PSEUDOMONAS OXALATICUS (OX 1) AND ITS FORMATE DEHYDROGENASE : A STUDY.

BY

THIAM YONG TAN

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TO MY MOTHER LEY CHEE AND

MY FATHER CHEE FONG

A B S T R A C T

ABSTRACT

PSEUDOMONAS OXALATICUS (OX 1) AND ITS FORMATE DEHYDROGENASE: A STUDY.

by Thiam Yong Tan

Under the supervision of Professor I. Suzuki

Formate dehydrogenase (formate:NAD^+ oxidoreductase, EC 1.2.1.2) from Pseudomonas oxalaticus (OX 1) was purified and by the criteria used, appeared close to homogeneity. Preliminary studies with various inhibitors indicate involvement of sulfhydryl groups and non-involvement of divalent metals in the active site(s) of the enzyme. NAD^+ enhanced the inhibition by cyanide, suggesting a mechanism of inhibition similar to that with the pea formate dehydrogenase (FDH) reported by Ohyama and Yamazaki (1975).

Kinetic studies of FDH were carried out. The reaction was determined to be Ordered Bi Bi (Cleland nomenclature) by the product inhibition patterns. Initial velocity formate plots were biphasic, resulting in two K_m's

for formate. Initial velocity NAD^+ plots were linear with only one K_m value for NAD^+. FDH appeared to be a "Hysteretic" enzyme as defined by Frieden (1970) requiring the presence of small amounts of NAD^+ or NADH for activation.

The behaviour of FDH when ultracentrifuged indicated that the active enzyme was an oligomer. Molecular weight was 151,000 ± 3,000 by Sephadex gel filtration and 157,000 ± 3,000 by sucrose density gradient centrifugation (in the presence of 0.01 M NAD^+). The number of subunits in the active oligomer is not known.

Growth studies of the organism on liquid and solid media indicated that major cellular reorganization was involved in the adaptation from glutamate to formate as sole carbon source. The ability of whole cells growing on oxalate to oxidize an external source of formate was studied. Electronmicrographs of ultra-sections of cells grown on various media are shown. Finally a speculative, hypothetical model of the <u>in vivo</u> formate oxidizing systems is presented in an attempt to accomodate most of the results obtained so far by various groups.

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ABBREVIATIONS

AMP	-	Adenosine monophosphate
ATP	-	Adenosine triphosphate
Barbital	-	5,5'-Diethylbarbituric acid
Bathocuproine	-	(2, 9-Dimethyl-4, 7-diphenyl-1, 10-phenanthroline)
B.S.A.	-	Bovine serum albumin
CCCP	-	Carbonyl-cyanide-m-chlorophenyl-hydrazone
CoA	-	Coenzyme A
DCPIP	-	2,6 Dichlorophenolindophenol
DEAE-cellulose	-	Diethylaminoethyl-cellulose
DNP	-	2-4, Dinitrophenol
DTNB	-	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	-	Dithiothreitol (Clelend's Reagent)
EDTA	-	Ethylenediaminetetraacetic acid (disodium salt)
FAD	-	Flavin adenine dinucleotide
FMN	-	Flavin mononucleotide
GMP	-	Guanosine monophosphate
GTP	-	Guanosine triphosphate
HEPES	-	(N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
MES	-	(2[n-Morpholino]ethane sulfonic acid)

NAD^+	-	Nicotinamide adenine dinucleotide
NADH	-	Reduced nicotinamide adenine dinucleotide
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Reduced nicotinamide adenine dinucleotide phosphate
NBT	-	Nitroblue tetrazolium
PCMB	-	p-hydroxymercuribenzoate (sodium salt)
PMS	-	Phenazine methosulfate
SDS	-	Sodium duodecyl sulfate
TCA	-	Trichloroacetic acid
TEMED	-	N, N, N', N' Tetramethylethylene-diamine
TRIS	-	Tris(hydroxymethyl)aminomethane
T-soy	-	Trypticase-soy

INTRODUCTION

INTRODUCTION

Although the bacterium Pseudomonas oxalaticus (OX 1) was first isolated from the gut of common Indian earthworms by Khambata and Bhat in 1953, no further studies were done on the organism until 1958 when Quayle and Keech demonstrated that P. oxalaticus growing on formate fixed CO_2 via ribulose 1:5-diphosphate in a cycle similar to that discovered earlier by Bassham et al (1954) to exist in Scenedesmus obliquus. In subsequent experiments Quayle with various coworkers from 1959 to 1968 examined P. oxalaticus grown on both formate and oxalate as sole carbon source. The paths of metabolism were charted and various enzymes were examined. It was discovered that the enzyme formate dehydrogenase (formate : NAD^+ oxidoreductase, EC 1.2.1.2) was a key enzyme in the growth of the organism on both formate and oxalate. An initial attempt was made at the purification of the enzyme, although it proved to be relatively unstable. However, a preparation was obtained which could be used for the enzymatic micro-estimation of formate (Johnson et al, 1964 b). Further work on the growth of the organism on various substrate mixtures brought to light some unusual and unexpected results with regard to the presence or absence in the cells of NAD^+-dependent formate

dehydrogenase (FDH) as opposed to the formate oxidizing capability of the cells (Blackmore and Quayle, 1968 b). This work was therefore begun in an attempt (1) to purify the FDH's from both oxalate-and formate-grown cells in order to determine if the enzymes were the same and (2) to elucidate and correlate the two "separate and distinct" systems which were capable of oxidation of formate; viz, the soluble NAD^+-dependent FDH and the particulate, non NAD^+-linked formate oxidase.

During the course of the work, results were published by Hoepner and Knappe (1970) and Hoepner and Trautwein (1971, 1972) which apparently conflicted with those obtained by Quayle and his coworkers and also differed with some of the data being gathered for this thesis. An attempt was therefore made in the final discussion to try to resolve the apparently divergent results by the postulation of a speculative, hypothetical model for the NAD^+-dependent FDH : formate oxidase systems. This thesis presents the results of FDH purification, the kinetics of the enzyme, molecular weight estimations and some inhibition studies. The results of some growth studies of P. oxalaticus on liquid and solid media are also presented.

H I S T O R I C A L

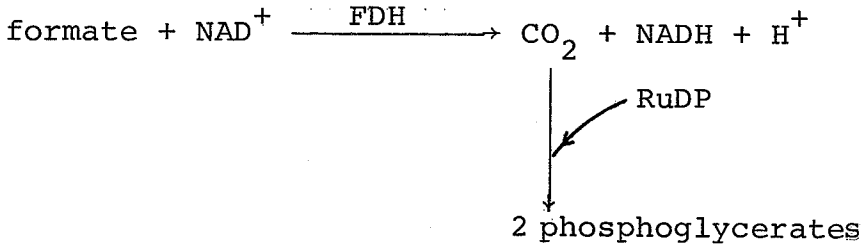
HISTORICAL

The bacterium Pseudomonas oxalaticus was originally isolated by Khambata and Bhat (1953 a) as a result of their studies with bacteria which were responsible for removal of oxalic acid in the soil, left behind by decaying plant material. In the course of their investigation, they isolated several strains of this organism which also had the ability to grow on formate. Just after this, they isolated a similar organism that they named Bacterium oxalaticum, which could grow better on formate than on oxalate as sole carbon source (Khambata and Bhat, 1953 b). Prior to this, other bacteria had also been isolated which could grow on oxalate and formate. The first was Pseudomonas ("Bacillus") extorquens which had a red pigment and was highly aerobic. It was isolated by Bassalik (1913). Bhat and Barker (1948) also isolated an organism, Vibrio oxalaticus, which could grow on oxalate as sole carbon source and on formate in the presence of yeast extract. Janota (1950) also did work on the utilization of oxalate for growth by Pseudomonas extorquens. She noted that the lag period before growth depended on the concentration of

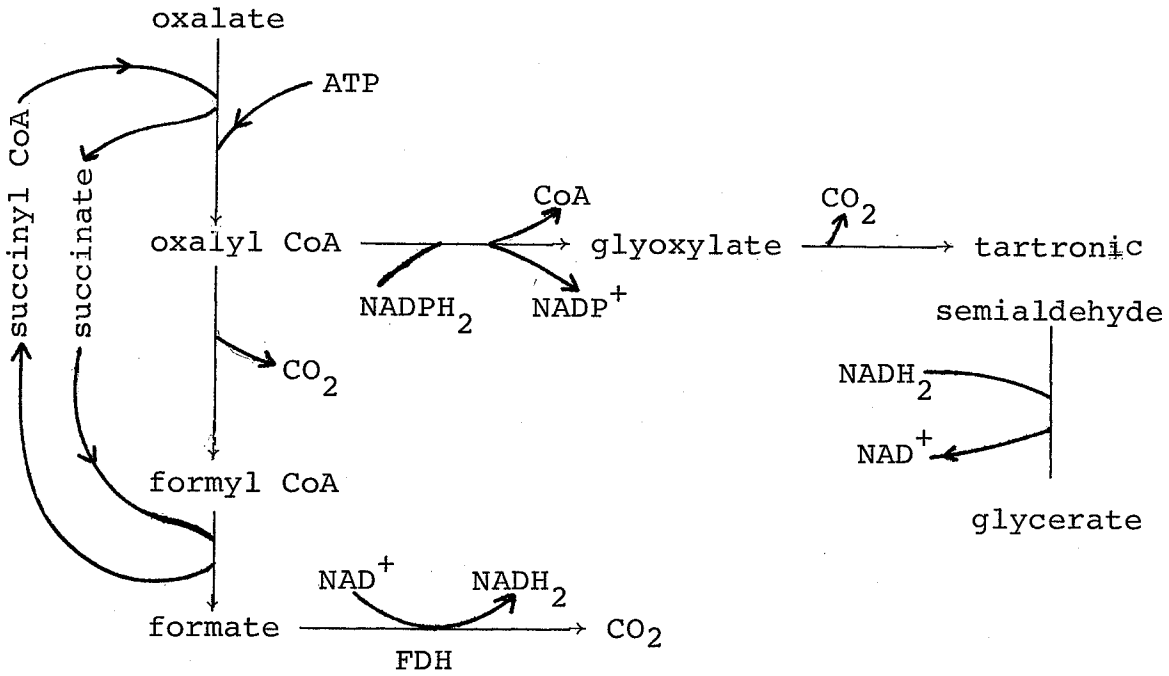
bacteria present. Jayasuriya (1954) also isolated an unnamed bacterium from soil which could grow on oxalate but not acetate or formate. It could, however, oxidize both acetate and formate.

It remained however for J. R. Quayle, with his various co-workers, to note the significance of formate being both carbon and energy source, and to elucidate the metabolic pathways and different mechanisms for energy production that Pseudomonas oxalaticus strain OX 1 employed whilst growing on formate and on oxalate. He found (Quayle and Keech 1958, 1959 a) that when formate was used as sole carbon source, the organism possessed a high level of ribulose diphosphate (RuDP) carboxylase ("carboxydismutase") EC 4.1.1.39, activity fixing carbon dioxide via ribulose 1:5 diphosphate. When oxalate served as sole carbon source however, the RuDP carboxylase activity was negligible. Johnson et al (1964), found that the enzyme formate dehydrogenase (FDH), formate: NAD^+ oxidoreductase EC 1.2.1.2, is the first enzyme in the carbon path of Pseudomonas oxalaticus in the "fixation" of carbon dioxide since the carbon dioxide came from a direct oxidation of the formate molecule by the enzyme. The path of carbon when Pseudomonas oxalaticus grows on formate is shown below,

below,



The path of carbon when the organism grows on oxalate (Quayle et al 1961 a, Quayle 1963 a, Chung et al 1971)



The enzyme FDH is also produced by the oxalate-grown organism (Quayle et al 1961 a).

In Nature, enzymatic oxidation of formate to carbon dioxide is known to be carried out in several ways. In peas (Mathews and Vennesland, 1950) and in some bacteria like Pseudomonas spp. (Quayle et al, 1961 a, Johnson and Quayle, 1964 a) and Rhodopseudomonas palustris (Yoch and Lindstrom, 1969), it is coupled to the reduction of NAD^+. In Escherichia coli however, it is not NAD^+ (or $NADP^+$)-dependent but is instead linked to cytochromes (b_1) probably via ubiquinones (Gest 1951, Wrigley and Linnane 1961, Itagaki, 1964). In animal tissues it is oxidized by a catalase-ferroxidase complex (Nakada and Weinhouse 1953, Rapporport et al 1956). In the case of the fungus Aspergillus niger, formate appears to be oxidized by molecular oxygen itself, which seems to be the only effective electron acceptor, resulting in the production of hydrogen peroxide and carbon dioxide as the reaction products (Hauge, 1957).

The enzyme FDH was initially discovered in French bean (Phaseolus vulgaris) seeds by Thunberg (1921). It was subsequently studied by Fodor and Frankenthal (1930), Andersson (1934) and Lichtenstein (1936) and found to require a cofactor, identified as Coenzyme I (NAD^+). This "plant" enzyme differed considerably from what was then known as the "bacterial" enzyme. This

"bacterial" enzyme was studied by Gale (1939), using the bacterium Escherichia coli grown anaerobically. He found that this formate dehydrogenase did not require NAD^+ as a cofactor and that it was inactivated by shaking with air in the presence of formate. Later work has shown that this soluble FDH of E. coli is a component of the inducible anaerobic formate-hydrogenlyase system (Gest and Peck 1955, Peck and Gest 1957). Gray and Gest (1965) proposed a two-enzyme model for this system consisting of the FDH and a particulate hydrogenase linked together by a cytochrome system. A formate: cytochrome oxidoreductase was found by Ishimoto et al (1954) in Desulfovibrio vulgaris. This enzyme has since been purified by Yagi (1969). The FDH is not NAD^+ or $NADP^+$-dependent, nor can it couple to ferredoxin. Its natural electron acceptor is a type c_3 cytochrome but it will couple to ferricyanide at pH 6 to pH 7. These enzymes, like the soluble FDHs in the Clostridium group all belong in the EC class 1.2.2. - and are basically part of anaerobic systems. Work on the clostridial systems has been done by Hug and Sagers (1957), Bradshaw and Reeder (1964), Li et al (1966). All the systems involving the FDH in Clostridium spp. seem to catalyse an overall reaction sequence in the direction of reduction of carbon dioxide to formate and thence to acetate (Lentz and Wood, 1955). Ferredoxin seems to be

the natural terminal electron acceptor for most of these systems (Brill et al 1964; Bradshaw and Reeder 1964). Although the FDHs themselves did not couple to NAD^+, formate did cause rapid NAD^+ reduction by the extracts. This was attributed to a NAD^+-specific, ferredoxin-dependent formate oxidase system (Brill et al 1964). However, Li et al (1966) demonstrated the presence of a $NADP^+$-dependent FDH catalyzing the oxidation of formate to carbon dioxide. Since the overall system catalysed the reverse of this reaction, it was only shown later how the organisms achieved this and overcame the thermodynamically unfavourable reaction of carbon dioxide reduction to formate. Andreesen and Ljungdahl (1971) demonstrated that the $NADP^+$-dependent FDH in Clostridium thermoaceticum was in fact driven against the thermodynamic gradient by being coupled with formyl tetrahydrofolate synthetase; carbon dioxide is thus reduced and ends up in the form of formyl-tetrahydrofolate.

A NAD^+-dependent, soluble FDH has also been shown in the facultative phototroph Rhodopseudomonas palustris by Yoch and Lindstrom (1969), although the specific activity of the enzyme in the extracts was relatively low compared with the enzyme in Pseudomonas oxalaticus. The optimum pH for NAD^+ reduction was pH 8.0 whereas the optimum for reduction of cytochrome c-552 (R.

palustris) and DCPIP by this enzyme was pH 6.8. Activity with respect to reduction of both NAD^+ and the cytochrome c was greatly increased by the addition of either FMN or FAD to the reaction mixture.

Adler and Sreenivasaya (1937) first reported that the FDH from peas was strongly inhibited by low concentrations of cyanide. Mathews and Vennesland (1950) reported that preparations from rat liver and kidney which could oxidize formate did not require NAD^+, but ATP or adenylic acid instead. Cyanide inhibition of this system was strong. However the preparation from peas ("Laxton's progress") was unaffected by ATP although the cyanide inhibition was even more pronounced. The specificity of the pea enzyme for NAD^+ and not $NADP^+$ was also noted. Using standard values for the thermodynamic data, the value for $\Delta G'$ at pH 8.0 (30^oC) for this reaction was calculated to be - 6.31 kcal per mole. The equilibrium of the reaction thus strongly favours the oxidation of formate to carbon dioxide.

Davison (1951) was the first to achieve a reasonable purification of this FDH. Using bean (Phaseolus multiflorus) seeds, she purified the enzyme 21-fold, confirmed the specificity for NAD^+, the inhibition by cyanide and reported that azide was also a strong inhibitor. Both cyanide and azide inhibitions

were reversible by dialysis, however. Reversal of the "normal" direction of the reaction (formate reduction) was also attempted by coupling the reaction to the oxidation of triose phosphate to phosphoglyceric acid (producing reduced NAD^+ and ATP), but no reduction of carbon dioxide to formate occurred. She also noted that although the enzyme could not couple with the cytochrome system directly, it could do so via a flavin enzyme.

Yamamoto (1954) largely confirmed these findings using bean (Vigna sesquipedalis) seeds but found that the cyanide inhibition was not reversible by dialysis in his preparation. He also observed activation of FDH by Fe^{++} and Co^{++}, although Fe^{+++} and Cu^{++} were strong inhibitors. His enzyme preparation was "sensitive" to oxygen but this sensitivity could be completely reversed by the addition of cysteine or partially reversed by ascorbic acid.

In bacteria, the presence of a NAD^+-dependent FDH was first demonstrated by Kaneda and Roxburgh (1959), when they showed that extracts of Pseudomonas PRL-W4 catalysed the reduction of NAD^+ in the presence of formate. Quayle et al (1961 a) mentioned the presence of a soluble, NAD^+-linked FDH in Pseudomonas oxalaticus (OX 1). Johnson et al (1964 a) showed that a soluble, NAD^+-dependent FDH was also present in extracts of

methanol-grown Pseudomonas AM1, Pseudomonas extorquens, Pseudomonas methanica and Protaminobacter ruber. The enzyme was quite labile unless protected by mercaptoethanol. Even then it was not very stable. Since the highest amount of FDH in this group was detected in Pseudomonas AM 1, purification was attempted from its extract. The FDH was purified three-fold only but its strict specificity for NAD^+ ($NADP^+$ did not work) and formate as substrates was demonstrated. At pH 7.5, no oxidation of methanol, formaldehyde, acetate, oxalate, succinate or malate by the enzyme preparation could be detected. The preparations had an optimum pH for enzyme activity of pH 8.4 and the K_m values obtained were 0.25 mM for formate and 0.09 mM for NAD^+. Some NADH oxidase activity was present in the preparation and inhibition of the enzyme by cyanide, azide (to a lesser degree than cyanide) and Fe^{++} and Cu^{++} salts were mentioned.

FDH from Pseudomonas oxalaticus strain OX 1, which has been an object of study in this work, was first noted by Quayle, as mentioned earlier. Subsequently his group purified it for use as an enzymatic tool for the micro-estimation of formate (Johnson et al, 1964 b) and found that the oxalate-grown cells apparently possessed a much higher level of FDH than formate-grown cells (Quayle, 1966). At the time, initial enzyme purification

procedures were mentioned together with some properties of the enzyme and the effects of various inhibitors. Preincubation of the enzyme for 30 minutes in the presence of a 1 mM concentration of the inhibitors resulted in 90% inhibition by azide, 100% by cyanide, 80% by fluoride and 80% by hypophosphite. The optimum pH for enzyme activity (25^{o}C) was determined to be pH 7.6 and the inherent instability of the enzyme *in vitro* was mentioned. The fact that incubation of FDH with formate in the cuvette before the addition of NAD^{+} to start the reaction produced a marked decrease in the rate of enzyme activity and hence the importance of starting the reaction by addition of either formate or enzyme last during enzyme assays, was also mentioned. Hoepner and Knappe (1970) presented a modified procedure for formate determination using the FDH from *Pseudomonas oxalaticus*. Much of this work substantiated that of Johnson *et al* (1964 b) and Quayle (1966), except the point that the formate-grown organism produced 3½ times more FDH, specific activity-wise than the oxalate-grown organism (Quayle had reported the opposite result). Conditions for growing the organism and collecting the cells were described in detail. The importance of collecting the cells early was stressed (just when turbidity was visible). An outline of their procedure for purification of the FDH was also given.

FDH has also been purified from mung bean (Phaseolus aureus) by Peacock and Boulter (1970) to about 50% purity and a kinetic study done on the enzyme. They postulated that the mechanism followed an ordered sequence of substrate addition to the enzyme, without a rate-limiting central ternary complex; that is, a Theorell-Chance mechanism. The molecular weight of the FDH, as determined by gel filtration was 92,000 ± 10,000 daltons.

