifiers. Since iodoacetate differentially affects the catalytic activities it might be interesting to examine the effects of this reagent on the concerted inhibition of the decarboxylation or reductive carboxylation.

With the sulfhydryl reagents proper, a diminished regulatory response was detected. The most immediate distinction between the various modifiers is the size of the residue introduced into the molecule, and this may be the basis for the different effects observed between the sulfhydryl reagents and the X-rays and peroxides. The complex and varied inhibition patterns (Fig. 21) however indicate an interplay of several factors. The relative effectiveness does not appear to correlate directly with steric factors or with the number of SH groups modified. NEM, smaller than the other reagents and modifying the least number of SH groups has the greatest effect on regulatory activities. DTNB, modifying 5 SH groups and introducing a larger residue, has a much less extensive effect. pCMB, also introducing a large residue and modifying 6 - 7 groups, is only slightly less effective than NEM.

Considering the effects of the chemical inactivators on the separate inhibitions it is clear that glyoxalate inhibition is more markedly affected than OAA inhibition (Figs. 22 and 23). With pCMB both of the separate inhibitions were slightly more affected than with DTNB tying in with their relative effectiveness on the concerted process. An interesting feature of the DTNB curve is that the glyoxalate inhibition is much more strongly affected than the concerted inhibition. Comparing the respective effects of these reagents on the separate and concerted inhibitions, it must be remembered that the binding might be different in the two types of inhibition.

It is apparent that pCMB, despite its relative bulk, reacts more extensively with ICDH than the other sulfhydryl reagents. Anomolies have been previously reported with pCMB and theories advanced that it reaches thiol groups inaccessible to other reagents because its high reactivity permits binding to a group which may be momentarily exposed during the constant dynamic changes which the enzyme is undergoing[107]. NEM, a smaller and less reactive residue, produces no large conformational changes[36,37] but may also reach certain essential groups. Its small size enables it to penetrate the molecule and its lower reactivity enables it to react only with the most reactive SH groups, and it is presumably this reaction which causes the alteration in regulatory properties. It is possible that, although DTNB and pCMB block almost the same number of sulfhydryls, their quite different reactivities might result in their blocking different sulfhydryl groups and that this is responsible

for their different effects on the regulatory properties.

The effects may of course be rationalized in other ways. The orientation of the chemical residue introduced into the enzyme may be important and the extent of the steric hindrance may not be directly related to the size of the residue inserted but partially to the manner in which it binds to the SH groups and the resultant bond angles. Differences might then be observed between the S-S bond with DTNB, the S-Hg bond with pCMB and the S-C bond with NEM.

While the effects of sulfhydryl reagents may be accounted for with such explanations, the lack of effect of peroxides and X-rays requires further comment. This result is interpreted to indicate that the regulatory site is more radioresistant than the catalytic site. While it is obvious that the regulatory site is not more radiosensitive, comparable radiosensitivities and simultaneous destruction of both sites may also be ruled out. X-ray inactivation is a random process and therefore with both sites of equal radiosensitivity, an irradiated sample would contain an equivalent number of molecules with catalytic and regulatory sites lost, but the molecules lacking catalytic activity would not all be the same as those lacking regulatory activity and consequently a loss of regulatory activity would be detected here also. The only case where this would not apply would be when destruction of one particular amino acid caused loss of both activities. The ability of SH reagents to produce catalytically active modified molecules and cause differential loss of catalytic and regulatory

activities suggests that the two sites are distinct and renders this alternative extremely improbable. The indirectness of the approach, (i.e. measurement of effector site via measurement of residual catalytic activity) for determining the intactness of the allosteric binding site renders the results more difficult to interpret.

The effects of the various reagents examined point to separate catalytic and regulatory sites. The differential inactivation of catalytic and regulatory activity by sulfhydryl reagents is strong evidence in support of this. The results are, however, insufficient to determine whether the regulatory site is completely distinct or whether it partially overlaps with the catalytic site.

While the amino acid residues involved in the regulatory site were not identified several possibilities exist. Since regulatory activity is affected by sulfhydryl modification the effectors may bind to (1) SH groups, (2) other residues adjacent to SH groups, or (3) other residues distant from the SH groups with sulfhydryl modification causing conformational changes which interfere with the binding of the effectors. If the third possibility was confirmed it would indicate that the regulation is allosteric.

The first possibility is thought to be the least likely because of the evidence for a separate catalytic and regulatory site. If the effectors bound through the catalytically essential sulfhydryl group then the observed differential losses would not be obtained. With no reagent was there complete destruction of

the regulatory activity. This levelling off effect is evidence against any proposal that the loss of regulatory activity is due to modification of one or more sulfhydryl groups of less reactivity than the groups involved in the loss of catalytic activity. If such were the case one would get significant allosteric activity when very little catalytic activity remains, as observed but the levelling off effect would not be explained. On the contrary, the patterns indicate loss of regulatory activity by modification of a very reactive residue but that once this residue is modified alterations in other residues have only a slight effect on the regulatory activity. This indicates that the regulators do not bind to the residue which undergoes modification. The changes produced by sulfhydryl binding initially result in diminished regulatory activity but the modification can affect only approximately 60% of the potency of the concerted inhibition. A point may be made here that only two concentrations of inhibitor were investigated and part of the apparent effect of these SH reagents may be to modify the effects of various concentrations of effectors on the enzyme activity (i.e. K_i may be altered). The levelling off cannot be attributed to modification of the binding site for one of the concerted inhibitors and not the other, since levelling off also occurs with the separate inhibitions. The greater effect on glyoxalate indicates that the greatest steric or electronic hindrance is to its binding site rather than to that for OAA.

Since it appears that the regulators do not bind to the catalytically essential SH groups, the lack of effect of X-rays may also be interpreted to indicate that the regulators do not bind to any SH group. With the randomness of X-ray inactivation and the extensive SH destruction, an effect on regulation would be expected unless the regulatory SH groups were very radio-resistant relative to the catalytic SH groups. Although the first four sulfhydryl groups destroyed are extremely radiosensitive (G value 1.6 - 2) the next four destroyed are still quite sensitive and therefore an effect would be observed if SH groups were involved. As further support for this the results with NEM in particular indicate that the diminished regulatory response results from modification of a highly reactive and therefore radiosensitive SH group. From this point of view the failure of X-rays and peroxides to modify the regulatory properties may be taken to suggest that the sulfur acids produced do not introduce sufficient steric hindrance to affect regulation.

A significant finding of the present work is that in ICDH, X-irradiation causes no diminution of the response of the enzyme activity to allosteric modifiers. Therefore, the regulatory function cannot be more radiosensitive than the catalytic functions. This is only the second enzyme so far studied in which no regulatory property is of greater radiosensitivity than the catalytic properties. It therefore seems that the suggestion of Sanner and Pihl that the allosteric properties of regulatory enzymes are more radiosensitive than the catalytic properties whilst a good generalization is not a strict rule[76,77].

Role of the Concerted Inhibition in the Metabolic Function of Mitochondrial NADP-ICDH

Since isocitrate is a metabolic branching point it is essential that its concentration be carefully regulated by control of the enzymic reactions which produce and utilize it. Intramitochondrial isocitrate oxidation may be by either the NAD-ICDH or NADP-ICDH with present trends tending to assign the NADP-ICDH a secondary role in energy linked electron transport[4,5]. In carrying out this particular function it is thought to operate via a transhydrogenase that is effective between NAD and NADPH to produce NADH to enter the respiratory chain. The activity of the transhydrogenase pathway is relatively low under normal conditions but is stimulated during periods of increased energy demands by the lowered ATP:ADP ratio. In this case NADP-ICDH may be controlled by energy needs indirectly via the transhydrogenase pathway. The concerted inhibition may further act as a control mechanism and open up a variety of functions for this enzyme relating it to the TCA cycle and to other metabolic pathways.

In microorganisms where isocitrate represents a branch point for reactions catalyzed by enzymes of anaplerotic sequences, metabolic significance of inhibition by glyoxalate and oxaloacetate in the integration of the TCA and glyoxalate cycles may be readily postulated [52,108]. It may then be suggested that in mammalian species where such anaplerotic sequences do not occur, the concerted inhibition may represent a fossil activity. It would

however, be preferable to propose a physiological role for this inhibition in ICDH — an enzyme of indeterminate function.