Hoepner again published work on the FDH from Pseudomonas oxalaticus (Hoepner and Trautwein, 1972). This time the purification procedures were carried out anaerobically, at pH 5.6. From cells grown on formate (with pyruvate added in small amounts as a cosubstrate), they obtained an enzyme preparation which could catalyse the oxidation of NADH by oxygen (forming hydrogen peroxide) and dyes in addition to catalysing the oxidation of formate by NAD^+ reduction, DCPIP and ferricyanide reduction, or the direct oxidation by molecular oxygen (resulting in formation of hydrogen peroxide). FDH was characterized as a flavoprotein with at least one mole FMN per mole of enzyme protein. It also contained non-heme iron (5-8 moles) and labile sulfide (7-8 moles). Mention was made of the slow but irreversible inhibition of FDH by formate and the existence of a particulate

fraction containing formate oxidizing activity without NAD^+ reduction (this particulate fraction was first noted by Quayle and Keech, 1959 d). These "formate oxidase" particles were extremely oxygen-labile and required the addition of formate to protect them against inactivation. They could not cause the release of any "soluble" FDH from these particles. This purified FDH exhibited two major and three minor protein bands when subjected to polyacrylamide gel electrophoresis (presumably at pH 5.6). Two molecular weight values for the enzyme were given. The main species being of 300,000 daltons and the minor one of 200,000 daltons. Both values were obtained by sucrose density gradient centrifugation. An NAD^+-dependent FDH has recently been purified to homogeneity (at least 95% pure by gel electrophoresis) from peas (Pisum sativum) by Ohyama and Yamazaki (1974). They have also published work clarifying the mechanism of inhibition of FDH by cyanide and azide and done some initial work on the kinetics of the enzyme (Ohyama and Yamazaki, 1975). They determined the molecular weight of the FDH as 70,000 ± 2,000 daltons by gel filtration and 72,000 ± 2,000 by sedimentation equilibrium. The FDH was colourless, contained virtually no iron, manganese or zinc and appeared to be a sulfhydryl enzyme. It was also dimeric in nature with subunits of

42,000 daltons as estimated by SDS-polyacrylamide gel electrophoresis.

Much work has been done on the organism, Pseudomonas oxalaticus (OX 1), itself. This has been almost entirely due to Quayle and his co-workers from 1958 to 1968. That the organism assimilated carbon dioxide (produced by the oxidation of formate through FDH) via part of the RuDP cycle (Bassham et al, 1954) was shown by Quayle and Keech (1958, 1959 a, c, d). Formate was shown also to double as the energy source. The NAD^+-linked, "soluble" FDH was thought to serve both functions (to provide NADH as well as to generate energy). When the organism grows on oxalate as sole carbon source, it utilizes the carbon path leading to glyoxylate and glycerate, as illustrated in the diagram at the beginning of this section. The necessary reducing power for the formation of glyoxylate and glycerate being generated via the oxidation of formate by the NAD^+-dependent FDH (Quayle and Keech, 1959 b; 1960 a, b; Quayle et al, 1961; Quayle and Taylor, 1961; Quayle, 1962, 1963 a, b, c). Since FDH was utilized by both formate and oxalate-grown cells for producing reducing power, this apparently accounted for the high levels of FDH found in oxalate-grown cell extracts (Quayle, 1966).

Blackmore and Quayle (1968 b) looked into the growth rate of Pseudomonas oxalaticus on various carbon sources. They separated the sources into "slow", "equi" and "fast"-growth substrates on the basis of whether the organism grew at a slower, the same or a faster rate on that substrate than it did on formate. Succinate, lactate and citrate were "fast"-growth substrates (mean generation times approximately 2 hours). Glycollate and glyoxylate were "equi"-growth substrates (mean generation times of 3½-4 hours) and malonate and glycerol were "slow"-growth substrates (mean generation times of 14 and 24 hours respectively). They then looked at the choice of mode of metabolism by the organism when presented with equimolar mixtures of formate and one of the other substrates and also checked the cell extracts for activity of "indicator" enzymes (e.g. FDH, RuDP carboxylase). The growth rate of the organism in a mixture always approximated the rate at which it grew on the "faster" of the substrates alone.

In mixtures of the "slow"-growth substrates and formate, the resulting cell extracts slways had high levels of FDH and RuDP carboxylase but in mixtures of "fast"-growth substrates and formate, these enzymes were apparently not synthesized (oxalate was not classified with the other substrates as FDH was present when the organism grew on either oxalate or formate alone, al-

though on the basis of mean generation time it could be included in the "equi"-growth group). This suggested that although formate or a derivative acts as an inducer for these enzymes, control by some form of metabolite repression must operate as well.

In an equimolar mixture of formate and oxalate the organism quickly employs the autotrophic mode as the major type of metabolism with the metabolism of oxalate as the minor one, regardless of whether the cells were previously grown on only formate or oxalate.

A rather interesting situation developed with the two "equi"-growth substrates. In a glyoxylate-formate mixture, the specific activity of FDH, as measured by NAD^+ reduction, was 60% of that obtained when the cells were grown on formate alone; even though cells grown on glyoxylate alone had a very low specific activity for FDH. RuDP carboxylase activity was negligible for both glyoxylate-formate and glycollate-formate cells whereas the level of glyoxylate carboligase (EC 4.1.1. b) was the same in both types of cells. With glycollate, the results were quite different. The levels of both RuDP carboxylase and FDH (as measured by NAD^+ reduction) activities were negligible in extracts of glycollate and glycollate-formate grown cells, although glyoxylate carboligase activity was high for

both types of cells. However, it was found that the cells grown in the glycollate-formate mixture had, in fact, utilized an extensive portion of the formate in the mixture. These cells could also oxidize formate (when tested manometrically) immediately whereas cells grown on glycollate alone could only oxidize formate after a lag period of 30 minutes. All attempts to demonstrate the presence of a NAD^+ (or $NADP^+$)-linked FDH proved unsuccessful however.

This same situation (the presence of "formate oxidase", but no FDH) was encountered with cells grown in mixtures of "fast"-growth substrates and formate. It therefore appeared that an extracellular presence of formate could induce the synthesis of a "formate oxidase" system that was being used solely as an energy source (albeit an ancillary one). Blackmore and Quayle (1968 a) also looked into the mode of metabolism employed by formate-grown cells just after transfer into an oxalate medium (Blackmore and Quayle, 1968 a). The organism utilized a mixed heterotrophic-autotrophic mode for a short time. The contribution by the autotrophic mode was only a minor one; the major mode utilized was the heterotrophic metabolism of the oxalate. Even this condition was essentially a temporary, transitional phase, as continued growth on oxalate saw the disap-

pearance of the key enzymes for the autotrophic pathway and the complete switch-over by the organism to heterotrophic metabolism.

Hoepner and Trautwein (1971) found that their Pseudomonas oxalaticus culture, of which there were three variants, was after all, unable to grow on formate as sole carbon source. The culture could only do so if pyruvate was present concomittantly as a co-substrate in small amounts (1:100 ratio of pyruvate: formate, approximately). The amount of pyruvate also affected the yield of bacteria from the culture (pyruvate was used at up to 5 mM concentrations, with 100 mM formate in the medium). Also, only one of the variants could grow on oxalate as sole carbon source, the other two could not, but all three variants could grow on glucose as sole carbon source however. They attributed Hoepner's previous results (Hoepner and Knappe, 1970) as probably due to bacterial contamination or impurities in the formate itself which was used to make up the medium. They could not at that time however, account for the difference in their organism and the previous work of Quayle's group. From the review of previous work and the information gathered in the present work, there has therefore been an attempt at a plausible explanation for the unusual OX 1 strain of Hoepner's and a reconciliation of the

apparently divergent results, of Quayle and Hoepner, regarding the FDH levels in the formate and oxalate-grown cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism

The bacterium used in the entire course of the work was Pseudomonas oxalaticus (ox 1), initially obtained from the American Type Culture Collection (ATCC 11883). On receipt of the culture, streaking was done on trypticase-soy agar plates. Two morphologically different colonies were observed. These were grown on T-soy slants supplemented with 10 mM sodium formate. Subsequent culture in minimal formate and oxalate broths revealed that one grew in these media much faster than the other. This culture was therefore used as source material for preparations of the enzyme, formate dehydrogenase. The other culture which grew more sluggishly on formate or oxalate was unfortunately lost at an early stage of this work. The remaining, faster growing (on formate and oxalate) culture was then lyophilized to prevent loss. The culture was also maintained on minimal formate agar slants under sterile paraffin oil at $2^{o}C$.

Lyophilization

The procedure followed was that suggested by Greaves (1960). The drying medium was made up as follows,

5% dextran (m.w. 200,000)

5% sucrose

1% sodium glutamate

with distilled water to a volume of 100 ml and autoclaved for 15 minutes at 121°C before use.

The organism was grown in T-soy slants supplemented with 10 mM formate and also on minimal formate slants, at 28°C. The resulting growth was washed off the surface of the agar with small volumes of sterile druing medium and put aseptically into sterile long-necked glass vials. The suspension was quick-frozen as a thin layer on the walls of the vials by rotating the vials rapidly whilst dipping them in liquid nitrogen. The frozen vials were hooked up to a Virtis Freeze-Mobile (Model 10-145 MR-BA) and left overnight. The vials were then sealed off at the neck with a torch under vacuum. The organism withstands lyophilization well as they remained viable in the vials after several years.

Growth Conditions

The bacterium was grown on a minimal salts medium as described by J. R. Quayle (1966). The composition of the medium is given below:

$(NH_4)_2SO_4$	2.5 g	
$MgSO_4.7H_2O$	0.2 g	
$CaCl_2.2H_2O$	1.0 mg	
$FeSO_4.7H_2O$	5.0 mg	Weight per
$MnSO_4.5H_2O$	2.5 mg	litre of
$Na_2MoO_4.2H_2O$	2.5 mg	medium.
$NaH_2PO_4.2H_2O$ *	7.8 g	
K_2HPO_4	8.7 g	

*KH_2PO_4 was substituted for whenever the carbon source used was a sodium salt.

Carbon source was added to á concentration of 10 mM. The pH of the medium after autoclaving (20 minutes per litre at 121°C) was between pH 6.7 - 6.8.

When large quantities of cells were required, 40 litre glass carboys were used. Growth was maintained at 28°C under vigorous forced air aeration through sintered glass spargers. Two different methods were employed in preparing inocula for large carboys. The first entailed growing one litre volumes of the medium in baffled Erlenmeyer flasks at 28°C (170 r.p.m.) until turbidity reached an O.D. reading of 0.02 - 0.04

absorbance units, as measured by a Klett-Summerson Colorimeter fitted with a green filter (≃ 540 nm). Two litres were used to inoculate each 40 litre carboy. The second method employed Roux bottles of trypticase-soy (or nutrient) agar supplemented with 10 mM sodium formate. The bacteria were grown at 28°C for 24 hours, then washed off with the appropriate minimal medium into the carboy. Two Roux bottles were used for each carboy. Either type of inoculum was suitable in obtaining cells for purification of formate dehydrogenase. The second method was used most frequently as the time interval was shorter.

There were two criteria which determined the level of FDH activity in the crude extract. One is that the cells were still in the mid-logarithmic phase of growth when harvested. The other is that the pH of the medium not be allowed to rise above pH 7.6. Therefore the pH of the medium in the carboys was monitored during the growth of the bacteria (after the medium became turbid) and allowed to rise from its initial pH to pH 7.6. It was then adjusted downwards to pH 6.8 with either formic or oxalic acid, depending on the carbon source the bacteria were growing on. Rapid growth was allowed to continue under vigorous aeration. When the pH again attained a value of pH 7.2 ± 0.1, the cells

were harvested using a steam-driven Sharples centrifuge. Speed was set at 50,000 r.p.m. (maximum) and flow rate adjusted for between 300-400 mls of effluent per minute. The cells were washed with 0.1 M potassium phosphate buffer (pH 7.0), spun down in a Sorvall refrigerated centrifuge at 5,000 x g and stored in a deep freeze at -76°C.

In experiments which required growth of the bacterium on solid medium, the agar plates were made up as follows:

Minimal salts and the carbon source were made up according to the liquid formula but in only 3/4 the volume of distilled water required. The remaining ¼ volume of water was used to make up Noble Agar for a final concentration (after mixing) of 1.5%. The two volumes were autoclaved separately for 20 minutes per litre (minimum time 15 minutes) at 121°C. It was important to cool the material before mixing the two volumes and pouring into petri plates. If the two volumes were mixed when very hot, the agar turned slightly brown.

Growth curves

The required volumes of T-soy and minimal salts (with appropriate carbon source) media were made up

and 70 ml portions were dispensed into 300 ml Nephelo-Culture flasks (Bellco). They were autoclaved for 15 minutes at 121^{o}C before use. The flasks were inoculated with a washed culture of young cells. The inoculum size was about 10% (v/v of a growing culture). The flasks were then shaken at 150 r.p.m. in a New Brunswick water bath shaker at 28^{o}C. Optical density readings were taken with a Klett-Summerson Colorimeter fitted with a green (540 nm) filter, via the side arm of the flask. At the same time as the optical readings, a small aliquot of the culture was also removed for viable counts. The pH, for the minimal salts media, was kept at 7.2 ± 0.1 by the addition of a sterile solution of 5% phosphoric acid.

Enzyme Assay Procedures

Formate dehydrogenase.

The enzyme was usually assayed by direct spectrophotometric method. The standard assay mixture contained 1.0 mM NAD^{+}, 1.0 mM sodium formate and 50 mM potassium phosphate buffer (pH 7.5) in a total volume of 1.0 ml. The mixture was made in Spectrosil cuvettes (Hellma) of 10 mm light path. Reaction was normally started by the addition of enzyme. The reduction of NAD^{+} was

followed at 340 nm with a Gilford 2200 recording spectrophotometer. FDH also uses methylene blue, 2,6-dichlorophenolindophenol, ferricyanide and molecular oxygen as electron acceptors besides NAD^+ (Hoepner and Knappe, 1970). In this work, except for oxygen, the other electron acceptors were measured via absorbance, with the Gilford. Standard solutions of potassium ferricyanide (20 mM) and DCPIP (1 mM) were made up. The reaction mixture was the same as for NAD^+ reduction except that NAD^+ was omitted. Either ferricyanide or DCPIP was added (from the standard solutions in amounts to bring absorbance close to 1.0 absorbance units at the respective wavelengths (420 nm for ferricyanide and 600 nm for DCPIP). In the case of DCPIP, PMS was added at half the concentration of DCPIP used. After addition of enzyme, the reaction was followed by the decrease of absorbance at the respective wavelengths. Molar extinction coefficients used for calculations were, ferricyanide = 1.0×10^3 and DCPIP = 19.6×10^3. Methylene blue required the use of anaerobic cuvettes and was used only a few times in the initial part of the work.

FDH assay (formate oxidase) with oxygen as terminal electron acceptor, was done using a Gilson Medical Electronics Oxygraph (Model KM) fitted with a

Clark oxygen electrode and a mixing chamber of 1.5 ml volume.

NADH-oxidase

This enzyme was also measured by direct spectrophotometric method at 340 nm. The reaction mixture contained 100 μM NADH and 50 mM potassium phosphate buffer (pH 7.5) in a volume of 1.0 ml using the same cuvettes as with FDH and the Gilford spectrophotometer.

All enzyme reactions were measured at 24-25°C.

Toluene-treatment of whole cells

In the experiments where whole cells were utilized, "toluene-treated" cells were prepared according to the method described by Kornberg and Reeves (1972). Whole cells were collected and suspended in 0.1 M potassium phosphate buffer (pH 7.2) with 5 mM $MgCl_2$ and thoroughly chilled in ice-water. The cold suspension was then placed in a test-tube and agitated vigorously on a Vortex mixer. Whilst agitating vigorously, 0.01 volume of toluene-ethanol (1:9, v/v) was added via micro-pipette and agitation continued for a further 1 minute. The suspension of toluene-treated cells was then kept in ice-water until use (up to 30 minutes).

Formate- and oxalate-oxidizing activity of whole cells

Cells grown in minimal broth were collected at the appropriate times by centrifugation at 2,000 x g for 5 minutes, washed once with sterile 0.1 M potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. The suspension was adjusted to give an O.D. reading of 1.5 units at 540 nm and kept in ice-water until use. 0.1 ml of the suspension was used in the Gilson Oxygraph as unit quantity of cells. Specific activity was defined as μ moles O_2 consumed per minute per unit cells (1 ml of 0.15 O.D. unit suspension) as measured by the Clark Oxygen electrode at 25°C. The challenge substrate was usually added last after the system had equilibrated in order to obtain a reliable endogenous rate for the resting cell suspensions.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis, basically as described by Ornstein (1964) and Davis (1964), was used to check the purity of enzyme preparations. The apparatus used was purchased from Buchler Instruments (Fort Lee, N.J.). Disc gels were run at a pH of 8.0 after it was discovered that the "standard" gels run at pH 9.3 were not suitable for FDH. The enzyme dissociated and/or denatured under those conditions. Compositions of the gels and buffers for running at pH 8.0 are listed below.

A	1 N HCl	48 mls
(pH 7.5)	Tris	6.85 g
	TEMED	0.46 mls
	Distilled water to	100 mls
B	1 N HCl	48 mls
(pH 5.5)	Tris	4.95 g
	TEMED	0.46 mls
	Distilled Water to	100 mls
C	Acrylamide	30.0 g
	BIS-Acrylamide	0.8 g
	Distilled Water to	100 mls
D	Acrylamide	10.0 g
	BIS-Acrylamide	2.5 g
	Distilled Water to	100 mls

E Riboflavin 4 mg/100 mls

Buffer (same for upper and lower chambers)

Barbital 5.52 g
Tris 1.0 g
Distilled water to 1 litre
Adjusted to pH 7.0 with HCl

Main (Lower) Gel

1 part of A
2 parts of C
1 part of distilled water

The above mixture was combined in a 1:1 ratio with a fresh solution of ammonium persulfate (0.14 g per 100 ml) and dispensed into the glass tubes, gently layered with distilled water and allowed to polymerize for 60 minutes. The water was removed with a capillary and the stacking gel layered on.

Stacking (Top) Gel

1 part of B
2 parts of D
1 part of E
4 parts of 40% sucrose (made up with distilled water)

The above mixture was made just before use and layered on top of the polymerized main gel (about 3-4 mm deep). Distilled water was again gently layered on top and the tubes polymerized with light for 15 to 20 minutes. The water was removed and the tubes put into the Buchler apparatus. The lower chamber was filled with the buffer and the buffer was added to the upper chamber to a depth of 6 to 8 cm. After checking for leaks from the upper chamber, 3 or 4 drops of the tracking dye (0.1% bromphenol blue) was mixed into the upper buffer and the protein samples (100-200 μg) in 40% sucrose were applied to the tubes. A voltage of 250 volts and a current of 3 to 4 milliamperes per tube was then applied. The samples were allowed to electrophorize until the tracking dye moved close to the bottom of the tubes. The gels were removed by rimming with the tip of a hypodermic needle and then injecting water between the gel and the glass.

The gels were fixed in a solution of 12.5% TCA and stained with Coomassie Brilliant Blue (0.05% in a solvent of 10% acetic acid - 20% methanol) for 1 hour (modified from the procedure of Chambrach et al, 1967) to show protein bands. Destaining was done using a solution of 10% acetic acid - 20% methanol, changed frequently in order to speed up the destaining process.

When specific staining for the location of FDH activity was desired, the gel, on immediate removal from the tube, was sliced longitudinally and one half incubated in a reaction mixture containing 0.5 mM NAD^+, 1 mM sodium formate, 2.5 mg PMS, 6 mg NBT and 50 mM potassium phosphate buffer (pH 7.5) in a total volume of 8 ml. The reaction tube was kept in the dark at 25°C and inspected every 5 minutes to ensure that there was no overstaining which results in wide bands (from diffusion). Activity of FDH appeared as a purple band. The other half of the gel was stained routinely for protein.

Analytical Procedures

Protein assays were routinely made using the colorimetric method described by Lowry et al (1951). Crystalline bovine serum albumin was utilized as the standard. Readings were taken using a Klett-Summerson photoelectric colorimeter fitted with a red filter (660 nm peak).

Formate was determined enzymatically using the procedures described by Johnson et al (1964) and Hoepner and Knappe (1970). Extinction was read using a Unicam SP 700 and a cuvette of 12 ml with 40 mm light path.

NADH concentration was determined spectrophotometrically by extinction at 340 nm, with precautions as noted by Fawcett et al (1961).

Sucrose Density Gradients

Molecular weight estimations of FDH using sucrose gradients were done according to the method described by Martin and Ames (1961). Density-grade, RNase-free sucrose was used to make up the solutions with "Standard buffer" (50 mM potassium phosphate pH 7.5). A linear gradient of 5% to 20% (w/v) sucrose was prepared using a Buchler gradient maker, with 4.8 mls 5% sucrose in the reservoir and 4.8 mls 20% sucrose in the mixing chamber. Two capillary tube outlets were connected to a peristaltic pump. The motorized vibrating stirrer was turned on in the mixing chamber and the solution flowed out at a regulated speed of about 20 minutes for the emptying of the chambers. The two resulting gradient tubes were run as duplicates with the same samples in each one.

The sample solution was layered on top of the gradient and the tubes centrifuged in a Beckman L2-65B ultracentrifuge with a SW 50.1 swinging bucket rotor. Centrifugation was done at 44,000 r.p.m. for 16 hours at 4°C. Two internal markers were used

(separately), yeast glucose-6-phosphate dehydrogenase and egg white ovalbumin. At the end of the run, the rotor was allowed to coast to a stop without applying the brake. The nitrocellulose tubes were removed and placed in a Buchler tube-puncturing device connected to a peristaltic pump. The tubes were punctured and contents flowed out slowly. Three-drop fractions were collected and assayed.

Electron-microscopy

Samples were prepared essentially according to the method of Kellenberger et al (1959). The acetate-veronal buffer was substituted with cacodylate (Dimethyl arsenate) buffer (pH 7.4) instead. The cells were collected by centrifugation and quickly suspended in a mixture of 3% acrolein and 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 hour. The suspension was then centrifuged at 1,000 x g for 5 minutes and the pellet was quickly mixed with warm (45^{o}C) 2% Noble agar and spread as a thin layer on an acid-washed glass slide. The solid agar was then cut into small blocks and put into a 1% osmic acid solution (in 0.1 M cacodylate buffer). The tube was immersed in an ice-bath for 2 hours. The osmic acid was rinsed off with cacodylate buffer and the agar blocks suspended in a 0.5% aqueous solution of uranyl acetate

overnight, dehydrated with ethanol and embedded (following the procedure of Hess, 1966). The agar blocks were dehydrated by immersing them for 10 minute periods in successive ethanol solutions of increasing strength; 30%, 50%, 70% and 90% ethanol. This was followed by two 30 minute periods in absolute ethanol. One or two agar blocks were put into each gelatin capsule (predessicated) for embedment with butyl methacrylate. Duplicate specimens were embedded in an ERL-4206 (vinyl cyclohexene dioxide) - based epoxy resin (Spurr, 1969). Use of this resin (hereby referred to as "Spurr" resin) required overnight infusion with a half-strength solution followed by a full-strength "Spurr" resin solution for 8 hours. The formula for "Spurr" resin is:

ERL-4206 (vinyl cyclohexene dioxide)	10 g
D.E.R. 736 (diglycidyl ether of propylene glycol)	6 g
NSA (noenyl succinic anhydride)	26 g
S-1 (dimethylaminoethanol or DMAE)	0.4 g

The compounds are measured by weight and mixed together in a beaker before use.

Gelatin capsules were also used with the "Spurr" resin. Polymerization of both butyl methacrylate and "Spurr" resin were by use of heat. For cross-linked

methacrylate the oven temperature was set at 50°C for 8 hours. For "Spurr" resin the conditions were 60°C for 10-12 hours. After cooling, the plastic pellets were cut into shape and sectioned in a LKB ultratome Model 4801A with a glass knife. The ultra-sections were placed on carbon film grids (200-400 mesh) obtained commercially, and viewed and photographed in an AEI model EM 6B electron microscope.

Reagents

All the chemicals used were reagent grade or better. Standard laboratory chemicals and reagents were obtained from J. T. Baker Chemical Co., Fisher Scientific and Matheson, Coleman and Bell. Special grades of ammonium sulfate (enzyme grade), guanidine hydrochloride, urea and sucrose (density-grade, RNase-free) were obtained from Mann Laboratories Inc. Commercial media (T-soy, nutrient) were obtained from Difco and BBL. Dialysis tubing (24 Å pore size, cellulose) was supplied by Canlab. Crystalline bovine serum albumin was bought from Nutritional Biochemicals Corporation. Polysciences, Inc. supplied all the reagents and material for the preparation of the samples for electronmicrography. DEAE-cellulose (Whatman microgranular DE32) was obtained from W. and R. Balston Ltd., "Good" buffers (Hepes, Mes) from Calbiochem. and NAD^+, NADH, $NADP^+$, NADPH, FAD, FMN and DTT from P-L Biochemicals Inc. The DNase, RNase, glucose-6-phosphate dehydrogenase, pronase (Streptomyces griseus protease), snake venom (Naja naja), ovalbumin and all the inhibitors were supplied by Sigma.

R E S U L T S

RESULTS

Morphology of the colonies

The morphology of Pseudomonas oxalaticus (0X 1) colonies growing on the various agar media is illustrated in plates 1 and 2. The photographs were taken using a Zeiss Jena Telaval inverted microscope with "MF" photomicrographic attachment. The colonies growing on T-soy or nutrient agar have very irregular edges, whereas those growing on minimal salts oxalate or formate agar have relatively smooth, even edges. However, when the organism was grown on LB agar which contains 1% NaCl in addition to yeast extract and tryptone, the resulting colonies had smooth, even edges. Consequently, T-soy and nutrient agar media were made up using 0.1 M sodium-potassium phosphate buffer (pH 7.0) in place of distilled water. The colonies that developed on these plates were identical to those on LB agar, with smooth, even edges. They are illustrated in plate 3.

Growth rates of the organism

Growth curves of the bacterium on the different carbon sources were obtained as described in "Methods".

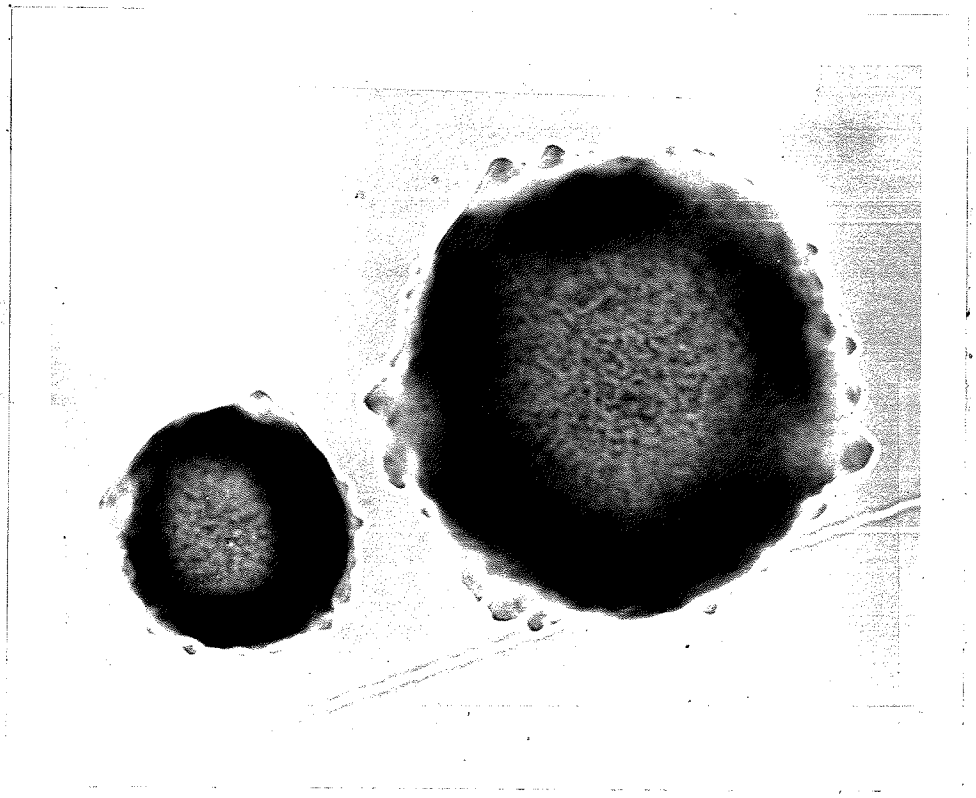
Plate 1. (a) Typical Pseudomonas oxalaticus colony on nutrient or T-soy agar.

(b) Typical P. oxalaticus colonies on minimal glutamate agar.

PLATE 1

Magnification x 63

(a)



(b)

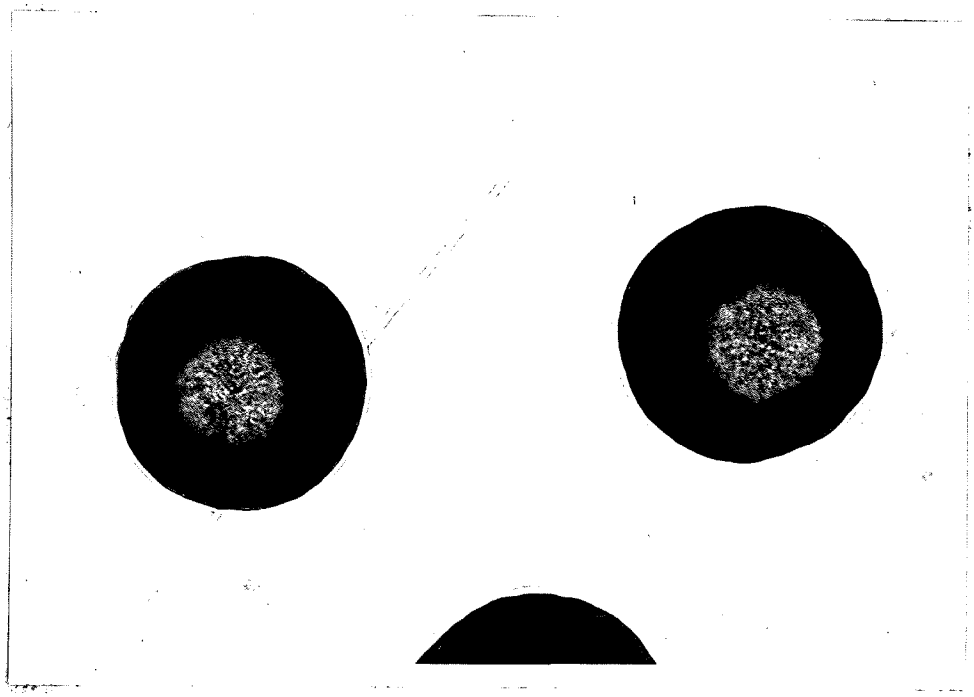


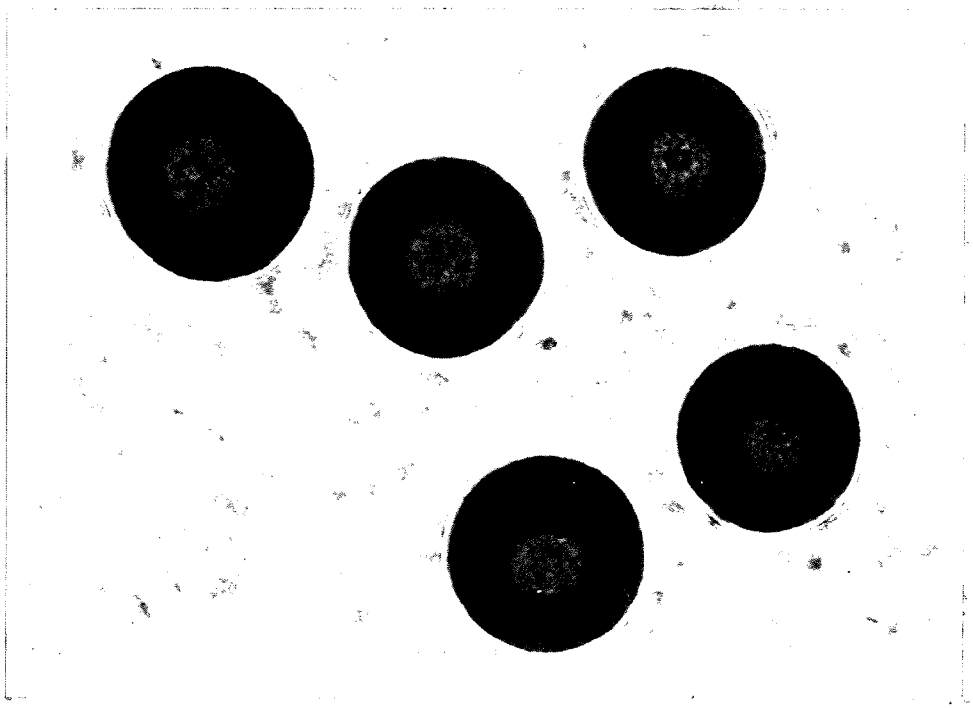
Plate 2. (a) Typical *P. oxalaticus* colonies on minimal oxalate agar.

(b) Typical *P. oxalaticus* colonies on minimal formate agar.

PLATE 2

Magnification x 63

(a)



(b)

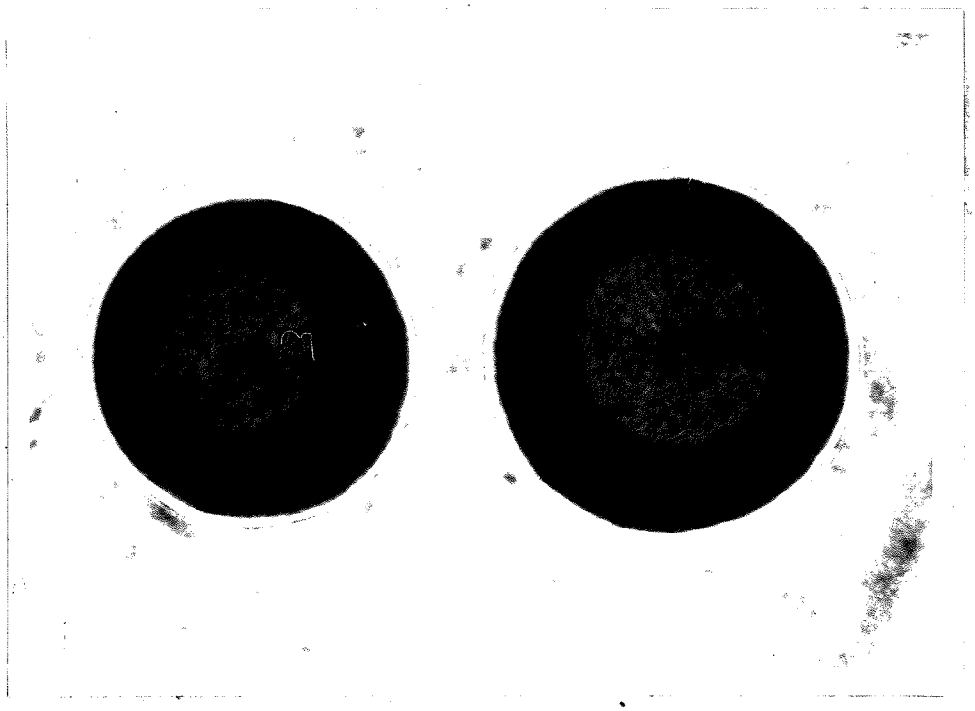


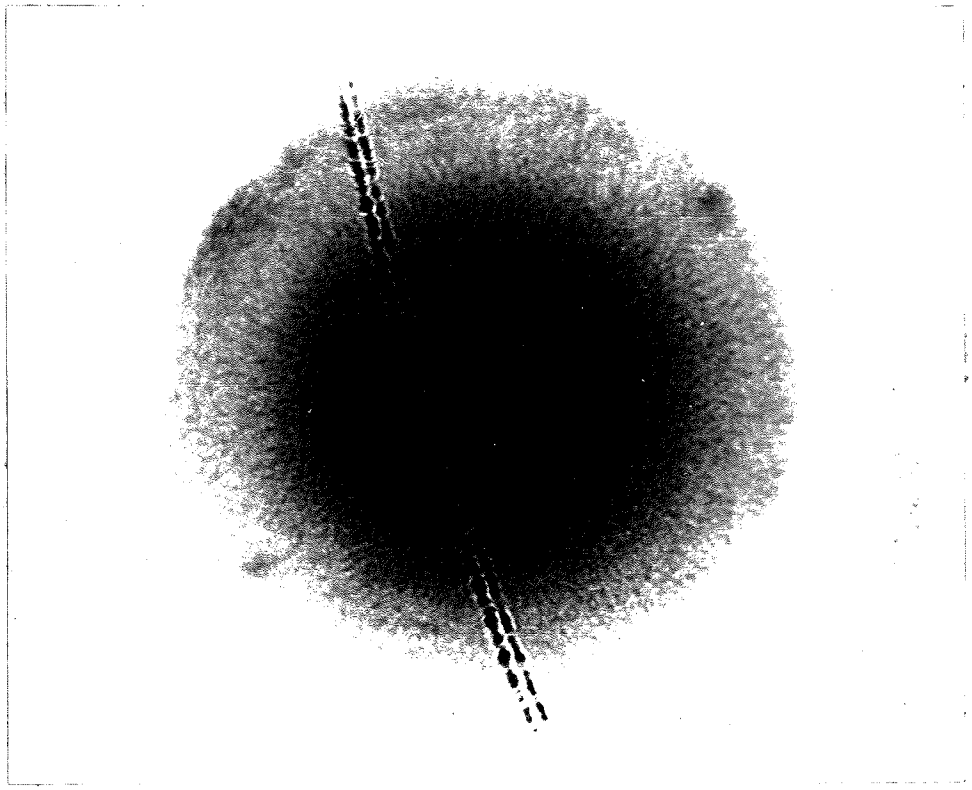
Plate 3. (a) Typical P. oxalaticus colony on LB agar.

(b) Typical P. oxalaticus colony on nutrient or T-soy agar with 0.1 M sodium-potassium phosphate (pH 7.0).

PLATE 3

Magnification x 63

(a)



(b)

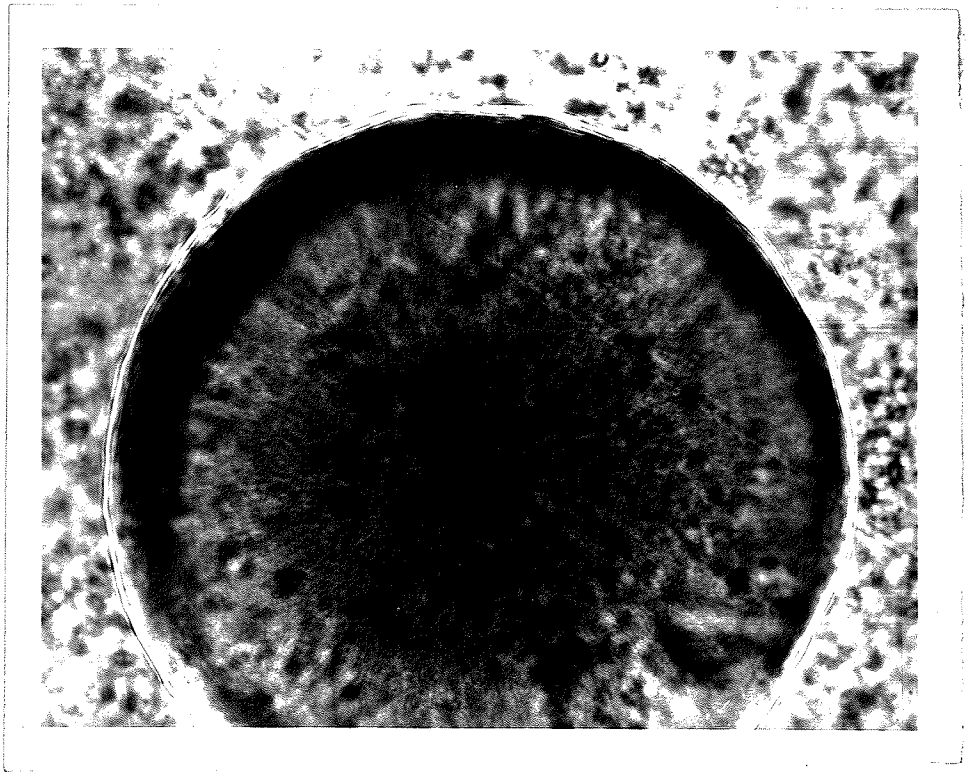


Figure 1. Growth curve for Pseudomonas oxalaticus on nutrient broth medium (28°C).

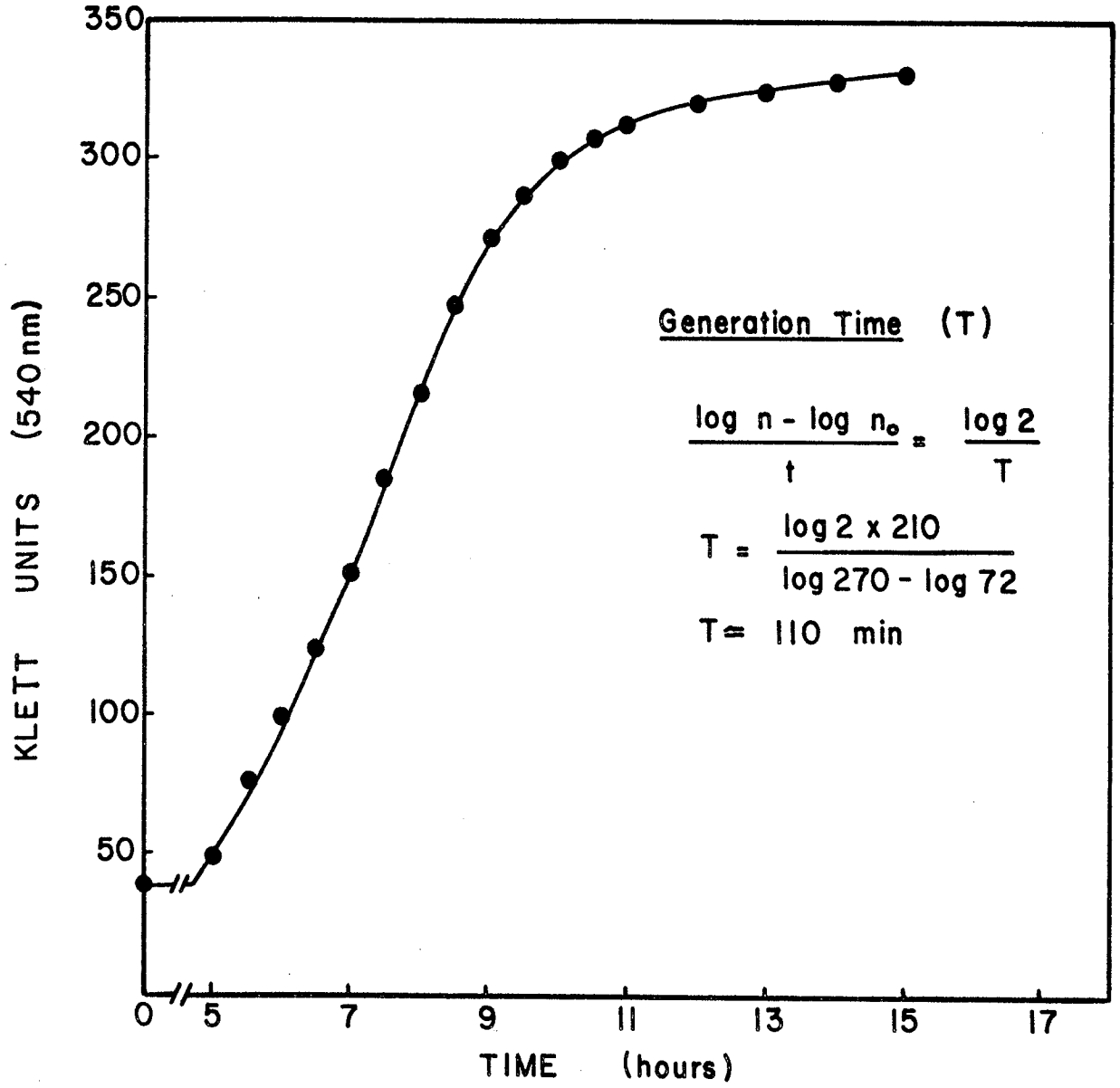

350
300
250
200
150
100
50
KLETT UNITS (540nm)
0
5
7
9
11
13
15
17
TIME (hours)
Generation Time (T)
log n - log n₀ / t = log 2 / T
T = log 2 x 210 / log 270 - log 72
T = 110 min

Figure 2. Growth curve for P. oxalaticus on minimal salts medium with glutamate as carbon source (28°C).