An interesting observation arising from the present work is the occurrence of the three enzymes, MDH, LDH and ICDH together in the commercial enzyme preparation. Based on correspondence of properties to those in the literature (isoelectric points[109,110], elution from CM cellulose[111], and effects of high OAA concentration[111]) the commercial ICDH preparation appears to contain at least one mitochondrial and one supernatant isoenzyme of MDH. It should be noted that it is the mitochondrial form of MDH which contaminates the ICDH after gel filtration on G-150.

Lactate dehydrogenase is suggested to be responsible for the observed glyoxalate reductase activity of the commercial sample of enzyme (Fig. 7) since both activities are eluted from the CM column together and LDH is thought to accept glyoxalate as substrate [95,96]. It is of course possible that there is another enzyme present which reduces glyoxalate. In either case it is interesting that an enzyme — ICDH — occurs in proximity with two other enzymes whose individual substrates, together inhibit the third enzyme. All the more intriguing is the fact that the enzymes which utilize these two metabolites as substrates are very much biased in favor of a coenzyme (NADH) which ICDH will not accept. This in itself may represent a form of control linked to the possible role of ICDH.

A parallellism of properties and location has been noted before in the case of MDH, ICDH and aspartate aminotransferase[109,112]. The observation that MDH and aspartate aminotransferase "copurify" was in fact used by Greville to suggest some special realtionship between these two enzymes[112]. Such reasoning may also be invoked to propose a special relationship between ICDH and MDH which were shown to exhibit a significant number of similarities and a tendency to "copurify" in the present work. The ICDH preparations used were not examined for aspartate aminotransferase activity.

Since pure ICDH was not obtained the question of a common reactant (OAA) for MDH and ICDH has not yet been settled. With regard to the observed NADPH linked OAA reduction, MDH is apparently unable to account for all of the activity (Figs. 2 and 3) suggesting that ICDH does in fact catalyze this reaction. Other explanations discounting ICDH are unlikely. There may be another enzyme which copurifies exactly with ICDH; however, no such enzyme has been reported. The only other possibility would arise from a complementary interaction of MDH and ICDH to form a complex with enhanced NADPH-OAA oxidoreductase activity. While interactions of multienzyme complexes are an established phenomenon, these possibilities seem remote here.

Unfortuantely MDH and ICDH were not separated although a tendency toward separation was observed and it is likely that

with a refinement of the techniques used here, or an investigation of different parameters, purification can be achieved. Colman has produced substantial evidence indicating that her purification scheme yields a homogeneous enzyme[21]. Her enzyme was obtained from Boehringer-Manheim and it is possible that this contains less mitochondrial MDH than the Sigma preparations used here. While this possibility was not fully examined, commercial enzyme from Boehringer was shown to have considerable MDH activity.

From a metabolic point of view ICDH may act as an integrator of sequences for which the concerted inhibition could represent a feed-back mechanism. α-Ketoglutarate links the metabolism of amino acids and the breakdown of carbohydrates and fats and is in fact related to both glyoxalate and oxaloacetate[113]. It takes part in a variety of transamination reactions. With aspartate and α-ketoglutarate as substrates, glutamate and oxaloacetate are produced. α-Ketoglutarate also reacts with glyoxalate in a reaction catalyzed by α-ketoglutarate:glyoxalate carboligase[114,115]. Thus the presence of both glyoxalate and oxaloacetate together would inhibit ICDH activity and α-ketoglutarate production, turning off these sequences.

The fact that one gets inhibition of the forward reaction at a much lower concentration of effectors than are required to inhibit the reverse reaction again correlates with such a proposed function. The forward reaction would be inhibited and α-ketoglutarate production cease so that excess build up of

glyoxalate and oxaloacetate would not occur. This suggests that under physiological conditions the concentration of glyoxalate and oxaloacetate may never reach the point where they may inhibit the back reaction. Thus ICDH, with dehydrogenation inhibited, could operate in a reverse direction to lower the concentration of α-ketoglutarate and consequently that of glyoxalate and oxaloacetate.