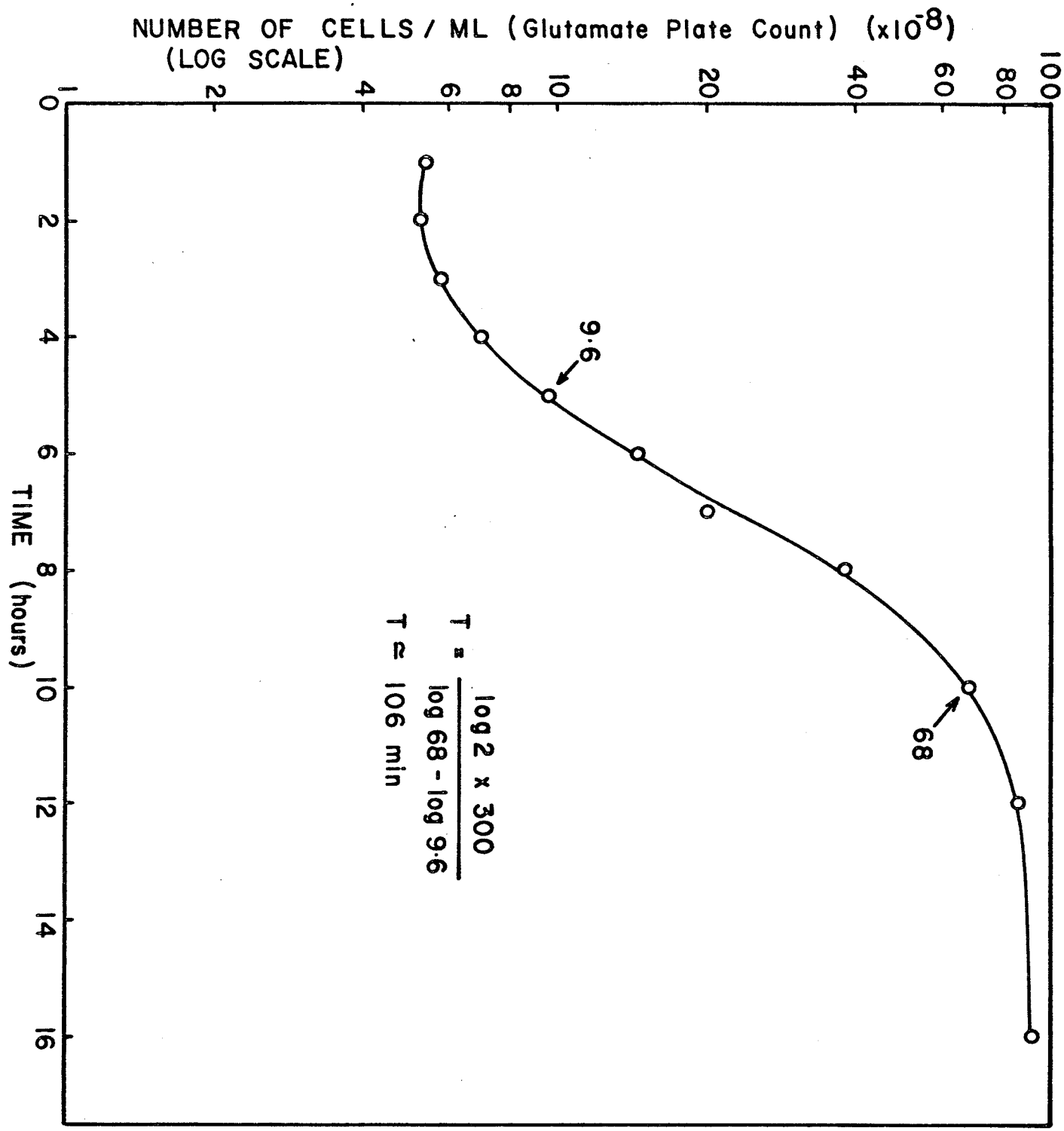

NUMBER OF CELLS / ML (Glutamate Plate Count) (x10^8)
(LOG SCALE)
1
2
4
6
8
10
20
40
60
80
100
0
2
4
6
8
10
12
14
16
TIME (hours)
9.6
68
T = log 2 x 300 / log 68 - log 9.6
T ≈ 106 min

Figure 3. Growth curve for P. oxalaticus on minimal salts medium with oxalate as carbon source (28°C).

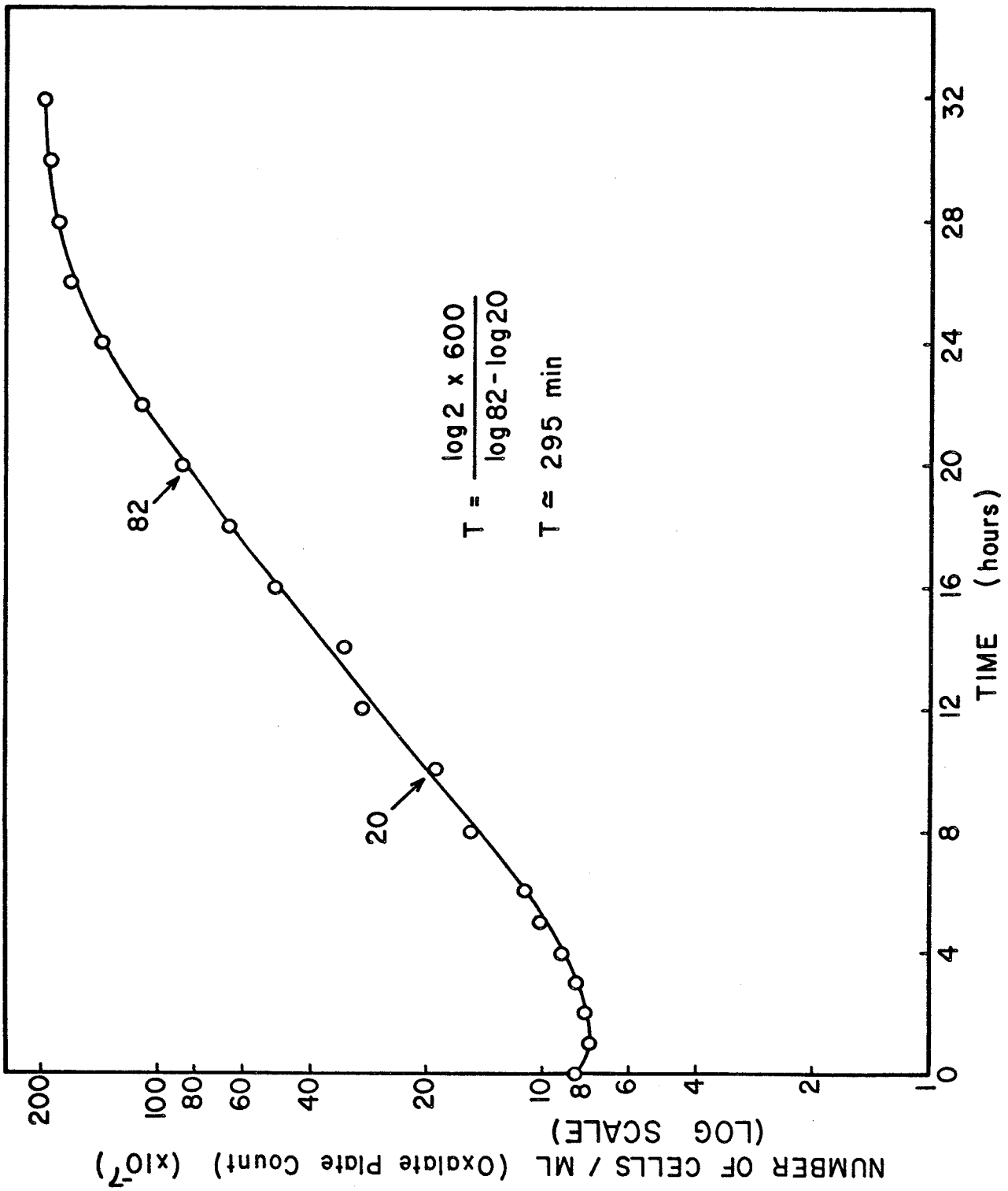
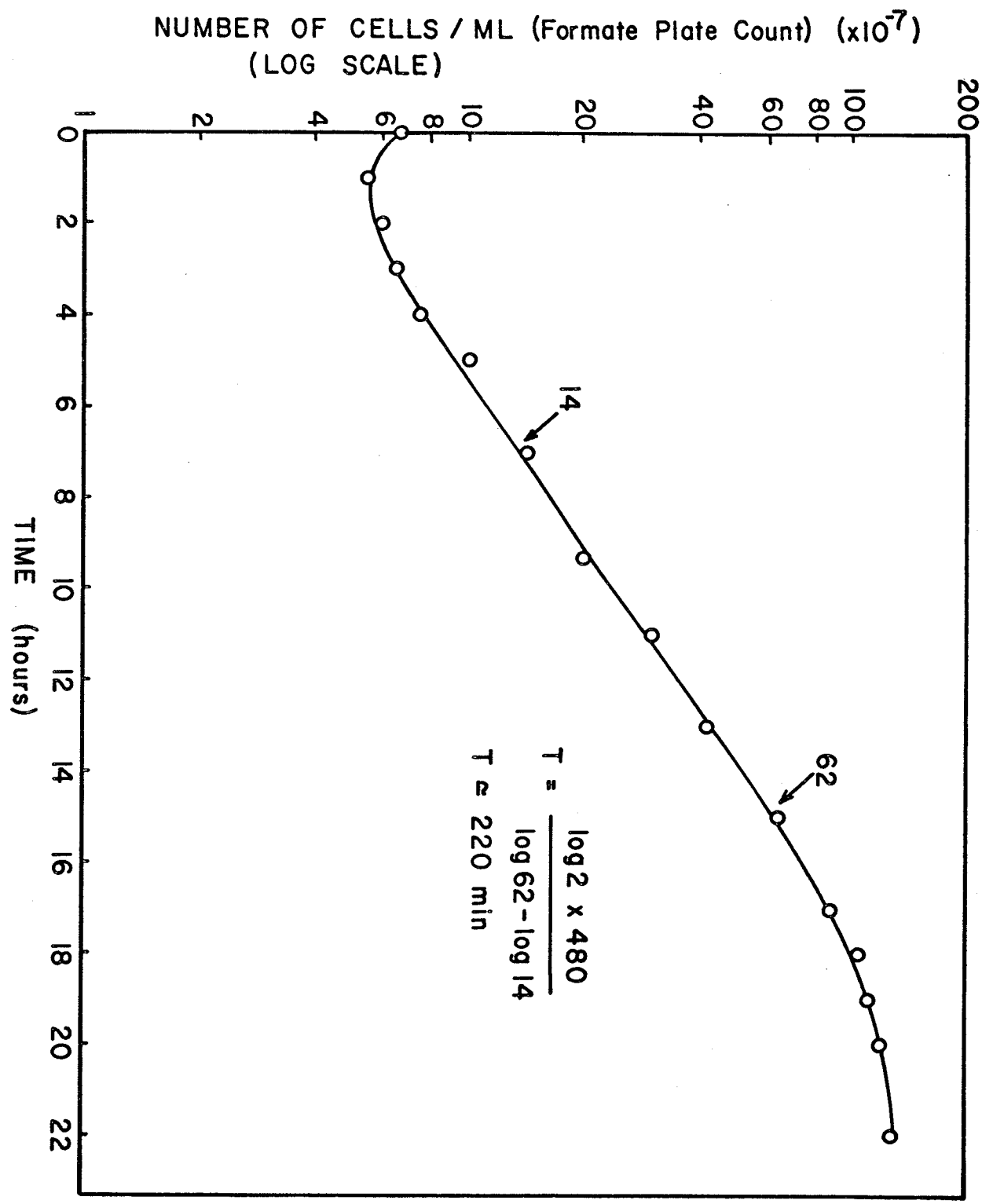

NUMBER OF CELLS / ML (Oxalate Plate Count) (x10⁷)
(LOG SCALE)
200
100
80
60
40
20
10
8
6
4
2
1
0
4
8
12
16
20
24
28
32
TIME (hours)
82
20
T = log 2 x 600 / log 82 - log 20
T ≃ 295 min

Figure 4. Growth curve for *P. oxalaticus* on minimal salts medium with formate as carbon source ($28^{o}C$).


NUMBER OF CELLS / ML (Formate Plate Count) (x10⁻⁷)
(LOG SCALE)
1
2
4
6
8
10
20
40
60
80
100
200
TIME (hours)
0
2
4
6
8
10
12
14
16
18
20
22
14
62
T = log2 x 480 / log 62 - log 14
T ≈ 220 min

These are illustrated in Fig. 1,2, 3,and 4 for growth of Pseudomonas oxalaticus on T-soy, glutamate, oxalate and formate respectively as sole carbon source. Using the formula given by Monod (1949), the mean generation times during the logarithmic phase were calculated. The values obtained were: 110 minutes for T-soy, 106 minutes for glutamate, 295 minutes for oxalate and 220 minutes for formate. The values for oxalate and formate are in close agreement with those mentioned by Blackmore and Quayle (1968 b).

Electron micrography of ultra-sections

Pseudomonas oxalaticus grown in liquid cultures of nutrient broth and minimal glutamate, oxalate and formate media at 28°C were collected, in the mid-logarithmic phase of growth, by centrifugation at 2,000 x g for 5 minutes. The cells were fixed and prepared for embedding essentially according to the method described by Kellenberger et al (1959). The agar blocks were stained (using osmic acid and uranyl acetate) and dehydrated following the procedure of Hess (1966), and finally embedded in cross-linked methacrylate and "Spurr" epoxy (see "Methods"). Ultra-sections were obtained using a LKB ultratome and viewed and photographed in an AEI model EM 6 B electron microscope. There were no visible differences between cells embedded with the

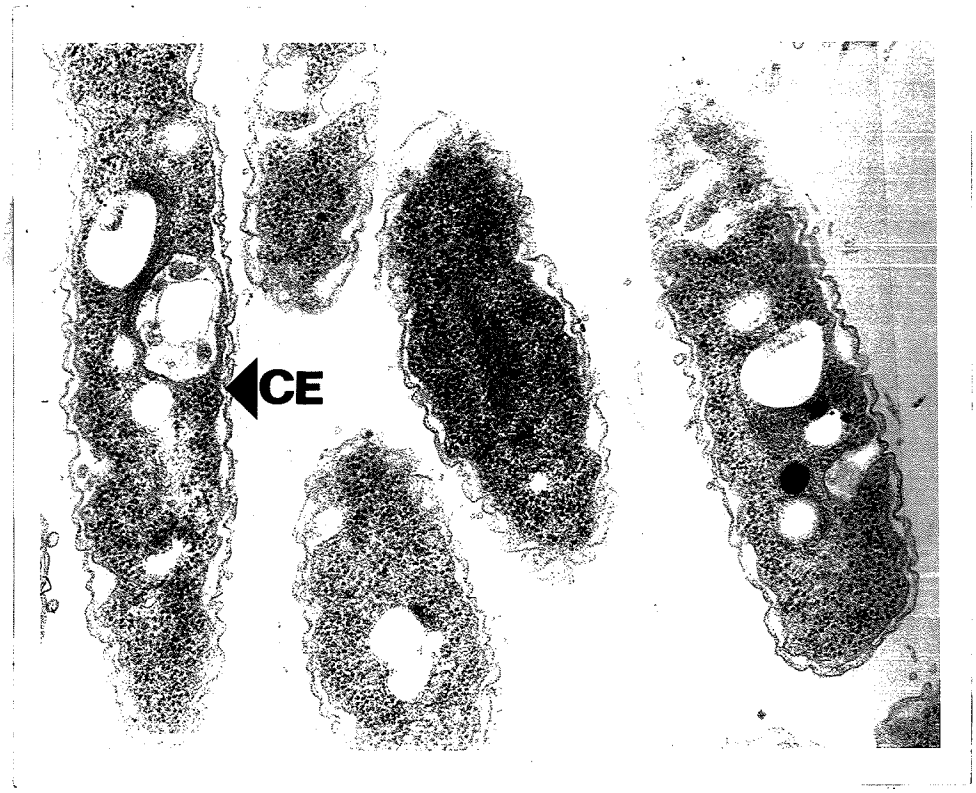
methacrylate and the "Spurr" epoxy. Plates 4 & 5 illustrate typical sections of the cells which were obtained. It is most evident that cells grown on T-soy or nutrient broth had cell-walls that withstood the rigours of fixing and dehydrating procedures very poorly. Using 20% sucrose in the buffer during fixation and washing of the cells did not help. The age of the cells was not a factor as cultures of 12, 18, 24, 32 and 48 hours (at 28^{o}C) were used for the sections with the same result. From the morphological change observed in the colonies grown on T-soy or nutrient agar when 0.1 M sodium-potassium phosphate was added to the media, it was then thought that the lack of salts in the broth media was the likely cause of the "weak" cell-walls. Cells were therefore grown in T-soy and nutrient broths made up with 0.1 M sodium-potassium phosphate buffer (pH 7.0) instead of distilled water and prepared for sectioning. Cells grown on LB broth were also used. Typical sections of these cells are illustrated in plate 6. The cell-walls appear morphologically "normal" and the sections resemble the glutamate and oxalate-grown cells very closely. Notable morphological differences occur only between the autotrophically (formate)-grown cell sections and all the heterotrophic cell sections. There is a slight but noticeable difference in the cell envelope. The space between the layers seems to be more developed and clearly defined in the autotrophic cell sections than in the heterotrophs.

Another difference is the presence of lightly-stained areas in all the heterotrophic cell sections. These might be storage vacuoles (containing poly-β-hydroxybutyrate ?) and are absent from the sections of autotrophic cells.