Considering other pathways lactate dehydrogenase has been implicated in glyoxalate metabolism and glyoxalate in turn in glycine oxidation, once again connecting ICDH with amino acid metabolism[95]. High concentrations of oxaloacetate on the other hand inhibit both MDH and ICDH. Other examples could be cited but these serve to indicate that the proposal of feed-back inhibition can be justified.

It would appear that the concerted inhibition is more significant than the separate inhibitions because of the much lower concentrations required. However, present knowledge of intracellular metabolite concentration, expecially with regard to compartmentalization and the possibility of pockets of high concentrations of specific metabolites, is insufficient to completely rule out a role for the separate inhibitors.

APPENDIX I

Enzyme samples, purified by the method of Colman, typically showed MDH levels around 30% of the ICDH activity when expressed in terms of the rate of nucleotide reduction/mg protein. Partially purified MDH from pig heart has been obtained with a specific activity of 322 μmolar units/mg[92]. Thus, the catalytic constant for MDH is at the very least 10 x that of ICDH, where maximum specific activity is about 29 μmolar units.

Thus, on a protein weight basis, "pure" ICDH contains less than 3% MDH. The specific activity of 322 μmolar units/mg for pig heart MDH is undoubtedly not the absolute specific activity of the enzyme. More highly purified samples of MDH from *B. subtilis*[116] and beef heart[109] exhibit specific activities of 810 and 1100 respectively. Thus, it would seem likely that "pure" pig heart ICDH contains <1% MDH contamination.

APPENDIX II

In order to express the radiation-induced damage to molecules quantitatively, the term G-value has been introduced. The G-value is defined as

$$G = \frac{\text{number of molecules damaged}}{\text{100 eV of energy absorbed}}$$

e.g.
$$G = \frac{(X \times 10^{-3} \text{ moles/ml})(6 \times 10^{23} \text{ molecules/mole})}{(D_{rads})(6.2 \times 10^{13} \text{ eV/rad/gm})} \times 100 \text{ eV}$$

$$= (0.98 \times 10^{9}) \frac{[X]}{D}$$

where [X] = concentration of damaged molecules (molar)

D = dose (rads).

With enzyme inactivation the number of molecules damaged cannot be directly measured. Therefore, in order to calculate a G-value another equation is used which involves measurable parameters, viz.

$$G = \frac{[E]}{D_{37} \times 10^{-9}}$$

where [E] = enzyme concentration

and D_{37} = dose required to cause 63% inactivation of the enzyme.

For the derivation of this equation see references 117 and 118.

With the indirect effect a further complication must be considered. Other substances present in the solution, buffer molecules in particular, absorb a considerable amount of the radiation energy so that the effective dose is less than the measured value. To correct for this a graph such as Fig. 11B is plotted and extrapolated to zero [E]. The D_{37} value at the intercept is the contribution of the buffer. This value is then subtracted from the observed D_{37} and G calculated using the corrected D_{37}.

REFERENCES

1. Martius, C. and Knoop, F., Hoppe-Seyl. Z. 246 1 (1937).
2. Martius, C., Hoppe-Seyl. Z. 257 29 (1939).
3. Alder, E., v. Euler, H., Gunther, G., and Plass, M., Biochem. J. 33 1028 (1939).
4. Plant, G.W.E., in "Current Topics in Cellular Regulation" (B.L. Horecker and E.R. Stadtman, eds.) v. 2, Academic Press (1970) p. 16.
5. Nicholls, D.G. and Garland, P.B., Biochem. J. 114 215 (1969).
6. Plant, G.W.E., in "The Enzymes", 2nd ed. (P.D. Boyer, H. Lardy, and K. Myrback, eds.) v. 7, Academic Press (1963) p. 105.
7. Ochoa, S., in "The Enzymes" 1st ed. (Sumner and Myrback, eds.) Vol. 2, part 2. Academic Press (1951) p. 929.
8. Kornberg, A. and Pricer, W.E. Jr., J. Biol. Chem. 189 123 (1951).
9. Ceithaml, J. and Vennesland, B., J. Biol. Chem. 178 133 (1949).
10. Charles, A.M., Can. J. Biochem. 48 95 (1970).
11. Barban, S. and Ajl, S., Bacteriol. 71 732 (1956).
12. Lowenstein, J.M. and Smith, S.R., Biochim. Biophys. Acta. 56 385 (1962).
13. Henderson, N.S., J. Exptl. Zool. 158 263 (1965).
14. Siebert, G., Dubuc, J. Warner, R.C. and Plant, G.W.E., J. Biol. Chem. 226 965 (1957).

15. Moyle, J. and Dixon, M., Biochem. J. 63 548 (1956).

16. Grafflin, A.L. and Ochoa, S., Biochim. Biophys. Acta. 4 205 (1950).

17. Illingworth, J.A. and Tipton, K.F., Biochem. J. 118 253 (1970).

18. Bell, J.L. and Baron, D.N., Biochem. J. 90 8p (1964).

19. Bell, J.L. and Baron, D.N., Enzymol. Biol. Clin. 9(5) 393 (1968).

20. Colman, R.F., J. Biol. Chem. 243 2454 (1968).

21. Colman, R.F., Szeto, R., and Cohen, P., Biochemistry 9 4945 (1970).

22. Rose, Z.B., J. Biol. Chem. 235 928 (1960).

23. Moyle, J. Biochem. J. 63 552 (1956).

24. Magar, M.E., and Robbins, J.E., Biochim. Biophys. Acta, 191 173 (1969).

25. Colman, R.F., Biochem. Biophys. Res. Commun. 28 222 (1967).

26. Ochoa, S., J. Biol. Chem. 159 243 (1945).

27. Ochoa, S. and Weisz-Tabori, E., J. Biol. Chem. 159 245 (1945).

28. Ochoa, S., J. Biol. Chem. 174 123 (1948).

29. Moyle, J. and Dixon, M. Biochim. Biophys. Acta, 16 434 (1955).

30. Siebert, G., Carsiotis, M. and Plant, G.W.E., J. Biol. Chem. 226 977 (1957).

31. Fisher, H.F., and Cross, D.G., Science 153 414 (1966).

32. Colman, R.F., Biochemistry 8 888 (1969).

33. Cleland, W.W., Ann. Rev. Biochem. 36 107 (1967).

34. Colman, R.F. and Chu, R., Biochem. Biophys. Res. Commun. 34 528 (1969).

35. O'Leary, M.H., Biochim. Biophys. Acta, 235 14 (1971).

36. Colman, R.F. and Chu, R., J. Biol. Chem. 245 601 (1970).

37. Colman, R.F. and Chu, R., J. Biol. Chem. 245 608 (1970).

38. Colman, R.F., Biochim. Biophys. Acta, 191 469 (1969).

39. Lotspeich, W.D. and Peters, R.A., Biochem. J. 49 704 (1951).

40. Barron, E.S.G. and Singer, T.P., J. Biol. Chem. 157 221 1945.

41. Koshland, D.E. Jr.,in "Current Topics in Cellular Regulation" (B.L. Horecker and E.R. Stadtman, eds.) Vol. 1 Academic Press (1969). p. 1.

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

43. Magar, M.E. and Homi, M.L., Biochem. Biophys. Res. Commun. 31 665 (1968).

44. D'Abramo, F., Romano, M. and Ruffo, A., Biochem. J. 68 270 (1958).

45. Kleinzeller, A., Biochem. J. 37 674 (1943).

46. Ruffo, A., Romano, M. and Adinolfi, A., Biochem. J. 72 613 (1959).

47. Ruffo, A., Testa, E., Adinolfi, A, and Pelizza, G., Biochem. J. 85 588 (1962).

48. Ruffo, A. and Adinolfi, A., Biochem. J. 89 50p (1963).

49. Ruffo, A.,Testa, E., Adinolfi, A., Pelizza, G. and Moratti, R., Biochem. J. 103 19 (1967).

50. Payes, B. and Laites, G.G., Biochem. Biophys. Res. Commun. 10 460 (1963).

51. Shiio, I. and Ozaki, H., J. Biochem. (Tokyo) 64 45 (1968).

52. Marr, J.J. and Weber, M.M., Biochem. Biophys. Res. Commun. 35 12 (1969).

53. Datta, P. and Gest, H. Nature, 203 1259 (1964).

54. Dertinger, H. and Jung, H. "Molecular Radiation Biology". Springer-Verlag, New York (1970) p. 70.

55. Okada, S. in "Radiation Biochemistry", vol. 1, Academic Press (1970) p. 103.

56. Alexander, P., and Lett, J.T.,in "Comprehensive Biochemistry" Vol. 27 (M. Florkin and E.H. Stotz, eds.) Elsevier, New York (1967) p. 267.