Plate 4. (a) Typical section of cells grown on nutrient or T-soy broth.
(CE) - cell envelope (x 35,000)

(b) Typical section of cells grown on minimal salts with glutamate.
(CE) - cell envelope
(V) - vacuole (x 35,000)

PLATE 4



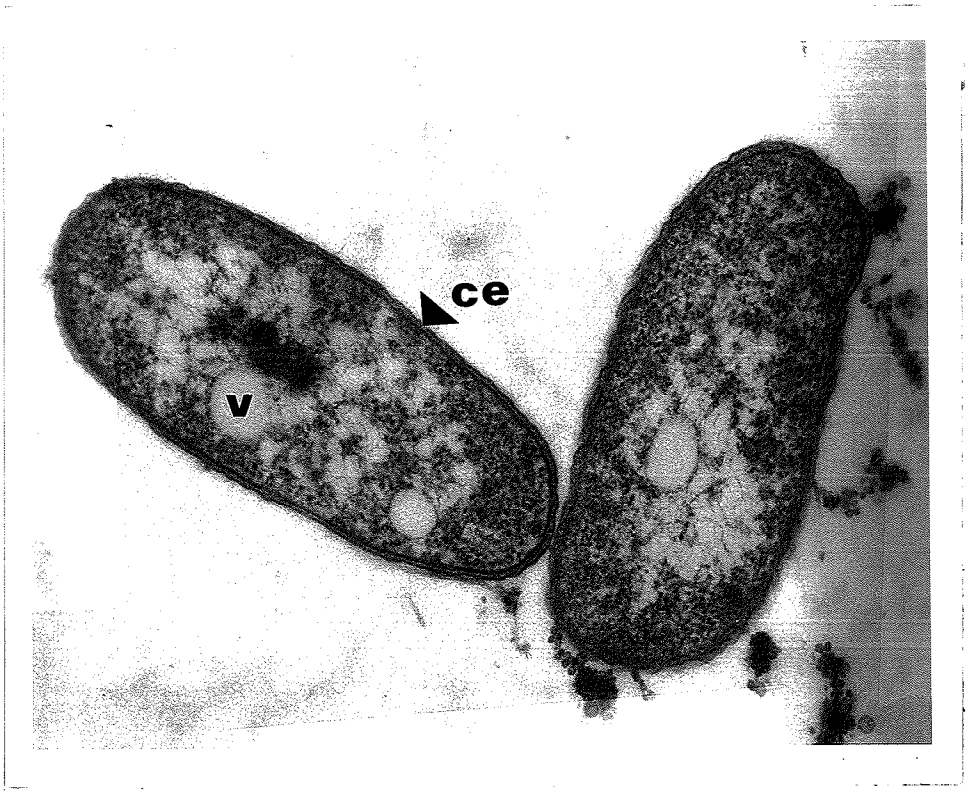
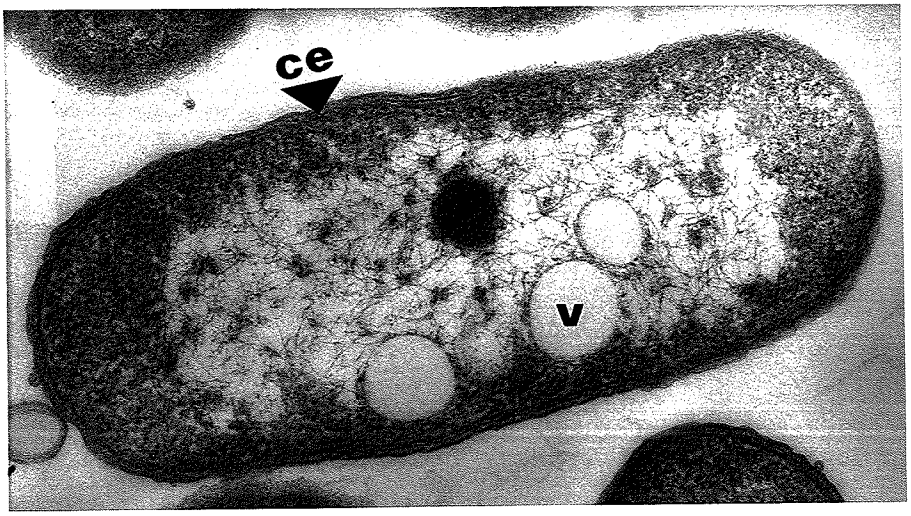


Plate 5. (a) Typical section of cells grown on minimal salts with oxalate. (x 35,000)
(CE) - cell envelope
(V) - vacuole

(b) Typical section of cells grown on minimal salts with formate (autotrophic). (x 40,000)
(CE) - cell envelope

PLATE 5

(a)



(b)

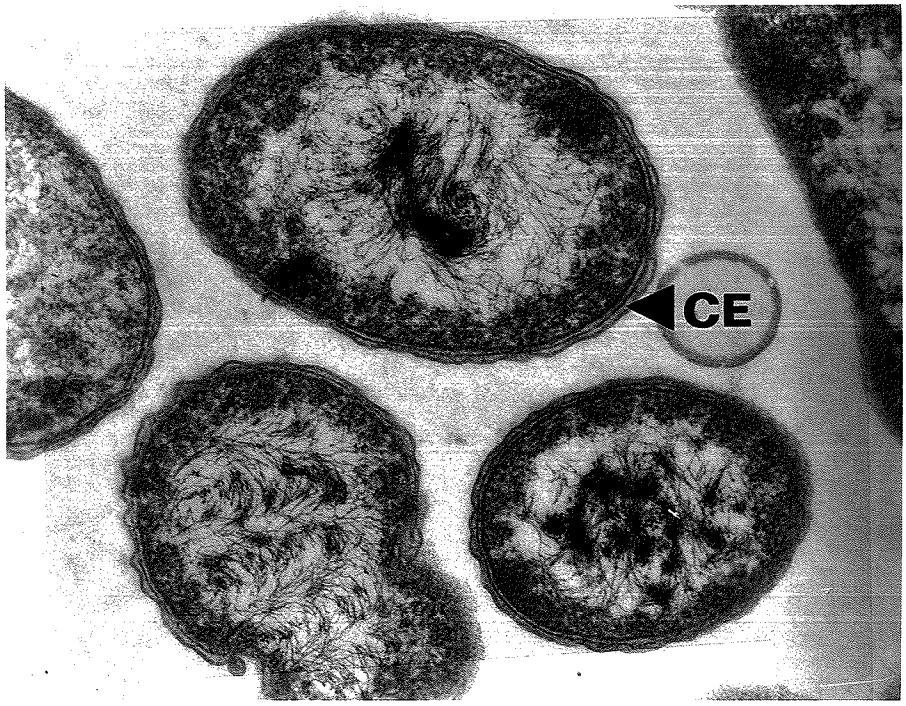
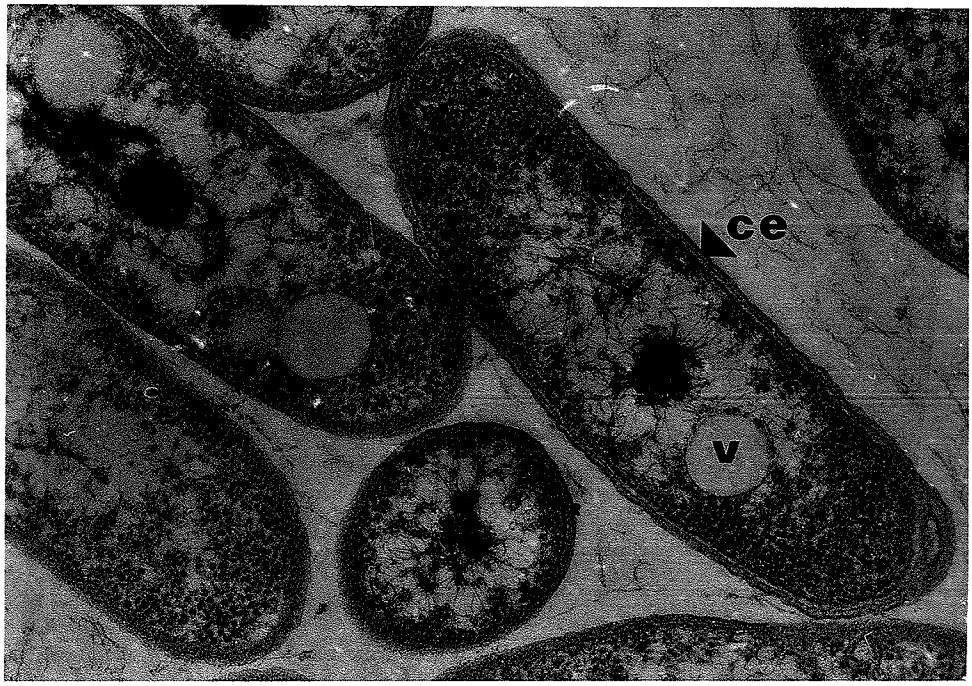


Plate 6. Typical section of cells grown in LB broth and nutrient or T-soy broth made up with 0.1 sodium potassium phosphate (pH 7.0)

(CE) - cell envelope

(V) - vacuole

PLATE 6



Magnification x 40,000

Purification of Formate Dehydrogenase (FDH)

Batches of frozen Pseudomonas oxalaticus cells (formate or oxalate grown) stored as described in "Methods", were thawed by suspending in four times their weight (v/w) of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 1 mM $MgCl_2$. The suspension was stirred slowly at 4°C until it was homogenous. Crystalline DNase and RNase were added to the suspension (approximately 500 µg each per 100 ml of suspension). The RNase was pretreated before use by dissolving it in the phosphate buffer, heating at 80°C for 5 minutes and holding it in ice-water until use. The suspended cells were passed twice through a French pressure cell (Aminco) at 14,000 p.s.i. The broken cells were centrifuged at low speed (5,000 x g) for 10 minutes. The supernatant was recentrifuged at high speed (120,000 x g) for 30 minutes using a 60 Ti rotor in a Beckman L2-65B ultracentrifuge. An aliquot of the resulting supernatant was taken for protein determination. This supernatant was defined as "crude extract". To it was added DTT to a final concentration of 3 mM and NADH to a concentration of 0.01 mM after the protein concentration was adjusted to 20 mg per ml using 0.05 M potassium phosphate buffer of pH 7.5 (hereafter referred to as

"standard buffer").

All steps before and following this were performed at 2-4^{o}C. In the following steps, buffers contained 0.1 mM EDTA, 3 mM DTT and 0.1 mM NADH unless otherwise indicated. Protamine sulfate (2% solution, pH 5.0) was added with constant stirring to the crude extract in the ratio of 0.07 mg/mg protein. After a further 10 minutes of stirring, the suspension was centrifuged at 15,000 x g for 20 minutes. The resulting supernatant was again treated with the same amount of protamine sulfate as before and the procedure repeated. It was advisable to do a trial run with a small amount of crude extract treated with protamine sulfate. This was because, with some batches of cells (oxalate grown), a significant amount of FDH was "precipitated" in the second protamine sulfate step. In that case, the ratio of protamine sulfate was merely lowered to 0.06 mg/mg protein.

The supernatant from the second protamine sulfate treatment (0.14) was then fractionated with a saturated solution of ammonium sulfate (pH 7.0, with 3 mM DTT and 0.01 mM NADH). The fraction precipitating between 30-40% saturation with ammonium sulfate was dissolved in the standard buffer in a volume 1/20 that of the original volume of the crude extract. This solution (at this point it was bright yellow) was put in a dialysis tubing

(pretreated as described in "Methods") and dialysed overnight against 100 times its volume of standard buffer. Any material that precipitated after dialysis was removed by centrifugation at 15,000 x g for 20 minutes. If NADH was not incorporated in the buffer it was at the dialysis stage that the enzyme apparently lost most of its activity. In effect, a lengthy lag period was created before the enzyme would function at maximum velocity. If the enzyme was left in that state for lengthy periods of time, activity was irreversibly lost. The lag period (produced in the absence of NADH or NAD^+) approximated five to six minutes. (See "Activation of FDH by NADH and NAD^+.") This lag could be completely eliminated by 0.01 mM NADH or from 0.005 mM to 0.01 mM NAD^+. Hence 0.01 mM NADH was incorporated into the buffer solutions.

The enzyme solution after dialysis was then applied onto a DEAE-cellulose column (2.5 x 35cm) prepared as described in "Methods" and equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). The adsorped enzyme was then washed with 100 ml of 0.05 M potassium chloride in the standard buffer and eluted with a linear gradient of potassium chloride in the same buffer. The mixing chamber contained 400 ml of 0.1 M KCl and the reservoir 400 ml of 0.4 M KCl to produce the gradient. Ninety-drop fractions were collected with a LKB Ultrovac 7000

fraction collector. The enzyme was recovered in the fractions corresponding to a KCl concentration of between 0.22 to 0.26 M. An elution profile for a DEAE-cellulose column can be seen in Fig. 5.

After the active fractions were pooled, they were concentrated by adding saturated ammonium sulfate to 60% saturation, stirring gently for 30 minutes and then centrifuging. The pellet obtained was extracted by stirring for 20 minutes successively with 40%, 35% and 30% ammonium sulfate solutions (in the standard buffer). After each extraction, the mixture was centrifuged at 15,000 x g for 10 minutes and the supernatant assayed for enzyme activity. The enzyme was recovered in the 30% ammonium sulfate extraction. Two extractions with 30% ammonium sulfate were done to recover most of the enzyme. The enzyme was then concentrated by adding saturated ammonium sulfate to 60% saturation again. The precipitateobtained on centrifugation was then dissolved in a minimal volume of 0.1 M potassium phosphate buffer (pH 7.5). This solution was light yellow in colour.

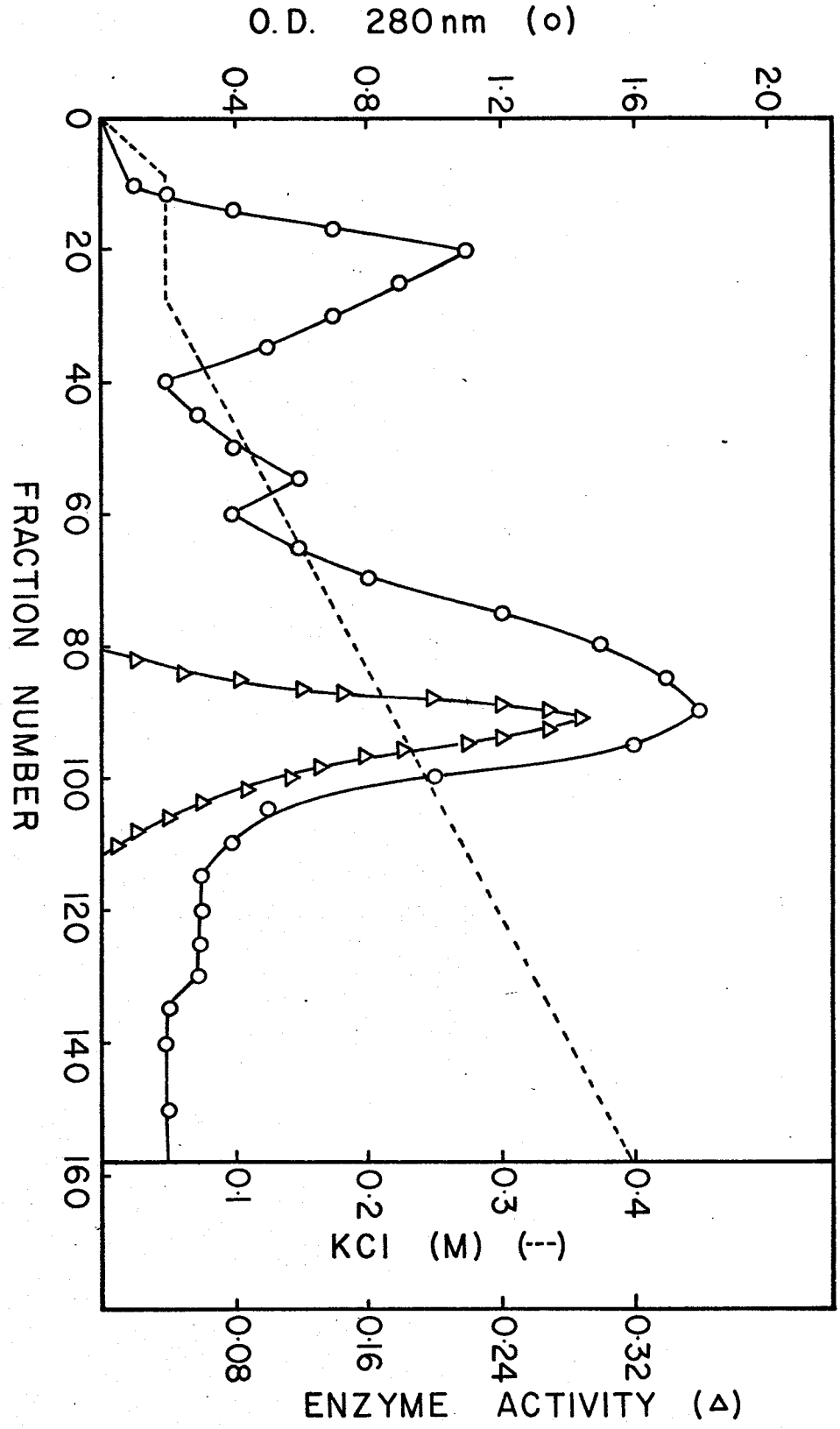
This fraction (hereby termed "reverse ammonium sulfate I") was applied onto a Sephadex G-100 (Pharmacia) column (2.5 x 100 cm). This column was packed under hydrostatic pressure of 20 cm, layered with 4 cm of G-25 (medium) and equilibrated with 0.1 M potassium phosphate

Figure 5. Elution pattern of FDH from DEAE-cellulose column. 5.8 ml fractions were collected at 2-4°C.

(-----) molarity of the KCl gradient

(O) optical density of fractions

(Δ) FDH activity measured spectrophotometrically as described in "Methods".


O.D. 280nm (o)
0·4
0·8
1·2
1·6
2·0
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
0·1
0·2
0·3
0·4
KCl (M) (---)
0·08
0·16
0·24
0·32
ENZYME ACTIVITY (Δ)

buffer (pH 7.5). The enzyme was eluted with the same buffer and 90-drop fractions were collected. FDH was recovered immediately after the first void volume as shown in the G-100 column elution profile in Fig. 6. The active fractions were pooled and the enzyme concentrated by adding saturated ammonium sulfate to 60% with slow stirring. The pellet obtained on centrifugation was extracted by the reverse ammonium sulfate procedure as described for the pellet obtained after the DEAE-cellulose column. Again, most of the FDH was extracted in the 30% ammonium sulfate. The two extractions were pooled and brought to 60% ammonium sulfate saturation to precipitate the enzyme. Again the precipitate was collected by centrifugation (15,000 x g for 15 minutes) and dissolved in a small volume of 0.1 M potassium phosphate buffer (pH 7.5). The purified enzyme preparation (reverse ammonium sulfate II) was divided into several small aliquots and stored frozen at $-20^{o}C$ for future use. A typical purification result of FDH is shown in Table I. FDH thus prepared was very faintly yellow at a protein concentration of between 5 to 10 mg per ml. Preparations obtained from formate-grown cells were consistently more yellow in colour than those from oxalate-grown cells used as source material. From the extinction at 450 nm of a FDH sample containing about 30 mg protein per ml and assuming a molecular weight of 150,000 daltons, the

resulting approximate calculation gave a value of less than 0.1 mole flavin per mole enzyme protein.

Before it was known, the enzyme purification was carried one step further. The fractionsrecovered after the G-100 step (reverse ammonium sulfate II) was passed through a 2.5 x 100 cm Sephadex G-150 column. Preparation of this column was the same as for the G-100 column except that the hydrostatic pressure was set at a maximum of 10 cm. With this column, the FDH was eluted at the end of the second void volume. The active fractions were pooled and the enzyme was precipitated with saturated ammonium sulfate to 60% saturation. The enzyme was collected by centrifugation at 15,000 x g and dissolved in a minimal volume of 0.1 M potassium phosphate buffer (pH 7.5). An elution profile of the G-150 column is shown in Fig. 7. However the specific activity of FDH did not increase, with the G-150 eluate, over that of reverse ammonium sulfate II. In fact, the specific activity decreased slightly but this was probably due to the instability of the enzyme under the conditions. The polyacrylamide disc gels of the fractions obtained from the G-100 column and the G-150 column were identical. Subsequently, enzyme purifications were terminated after the reverse ammonium sulfate II.

Figure 6. Elution pattern of FDH from G-100 Sephadex column. 5.8 ml fractions were collected at 2-4°C.

(●) protein concentrations in mg per ml.

(▲) FDH activity per 10 µl enzyme measured spectrophotometrically as described in "Methods".

(⊙) Optical density at 620 nm for dextran blue to measure the void volume.

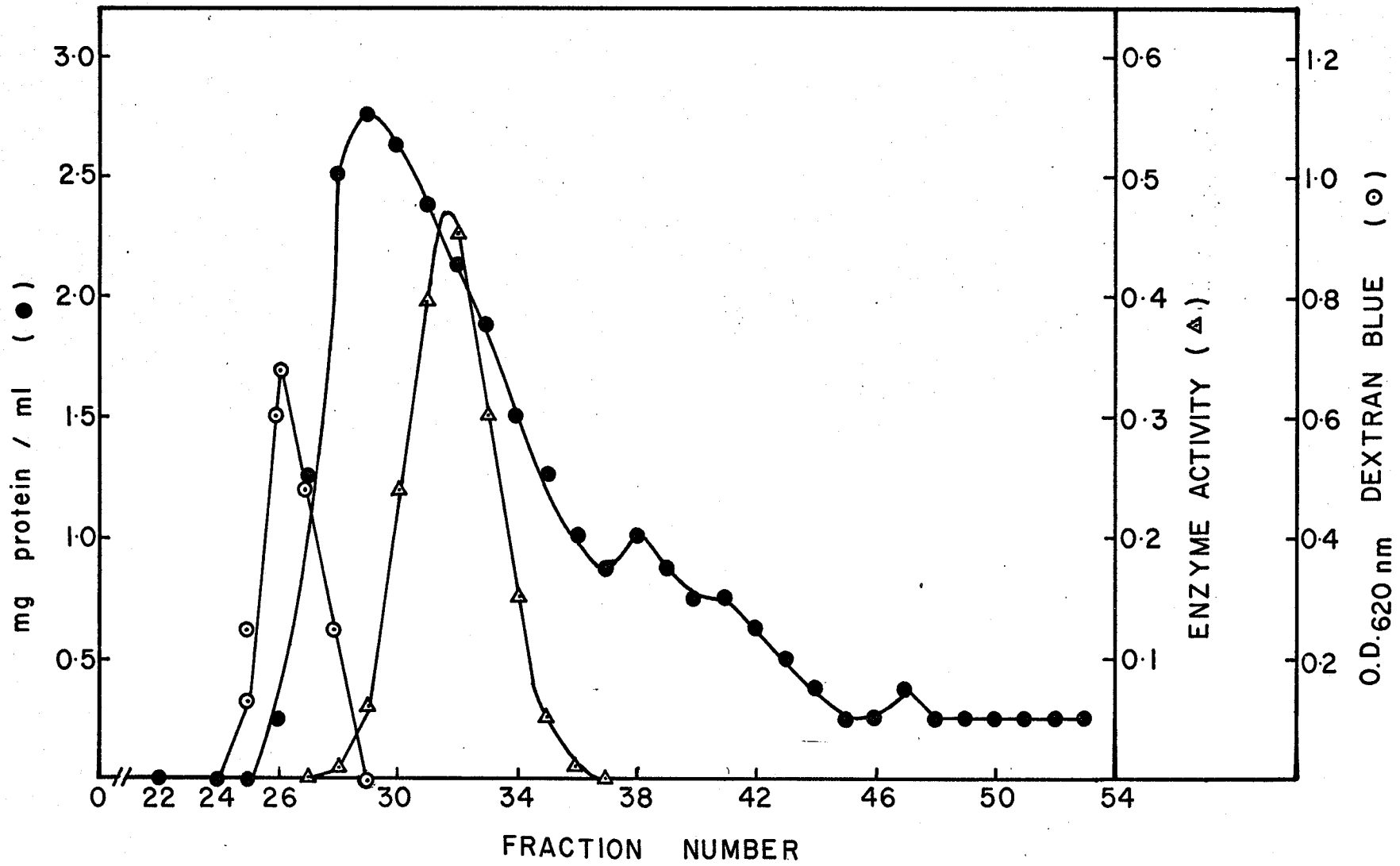

3·0
2·5
2·0
1·5
1·0
0·5
mg protein / ml (●)
0·6
0·5
0·4
0·3
0·2
0·1
ENZYME ACTIVITY (△)
1·2
1·0
0·8
0·6
0·4
0·2
O.D.620 nm DEXTRAN BLUE (⊙)
0
22
24
26
30
34
38
42
46
50
54
FRACTION NUMBER

Figure 7. Elution pattern of FDH from G-150 Sephadex column. 5.8 ml fractions were collected at 2-4°C.

(⊙) protein concentration in mg per ml

(▲) Optical density at 620 nm for dextran blue to measure the void volume.

(●) FDH activity measured spectrophotometrically as described in "Methods".

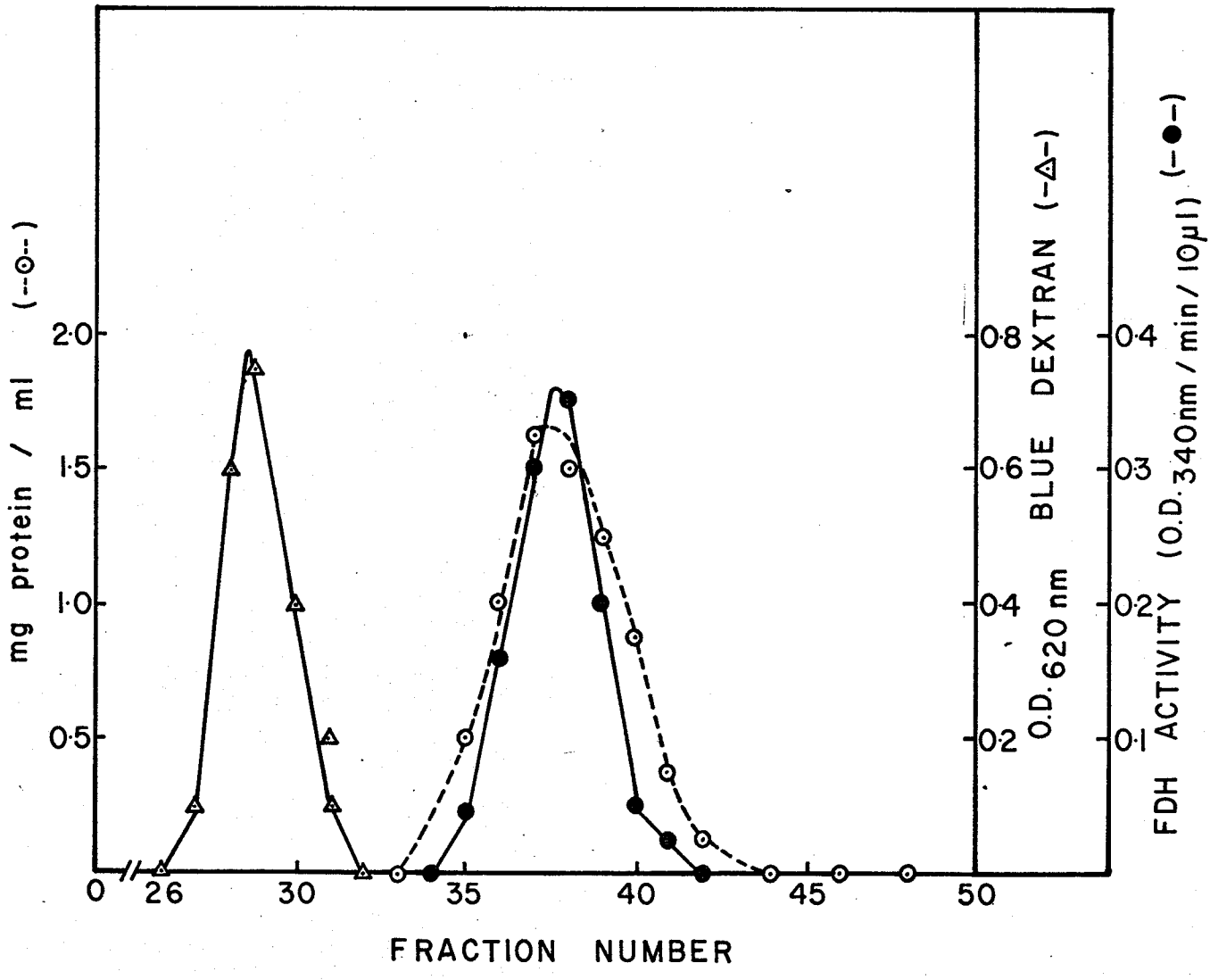

mg protein / ml (--⊙--)
2·0
1·5
1·0
0·5
0
26
30
35
40
45
50
FRACTION NUMBER
O.D. 620 nm BLUE DEXTRAN (-Δ-)
0·8
0·6
0·4
0·2
FDH ACTIVITY (O.D. 340nm / min / 10µl) (-●-)
0·4
0·3
0·2
0·1

TABLE I

A SUMMARY OF THE PURIFICATION PROCEDURE FOR FORMATE DEHYDROGENASE

Step	Volume in ml	Total units* of Enzyme	Total Protein in mg	Specific Activity+	Purification	Yield (%)
1. "Crude extract", 120,000 x g supernatant	800	3,460	16,800	0.206	1.00	100
2. 1st Protamine sulfate (0.07)	845	3,564	16,800	0.212	1.03	103
3. 2nd Protamine sulfate (0.14)	880	3,668	16,530	0.222	1.08	106
4. Ammonium sulfate (0.3-0.4)	50	2,558	2,950	0.867	4.21	73.9
5. Dialysis and centrifugation	52	1,924	2,100	0.916	4.45	55.6
6. DEAE-cellulose	104	1,082	228	4.746	23.04	31.3
7. Reverse Amm. sulfate I (30%)	8	624	125	4.992	24.23	18.0
8. Sephadex G-100	29	478	61	7.844	38.08	13.8
9. Reverse Amm. sulfate II (30%)	10	320	39	8.205	39.83	9.2

* Units = μ moles NADH produced per minute.

+ Specific Activity = units per mg protein.

Comments

When formate-grown cells were used as source material for FDH, the crude extract sometimes contained apparently low levels of the enzyme activity as measured by NAD^+ reduction. The time for harvesting these cells seemed to be more critical than for those grown on oxalate in obtaining high levels of FDH in the extract. Whereas oxalate-grown cells produced a crude extract showing high FDH activity after sonication in a Raytheon Sonicator unit (set at 10 Kilohertz), the formate-grown cells treated thus invariably produced extracts with low FDH activity. NADH oxidase activity was always checked concomittantly with FDH activity. The French Press was the method used for breakage of formate-grown cells at all times. Batches of cells which resulted in crude extracts with low FDH activity were not used for FDH purification.

Storage and stability of the enzyme

The enzyme preparation, Reverse Ammonium Sulfate II, undiluted (approximately 3-5 mg, or higher, protein per ml) and in the presence of 3 mM DTT and 0.01 mM NADH was stable at $2^{\circ}C$ for 3 days. The same enzyme preparation was stable for periods uptto 2 months when kept frozen at $-20^{\circ}C$. The purified enzyme was much more

stable than the crude enzyme. It could be held at 24-25°C for an hour without noticeable loss of activity, as measured by extinction at 340 nm.

Effect of pH on Enzyme Activity

The effect of hydrogen ion on the maximum velocity of FDH was investigated. A combination of buffers was used, namely MES, potassium phosphate and Tris-HCl. The overlapping values were used as reference for extrapolation of values in order to obtain the smooth curve illustrated in Fig. 8. The optimum pH obtained is pH 7.5 at 25°C.

Effect of Heat

The enzyme FDH is extremely sensitive to heat, being rapidly inactivated at temperatures above 30°C. Heating at 55°C for two minutes completely inactivated the enzyme.

Purity of the Enzyme

The enzyme FDH is considered to be near a state of homogeneity even though the polyacrylamide gels obtained by electrophoresis (see "Methods") showed more than a single protein band as illustrated in plate 7. There was one large band trailed by two minor

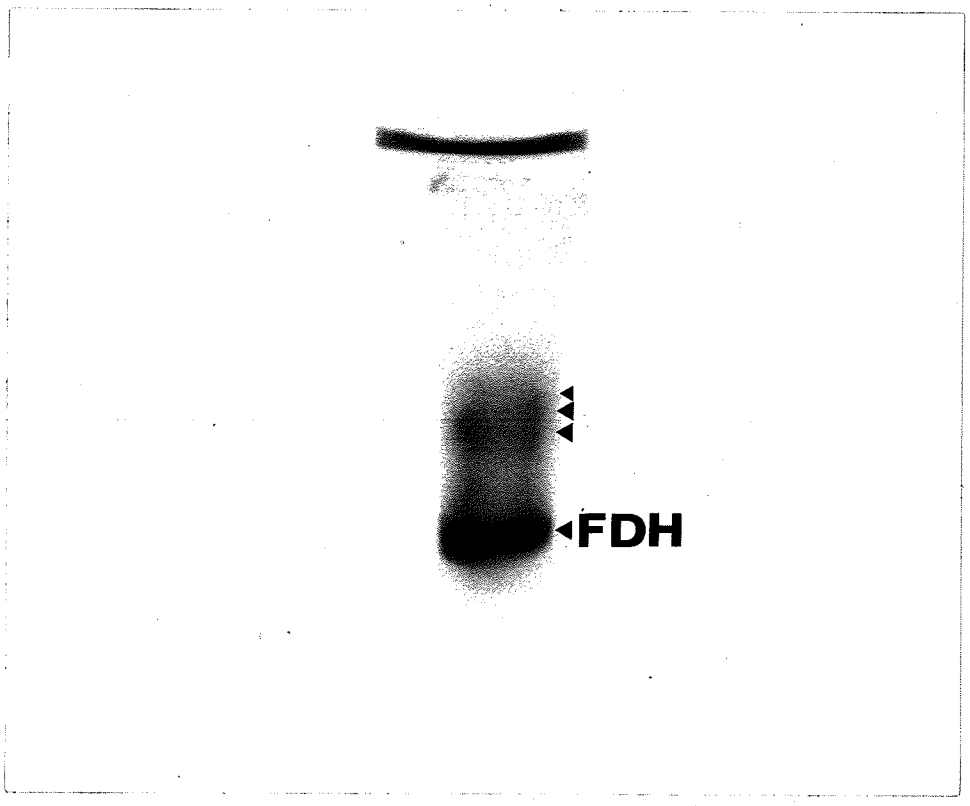
ones. However, these minor bands represented only less than a quarter the intensity of the major band. Finally there was a very faint band moving behind the two minor bands.

In one experiment, a gel was sliced longitudinally after electrophoresis. One half was stained with Coomassie Brilliant Blue (as usual, for protein) and the other half was specifically stained with phenazine methosulfate and nitroblue tetrazolium (see "Methods"). The activity band obtained corresponded with the major protein band. Plate 8 illustrates gels stained for activity.

A possible point of interest regarding the activity staining of polyacrylamide gels involves the "crude extracts" from oxalate-grown and formate-grown cells. After electrophoresis and staining for activity, only one large activity band was evident from the oxalate-grown cell preparation whereas there were three bands from the formate-grown cell preparation. After purification of the FDH, only one band was seen in preparations from either type of cells.

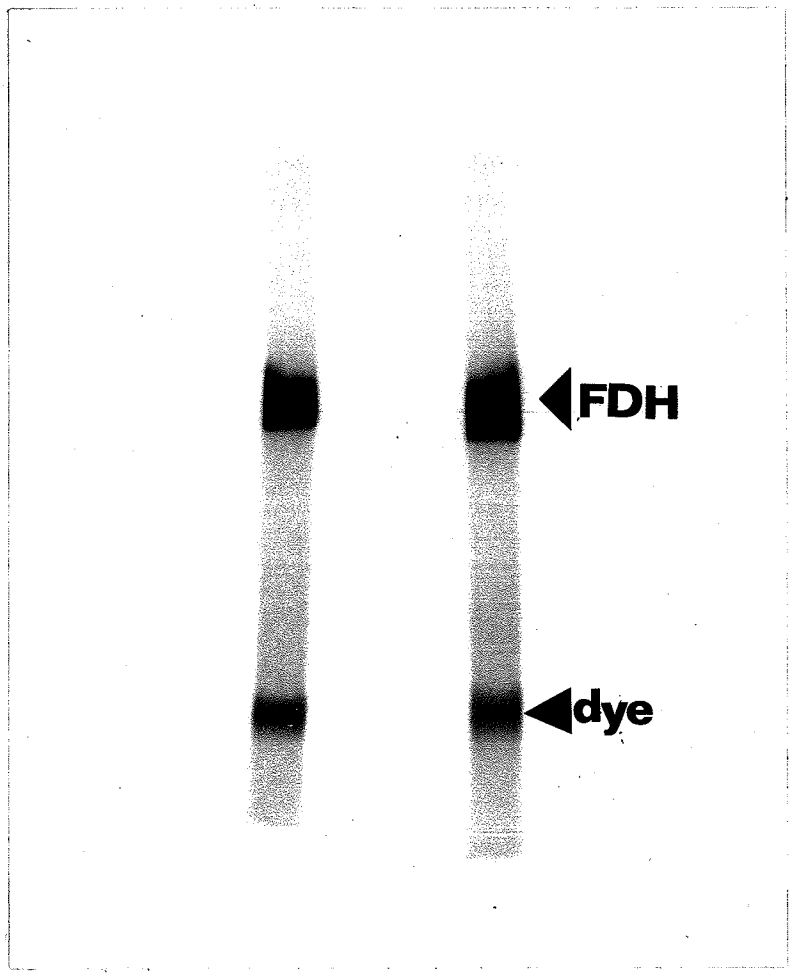
Centrifugation of the purified FDH was attempted in the Beckman Model E analytical ultracentrifuge (see "Molecular Weight Determinations") but no distinct peak was observed with Schlieren optics.

Plate 7



Appearance of FDH in polyacrylamide gels after electrophoresis and staining with Coomasie blue (see "Methods"). 100-200 µg of enzyme was used.

Plate 8



Appearance of FDH in polyacrylamide gels after electrophoresis and specific staining for activity (see "Methods"). 100-200 µg of enzyme was used.

Figure 8. Effect of pH on FDH activity.

(●) FDH activity measured spectrophotometrically as described in "Methods". MES buffer used for pH 5.0 to pH 6.0. Potassium phosphate buffer used for pH 6.0 to pH 8.5. Tris-HCl buffer used for pH 8.0 to pH 9.0.

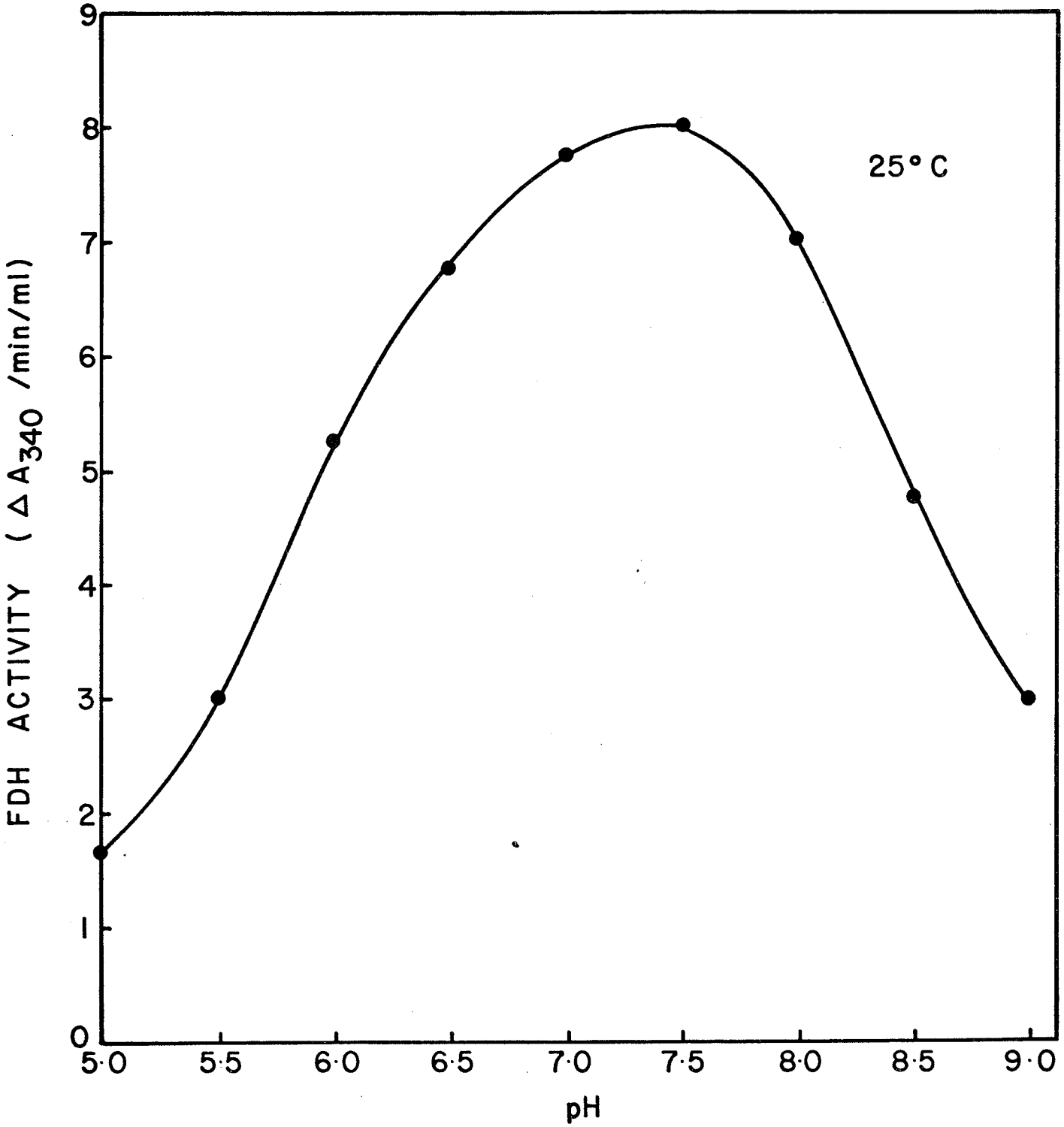
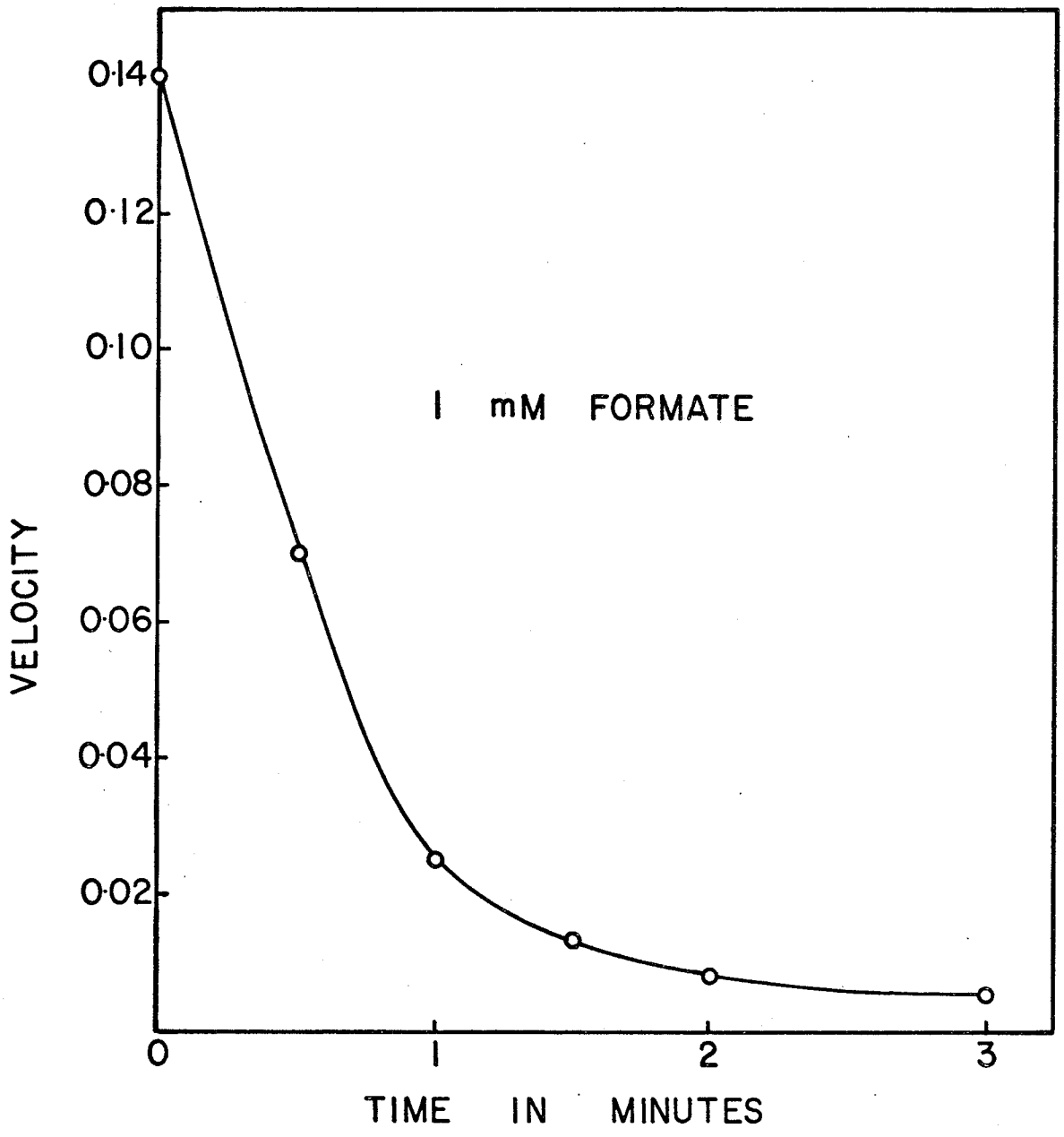

25° C
FDH ACTIVITY (ΔA340 /min/ml)
pH
0
1
2
3
4
5
6
7
8
9
5·0
5·5
6·0
6·5
7·0
7·5
8·0
8·5
9·0

Figure 9. FDH inactivation by formate in the absence of NAD^+. Enzyme velocity measured spectrophotometrically as described in "Methods".