57. Moseley, B.E.B.,in "Advances in Microbial Physiology" (A.H. Rose and J.F. Wilkinson, eds.) Vol. 2, Academic Press, New York (1968) p. 173.

58. Howard-Flanders, P., Ann. Rev. Biochem. 37 175 (1968).

59. Okada, S.,in "Radiation Biochemistry", vol. 1, Academic Press (1970) p. 151.

60. Okada, S. and Fletcher, G.L., Radiat. Res. 5 588 (1956).

61. Braams, R.,Radiat. Res. 27 319 (1966).

62. Scholes, G., Shaw, P., Willson, R.L., and Ebert, M.,in "Pulse Radiolysis" (M. Ebert, J.P. Keene,A.J. Swallow, and J.H. Baxendale, eds.) Academic Press, London and New York (1965) p. 151.

63. Mee, L.K. and Adelstein, S.J., Radiat. Res. 32 93 (1967).

64. Gaucher, G.M., Mainman, B.L., Thompson, G.P., and Armstrong, D.A., Radiat. Res. 46 457 (1971).

65. Phil, A. and Sanner, T., Radiat. Res. 19 27 (1963).

66. Giovannozzi-Sermanni, G. and DiMarco, G., Radiat. Res. 26 63 (1965).

67. Lange, R. and Pihl, A.,Intern. J. Radiation Biol. 2 301 (1960).

68. Barron, E.S.G., and Johnson, P., Arch. Biochem. Biophys. 48 149 (1954).

69. Barron, E.S.G., Dickman, S., Muntz, J.A. and Singer, T.P., J. Gen. Physiol. 32 537 (1949).

70. Adelstein, S.J., and Mee, L.K., Biochem. J. 80 406 (1961).

71. Adelstein, S.J., Biochemistry 4 891 (1965).

72. Mounter, L.A., Radiat. Res. 12 487 (1960).

73. Lynn, K.R., Radiat. Res. 46 268 (1971).

74. Mee, L.K., Radiat. Res. 9 151 (1958).

75. Okada, S. and Fletcher, G.L., Radiat. Res. 16 646 (1962).

76. Kleppe, K., Sanner, T. and Pihl, A., Biochim. Biophys. Acta, 118 210 (1966).

77. Damjanovich, S., Sanner, T. and Pihl, A., Eur. J. Biochem., 1 347 (1967).

78. Kleppe, K. and Spaeren, U., Biochemistry 6 3497 (1967).

79. Little, C., Sanner, T. and Pihl, A., Biochim. Biophys. Acta 178 83 (1969).

80. Little, C., Olinescu, R., Reid, K.G. and O'Brien, P.J., J. Biol. Chem. 245 3632 (1970).

81. DiMarco, G., Sanner, T. and Pihl, A., Biochim. Biophys. Acta, 220 1 (1970).

82. Sanner, T. and Pihl, A., Scand. J. Clin. Lab. Invest. (Suppl. 106) 22 53 (1968).

83. Ochoa, S., J. Biol. Chem. 174 115 (1948).

84. Little, C. and O'Brien, P.J., Biochem. J. 106 419 (1968).

85. Ochoa, S., in "Methods in Enzymology", 1, 699 (1955).

86. Lowry, O.H., Rosebrough, N.V., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193 265 (1951).

87. Illingworth, J.A. and Tipton, K.F., FEBS Letters, 7 157 (1970).

88. Kornberg, A.,in "Methods in Enzymology", 1 441 (1955).

89. Racker, E.,in "Methods in Enzymology" 2 723 (1955).

90. Schwert, G.W., and Takenaka, Y., Biochim. Biophys. Acta, 16 570 (1955).

91. Bessey, D.A., Lowry, O.H. and Brock, M.J., J. Biol. Chem. 164 321 (1946).

92. Mehler, A.H., Kornberg, A., Grisolia, S. and Ochoa, S., J. Biol. Chem., 174 961 (1948).

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