0·14
0·12
0·10
0·08
0·06
0·04
0·02
VELOCITY
1 mM FORMATE
0
1
2
3
TIME IN MINUTES

On preincubation at 25°C with pronase (protease from Streptomyces griseus) for 3 minutes, all enzyme activity was lost. However preincubation with snake venom phospholipase (Naja naja) at 25°C for 5 minutes caused no noticeable loss of activity. It thus appears that the enzyme contained no essential phospholipid.

Inactivation of FDH by Formate

The purified enzyme is extremely sensitive when exposed to formate in the absence of NAD^+ or when the concentration of NAD^+ is low (less than 0.1 mM). FDH was preincubated in the reaction cuvette with 1 mM formate (in the standard buffer) for various lengths of time after which the reaction was started by the addition of NAD^+ (40 to 50 µg of the enzyme in the cuvette). Fig. 9 shows that formate inactivates FDH rather quickly at the concentration of 1 mM. The inactivation appears to be irreversible.

Activation of FDH by NADH and NAD^+

Initially, much loss of activity of FDH was encountered when the enzyme was dialysed overnight. In actual fact, during dialysis, a lengthy lag period was created. On starting the reaction in a cuvette with the standard

reaction mixture (see "Methods"), no optical density increase was observed at 340 nm for a period of about 5 minutes. After this lag period, the enzyme reaction would begin and increase to a maximum, producing a linear trace in the recorder. The lag could also be reproduced if FDH was diluted in the cuvette (1:100 dilution) and allowed to stand for 10-15 minutes before starting the reaction with the addition of the substrates, NAD^+ followed by formate. It was found that the lag could be completely eliminated if 0.01 mM NADH or from 0.005 mM to 0.01 mM NAD^+ was added to the enzyme (with "lag") in the cuvette and further incubated 5 minutes before starting the reaction. A representation of the recorder tracings is shown in Fig. 10 and Fig. 11. NAD^+ at higher concentrations (0.3 mM) did not remove the lag although it was shortened to about 2 minutes. Reduction of the lag by low NAD^+ concentrations was not absolute, as it left a very slight lag of about 10 seconds. Addition of DTT and B.S.A. did not affect the lag period but increased the r_{max} at the end of the lag. They seemed to protect the enzyme against oxidation during the lengthy period it was left in the cuvette (at least 15 minutes) before reaction was started. As illustrated in Fig. 11, NADH eliminates the lag absol-

Figure 10. The effect of NAD^+ on the lag period of FDH.

(1) ● FDH assayed normally (see "Methods")

(2) ○ Enzyme incubated alone in cuvette for 10 min.

(3) □ Incubation with 1 mM DTT or 2 mg BSA for 5 min. after (2)

(4) △ Incubation with 0.3 mM NAD^+ for 5 min. after (2)

(5) ■ Incubation with 0.3 mM NAD^+ + 2 mg BSA for 5 min. after (2)

(6) ▲ Incubation with 0.005 mM NAD^+ for 5 min. after (2)

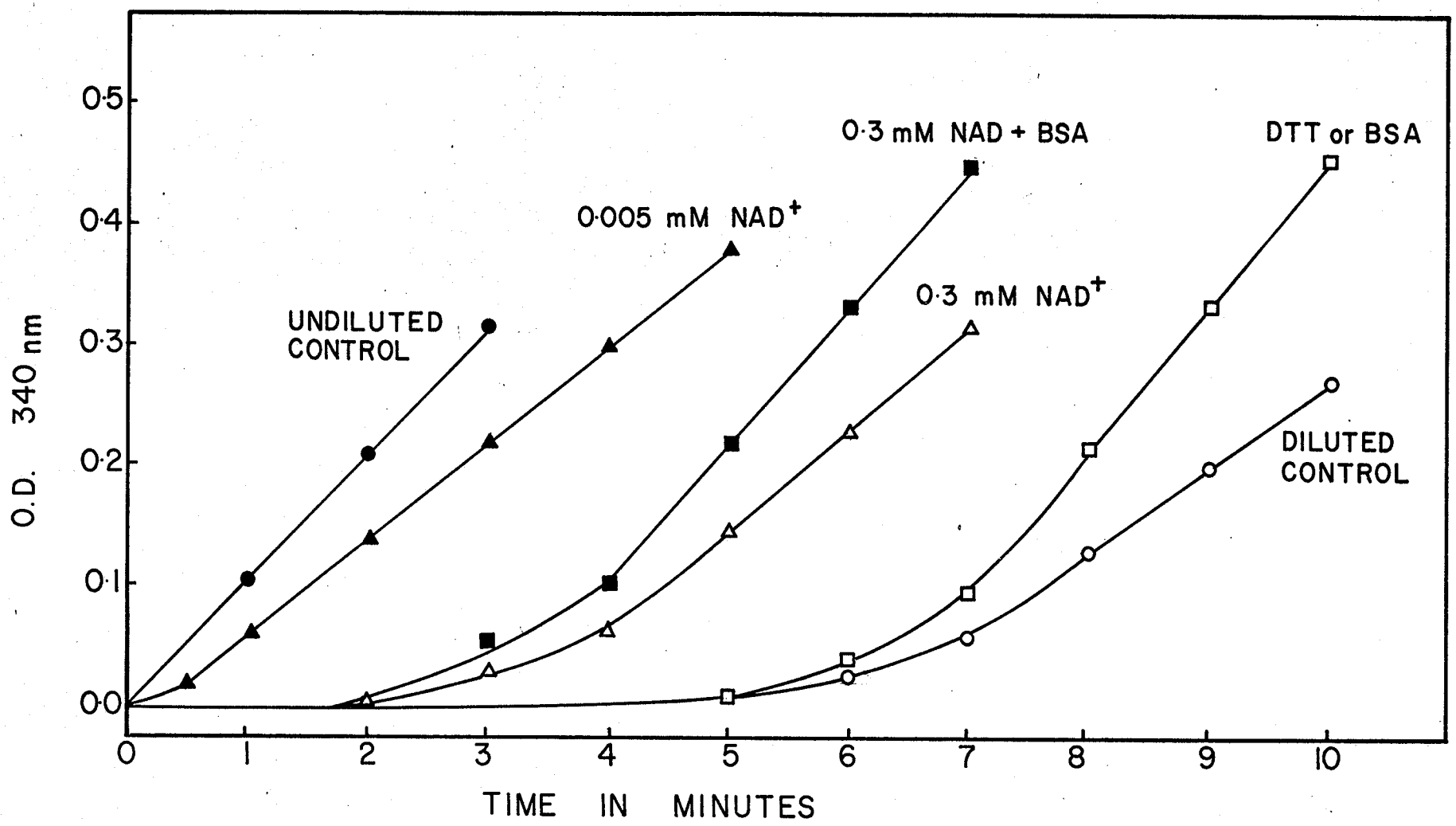

0·5
0·4
0·3
0·2
0·1
0·0
O.D. 340 nm
0·3 mM NAD + BSA
DTT or BSA
0·005 mM NAD+
0·3 mM NAD+
UNDILUTED CONTROL
DILUTED CONTROL
0
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES

Figure 11. The effect of NADH on the lag period of FDH.

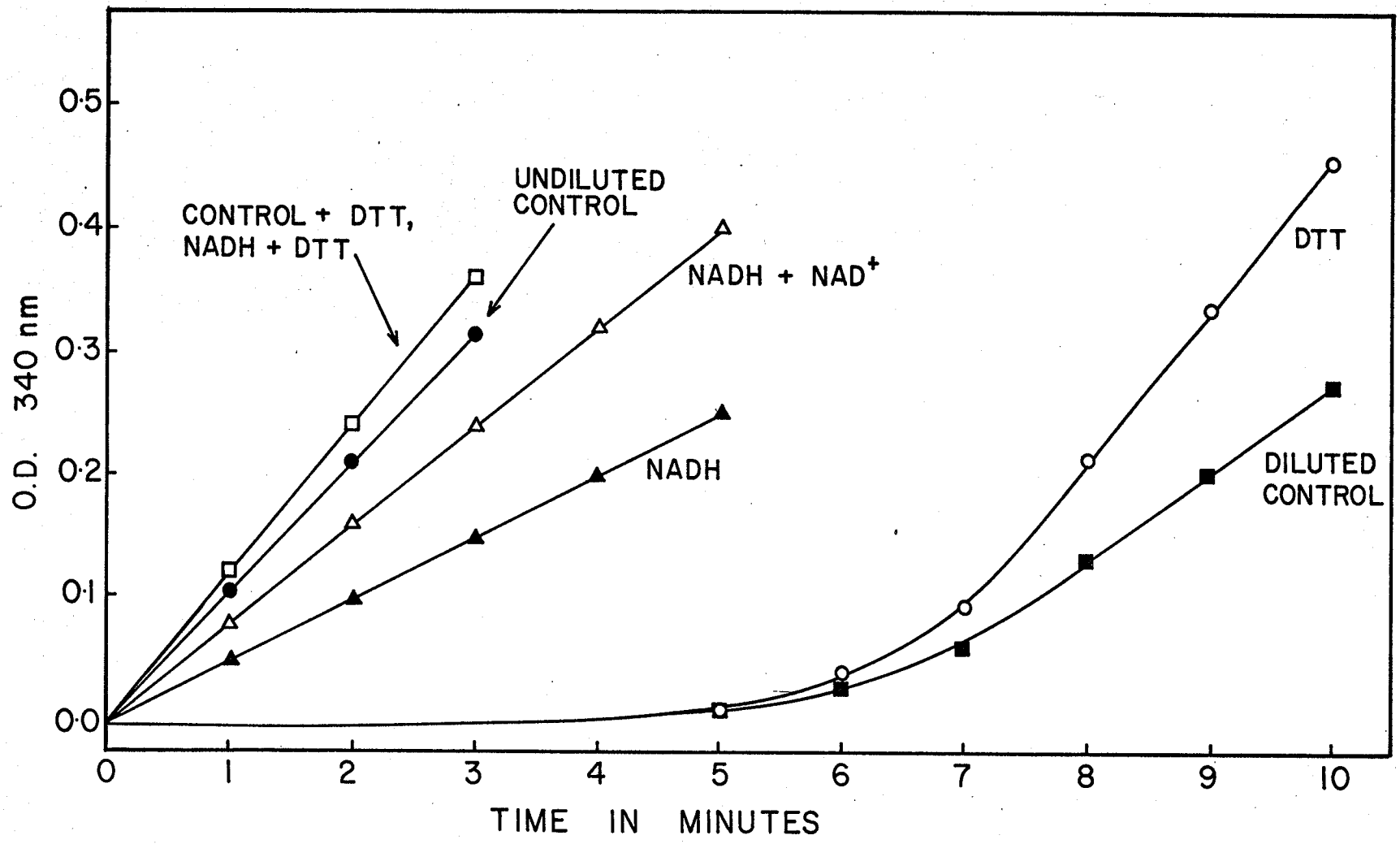
(1) ● FDH assayed normally (see "Methods")

(2) ■ Enzyme incubated alone in cuvette for 10 min.

(3) ▲ Incubation with 0.01 mM NADH for 5 min. after (2)

(4) △ Incubation with 0.01 mM NADH and 0.3 mM NAD^+ for 5 min. after (2)

(5) □ Incubation with 0.01 mM NADH and 1 mM DTT after (2) or like (1) + 1 mM DTT, for 5 min.


0·5
0·4
0·3
0·2
0·1
0·0
O.D. 340 nm
0
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES
CONTROL + DTT,
NADH + DTT
UNDILUTED
CONTROL
NADH + NAD+
NADH
DTT
DILUTED
CONTROL

utely. It was therefore incorporated into all buffer solutions routinely during purification of FDH. Other compounds were also used in attempts to eliminate the lag period. They were $NADP^+$ (H), Acetyl NAD^+, FMN, FAD, AMP, GMP, ATP and GTP. The lag period was unaffected by any of these compounds.

Molecular Weight of FDH

The enzyme exhibited polydispersive properties when centrifuged at high speed in a Beckman Model E analytical ultracentrifuge. Thus no distinct peak or peaks were observed with Schlieren optics. Different runs were made at various pHs and in the presence of sucrose (10%) and bovine serum albumin (1%) without resulting in any distinct peak formation for FDH. Molecular weight estimations were therefore attempted using a calibrated Sephadex G-200 column and sucrose density gradients (under appropriate conditions).

Sephadex G-200

A column was prepared and calibrated as described in "Methods". The method for estimating molecular

weights was basically that as described by Andrews (1964). Proteins of known molecular weight were used as calibrating standards. These included chymotrypsinogen A (bovine pancreas), M.W. 25,000; ovalbumin (egg white), M.W. 45,000; aldolase (rabbit muscle), M.W. 158,000 and glucose-6-phosphate (G-6-P) dehydrogenase (yeast), M.W. 102,000 (this value taken from Yue (1967), for the crystalline $NADP^+$-free apoenzyme). K_{av} values were calculated as described in the Calibration Kit manual by Pharmacia Ltd. The K_{av} values were plotted against the molecular weights (on the logarithm scale) as shown in Fig. 12. From the K_{av} obtained experimentally for FDH, its molecular weight is estimated to be 151,000 ± 3,000 daltons.

Sucrose Density Gradients

Sucrose gradients were prepared as described in "Methods". FDH was sensitive to the shear forces when centrifuged since no activity peak could be detected. The protein profile showed no distinct peaks either. However, when 0.01 M NAD^+ (20 times normal substrate levels for assay) was incorporated into the sucrose-buffer system, the enzyme remained intact and active during ultracentrifugation. Two internal-marker standards were used, ovalbumin (egg white) and yeast

glucose-6-phosphate dehydrogenase (dialysed, $NADP^+$-free). The activity and optical density profiles obtained from the gradients are shown in Figs. 13 and 14. The lower fraction numbers being closer to the bottom of the tube. Molecular weight of FDH was estimated according to the method of Martin and Ames (1961). The formula given is:

$$\left(\frac{\text{M.W. FDH}}{\text{M.W. STD.}}\right)^2 = \left(\frac{\text{FDH Fraction \# From Top of Tube}}{\text{STD. Fraction \# From Top of Tube}}\right)^3$$

From the experimental values obtained, the M.W. of FDH is calculated to be:

With G-6-P dehydrogenase as marker

(a) $\frac{\text{M.W. FDH}}{\text{M.W. STD.}} = \left(\frac{40.9}{30.4}\right)^3$

M.W. FDH = 1.560 x 102,000 = 159,150

(b) M.W. FDH = 1.582 x 102,000 = 161,140

Mean M.W. FDH = 160,140 ± 1,000

With ovalbumin as marker

M.W. FDH = 3.423 x 45,000 = 154,050

Therefore the mean molecular weight of FDH as obtained by sucrose density gradient centrifugation was calculated to be 157,000 ± 3,000 daltons.

Figure 12. Table showing K_{av} values of standards and FDH for molecular weight estimation by Sephadex G-200.

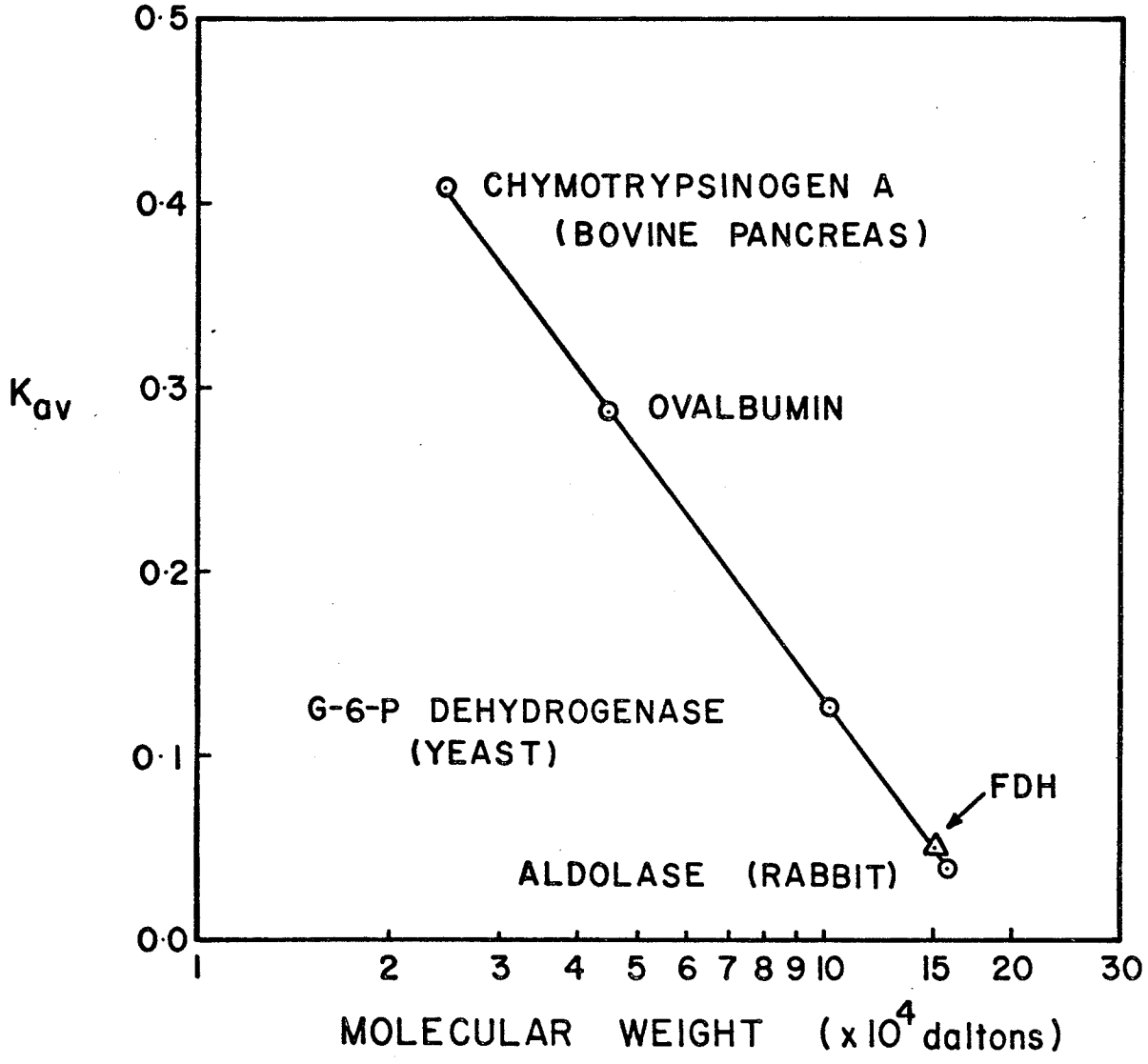

0·5
0·4
0·3
0·2
0·1
0·0
K_{av}
CHYMOTRYPSINOGEN A
(BOVINE PANCREAS)
OVALBUMIN
G-6-P DEHYDROGENASE
(YEAST)
FDH
ALDOLASE (RABBIT)
1
2
3
4
5
6
7
8
9
10
15
20
30
MOLECULAR WEIGHT ($\times 10^4$ daltons)

Figure 13. Estimation of molecular weight of FDH by sucrose density gradient centrifugation in the presence of 0.01 M NAD^+ with glucose-6-phosphate as marker.