94. Vesterberg, O. and Svensson, H., Acta Chem. Scand. 20 820 (1966).

95. Nakada, H. and Weinhouse, S., Arch. Biochem. Biophys. 42 257 (1953).

96. Sawaki, S. and Yamada, K., Nature 210 91 (1966).

97. Bacq, Z.M. and Alexander, P. in "Fundamentals of Radiobiology", Pergamon Press (1966) p. 63.

98. Casarett, A.P.,in "Radiation Biology", Prentice-Hall Inc., Englewood Cliffs, New Jersey (1968) p. 139.

99. Green, R.C., Little, C. and O'Brien, P.J., Arch. Biochem. Biophys. 142 598 (1971).

100. Vogel, W.H., Snyder, R. and Schulman, M.P., Biochem. Biophys. Res. Commun. 10 97 (1963).

101. Snyder, R., Vogel, W.H., and Schulman, M.P., J. Biol. Chem., 240 471 (1965).

102. Vonderschmitt, D.J., Vitols, K.S., Huennekens, F.M. and Scrimgeour, K.G., Arch. Biochem. Biophys. 122 488 (1967).

103. Johns, D.G., Sperti, S. and Burger, A.S.V., J. Clin. Invest. 40 1684 (1961).

104. Miles, D.W., Urry, D.W., and Eyring, H., Biochemistry 7, 2333 (1968).

105. Kaplan, N.O.,in "The Enzymes", 2nd ed. (P.D. Boyer, H. Lardy, K. Myrbach, eds.) Vol. 3, Academic Press (1960) p. 105.

106. Guardiola, A.L., Paretsky, D. and McEwan, W., J. Am. Chem. Soc. 80 418 (1958).

107. Boyer, P.D., Brookhaven Symposia No. 13 "Protein Structure and Function", Brookhaven National Laboratory, Upton, N.Y. (1960) p. 1.

108. Kornberg, H.L.,in "Essays in Biochemistry", Vol. 2 (P.N. Campbell and G.D. Greville, eds.) Academic Press (1966) p.1.

109. Grimm, F.G. and Doherty, D.G., J. Biol. Chem. 236 1980 (1961).

110. Thorne, C.J.R., Grossman, L.I., and Kaplan, N.O., Biochim. Biophys. Acta 73 193 (1963).

111. Thorne, C.J.R., Biochim. Biophys. Acta 42 175 (1960).

112. Greville, G.D.,in "Citric Acid Cycle Control and Compartmentation" (J.M. Lowenstein, ed.) Marcel Dekker, New York and London (1969) p. 42.

113. Tager, J.M., DeHaan, E.J. and Slater, E.C., in "Citric Acid Control and Compartmentation" (J.M. Lowenstein, ed.) Marcel Dekker, New York and London (1969) p. 227.

114. Okuyama, M., Tsuiki, S., and Kikuchi, G., Biochim. Biophys. Acta 110 66 (1965).

115. Kawasaki, H., Okuyama, M. and Kikuchi, G., J. Biochem. (Tokyo) 59 419 (1966).

116. Yoshida, A., J. Biol. Chem. 240 1113, 1118 (1965).

117. Okada, S., in "Radiation Biochemistry", Vol. 1, Academic Press, New York and London (1970) p. 327.

118. Okada, S., in "Radiation Biochemistry", Vol. 1, Academic Press, New York and London (1970) p. 31.

PUBLICATION OF WORK ARISING FROM THIS THESIS ——

1. X-ray inactivation of isocitrate dehydrogenase.
   Holland, P. and Little, C., Proc. Can. Fed. Biol. Soc. 13 120 (1970).

2. Effects of X-irradiation on the various enzymic functions of NADP-linked isocitrate dehydrogenase.
   Holland, P. and Little, C., Can. J. Biochem. 49 510 (1971).