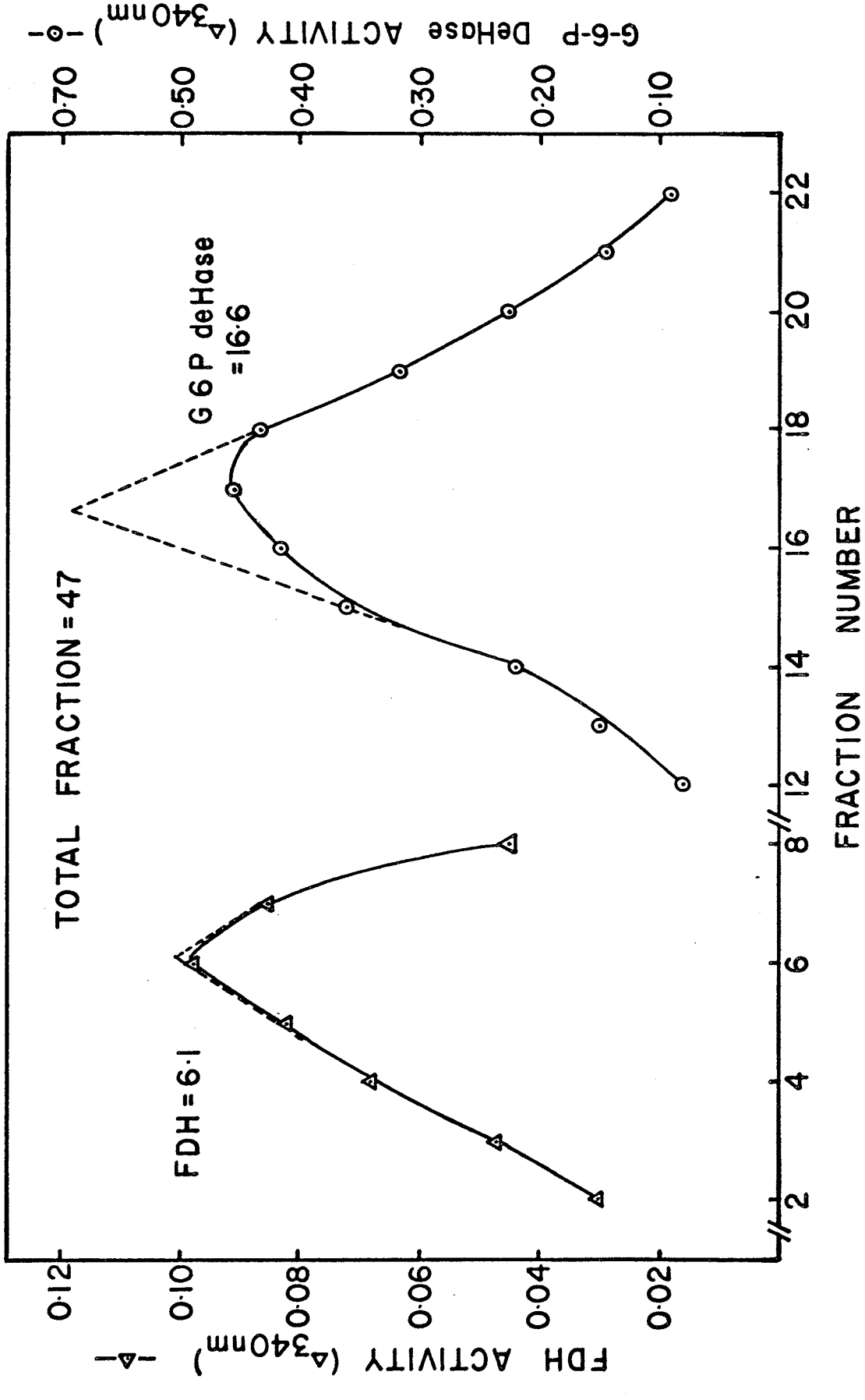
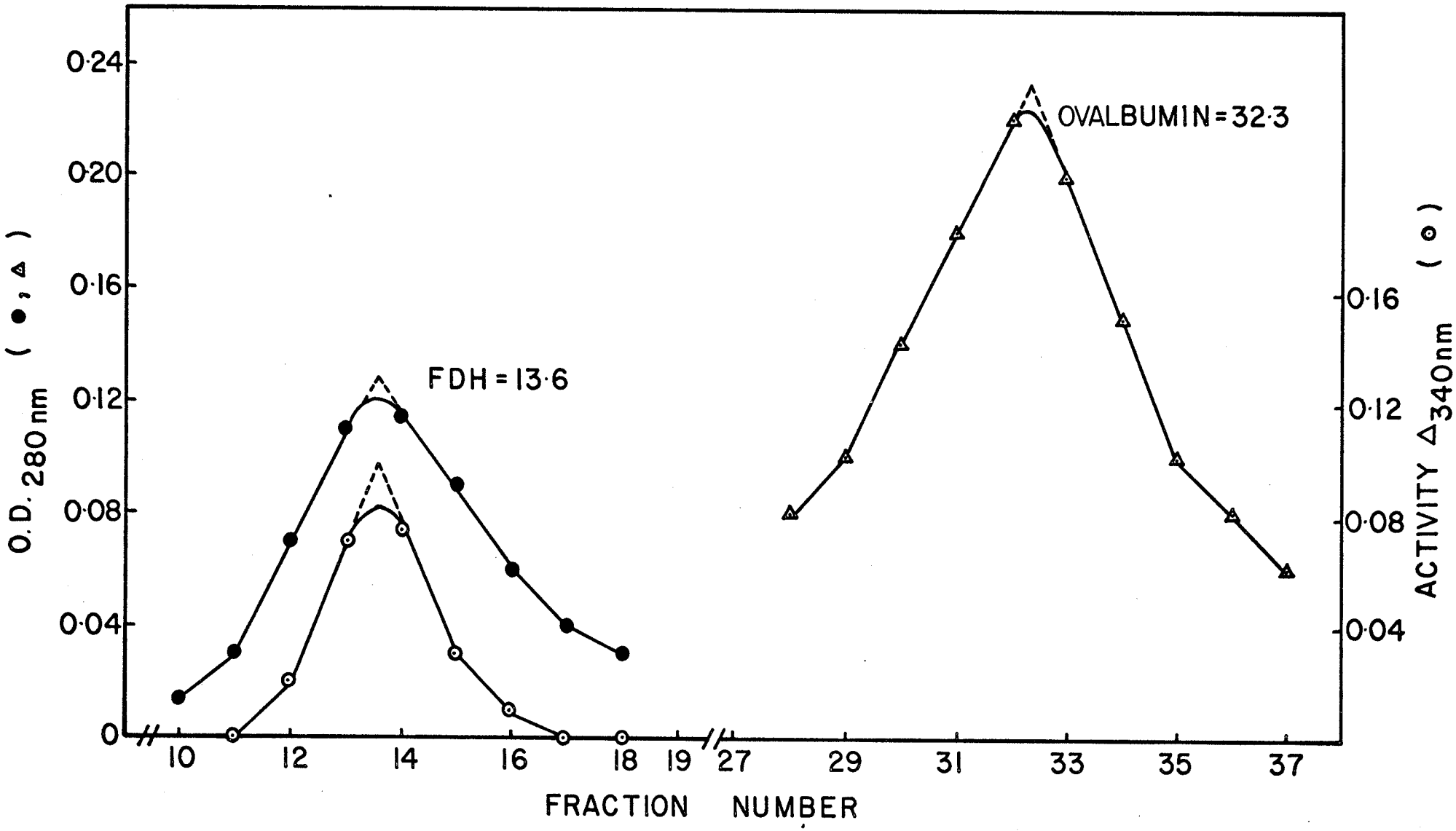

FDH ACTIVITY (Δ_{340nm}) -▲-
G-6-P Dehase ACTIVITY (Δ_{340nm}) -⊙-
0·12
0·10
0·08
0·06
0·04
0·02
0·70
0·50
0·40
0·30
0·20
0·10
2
4
6
8
12
14
16
18
20
22
FRACTION NUMBER
TOTAL FRACTION = 47
FDH = 6·1
G 6 P deHase
=16·6

Figure 14. Estimation of molecular weight of FDH by sucrose density gradient centrifugation in the presence of 0.01 M NAD^+ with ovalbumin as marker.


0·24
0·20
0·16
0·12
0·08
0·04
0
O.D. 280 nm (● , △)
FDH = 13·6
OVALBUMIN = 32·3
0·16
0·12
0·08
0·04
ACTIVITY Δ340nm (⊙)
10
12
14
16
18
19
27
29
31
33
35
37
FRACTION NUMBER

Kinetic Studies

Notations and terminology as proposed by Cleland (1963 a,b,c) are used throughout. Initial velocity studies were performed by determining the relative difference in velocity using varying concentrations of the primary substrate and fixed variable concentrations of the secondary substrate. All initial velocity and product inhibition studies are presented in the double-reciprocal form of Lineweaver and Burk (1934). The kinetic constants used and the analyses of replots were based on the assumption that the mechanism was an Ordered Bi Bi (Cleland, 1963 a) with NAD^+ as the primary substrate (A) and formate the secondary substrate (B) as described in detail in "Discussion".

NAD^+ : Formate

When NAD^+ was the variable primary substrate and formate the secondary, fixed variable substrate, the reaction followed classic Michaelis-Menten (1913) kinetics with all the lines intersecting on the X-axis. The apparent K_m obtained for NAD^+ was 0.025 mM. The plots can be seen in Fig. 15.

Formate : NAD^+

With formate as the primary variable substrate, however, the resulting reciprocal plots were biphasic in nature, although they were still linear in each phase with all lines intersecting on the X-axis. As can be seen in Fig.16, there is an apparent activation occurring at higher concentrations of formate. This resulted in two apparent K_m values for formate being obtained on extrapolation. They have been arbitrarily designated K_{m1} and K_{m2}. The value obtained for K_{m_1} was 0.12 mM and for K_{m_2} was 0.33 mM formate.

Initial velocity replots

Values of intercepts and slopes in Fig. were replotted against the reciprocal of the respective formate concentrations as illustrated in Fig.17. From the intercepts replot we obtain two true K_ms for formate (K_b), the values being 0.36 mM (K_2) and 0.10 mM (K_1) formate, agreeing fairly closely with the apparent K_m values obtained in Fig. 16. Two values indicate two possible sites for formate binding. Extrapolation also produced two V_1 values, 0.25 and 0.16, accounting

Figure 15. Double reciprocal plots of initial velocity against NAD^+ concentrations in the presence of different fixed levels of formate as indicated below:

A = 0.01 mM formate

B = 0.033 mM formate

C = 0.067 mM formate

D = 0.10 mM formate

E = 0.33 mM formate

F = 1.0 mM formate

G = 6.7 mM formate

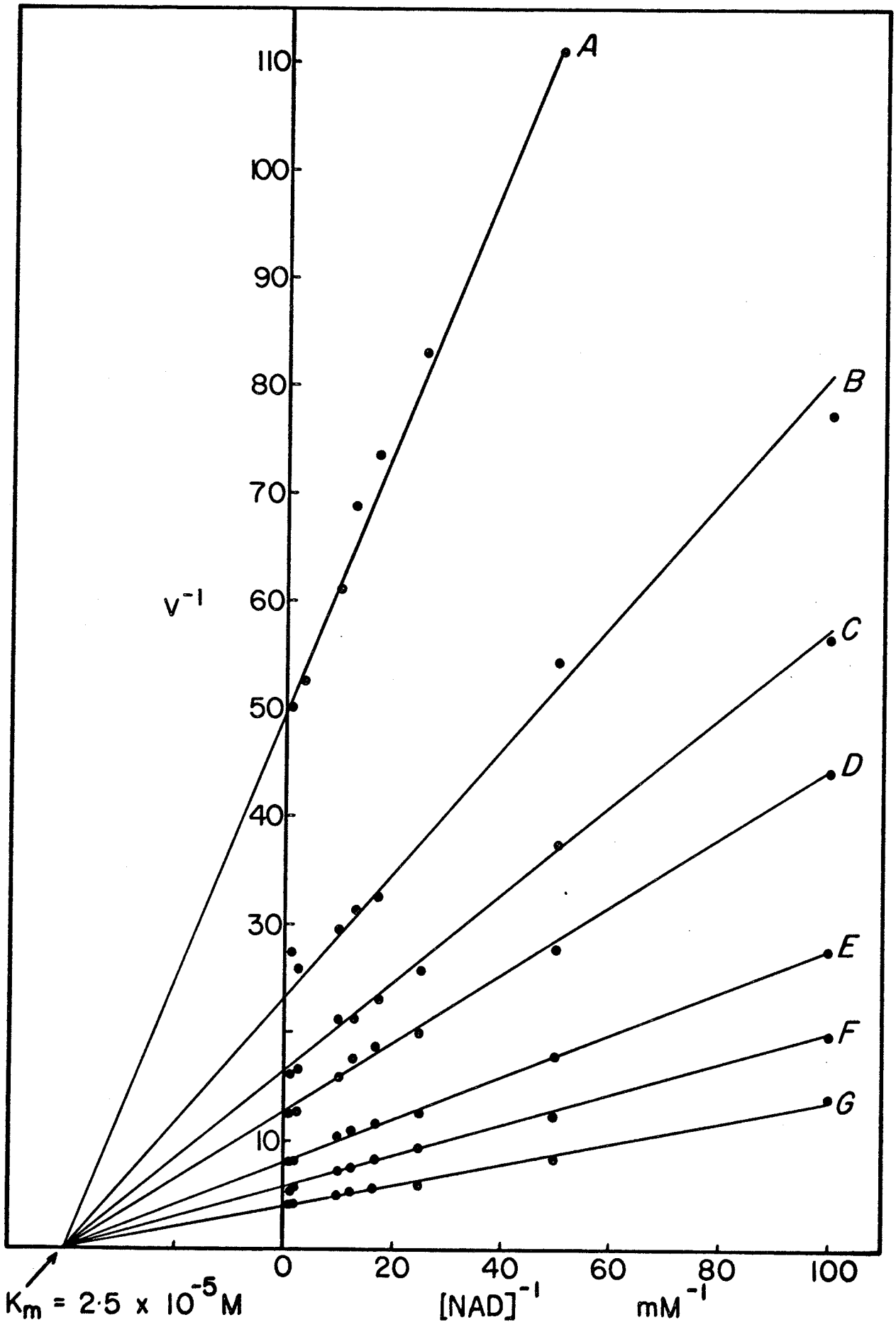

A
B
C
D
E
F
G
110
100
90
80
70
60
50
40
30
10
v^{-1}
0
20
40
60
80
100
$[NAD]^{-1}$
mM^{-1}
$K_m = 2.5 \times 10^{-5} M$

Figure 16. Double reciprocal plots of initial velocity against formate concentrations in the presence of different fixed levels of NAD^+ as indicated below:

A = 0.01 mM NAD^+

B = 0.02 mM NAD^+

C = 0.04 mM NAD^+

D = 0.06 mM NAD^+

E = 0.10 mM NAD^+

F = 0.50 mM NAD^+

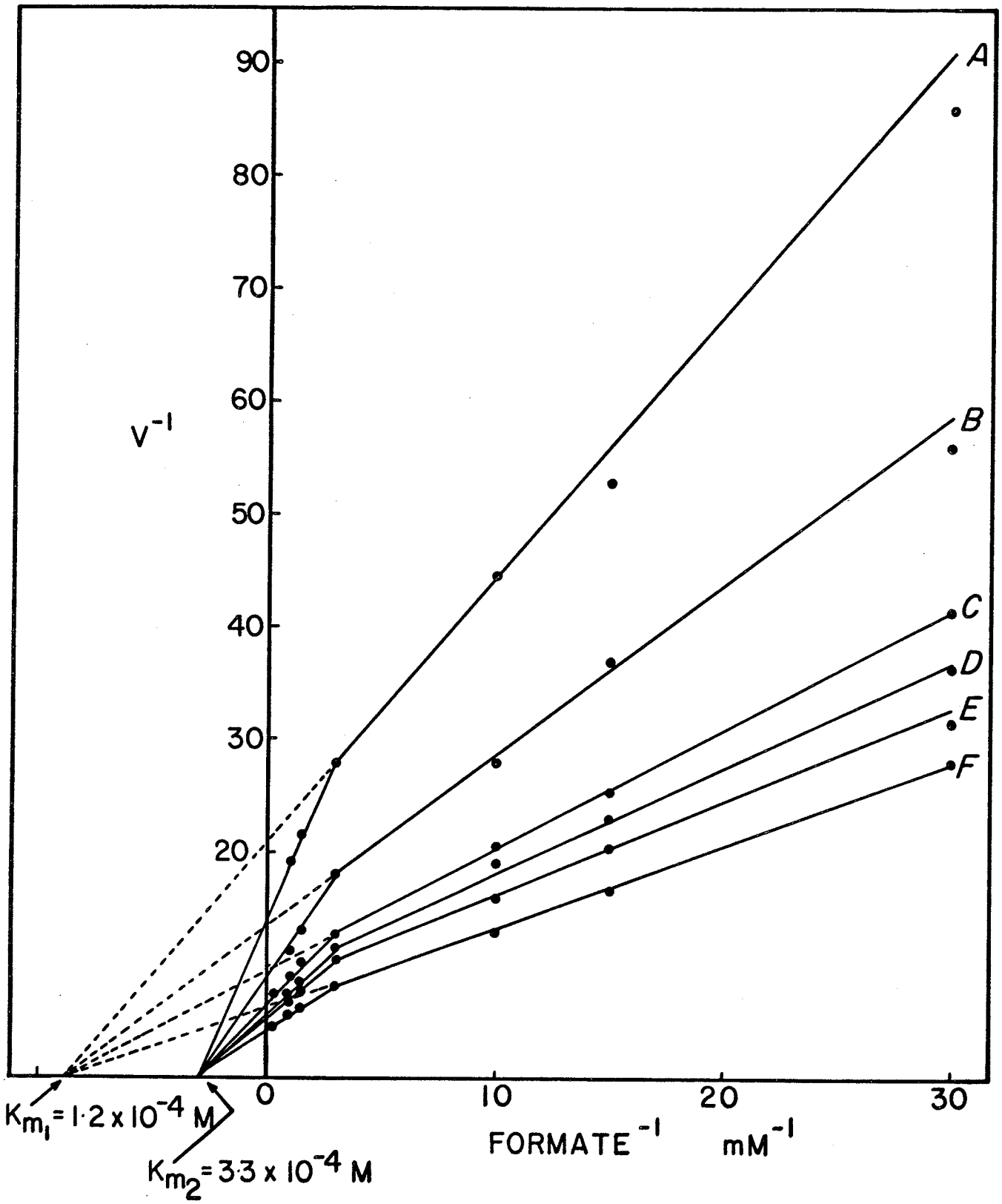

90
80
70
60
50
40
30
20
V^{-1}
A
B
C
D
E
F
0
10
20
30
FORMATE^{-1} mM^{-1}
K_{m_1} = 1·2 x 10^{-4} M
K_{m_2} = 3·3 x 10^{-4} M

Figure 17. Replots of intercept and slope values from Fig. 15 against the reciprocal of the respective formate concentrations.

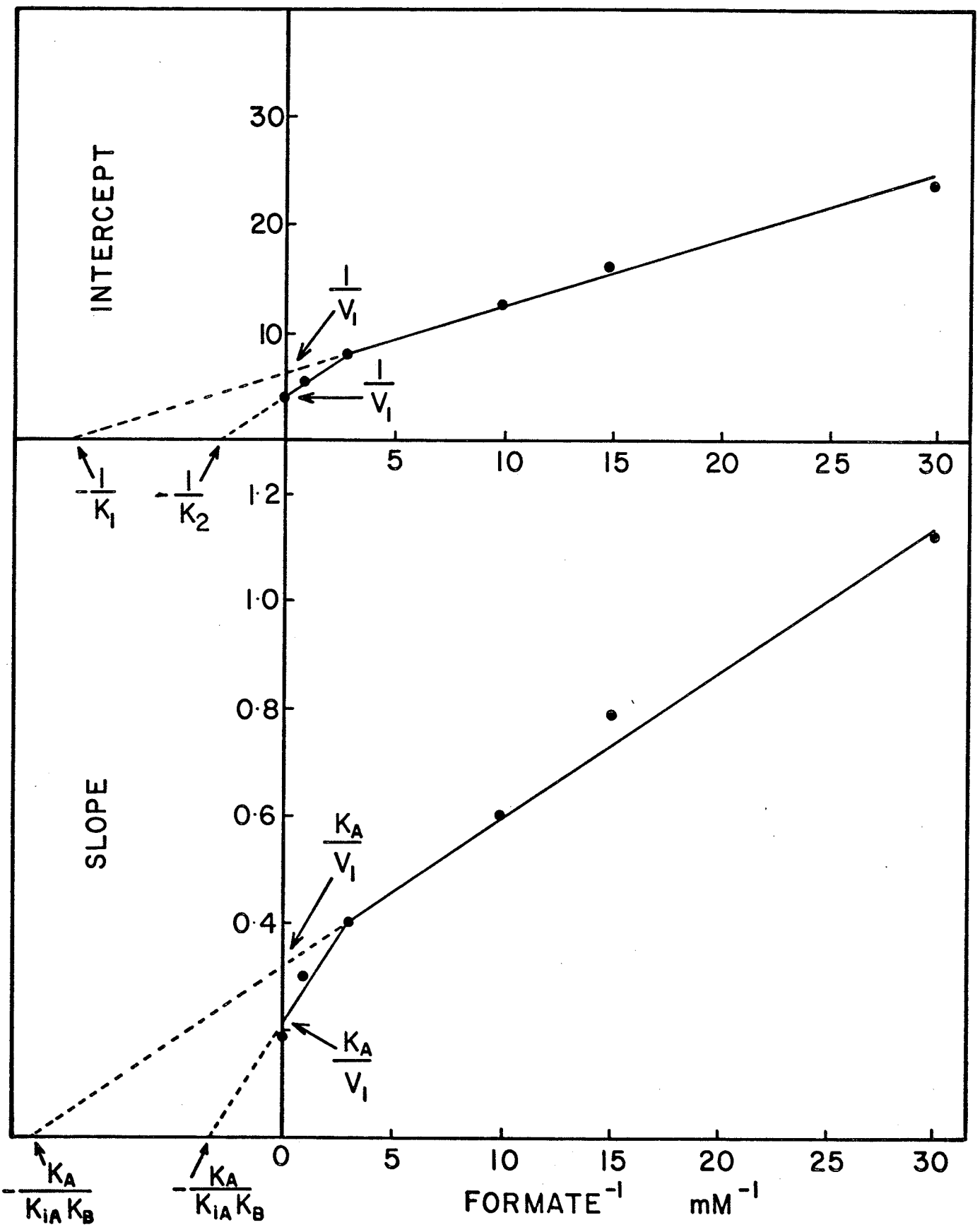

INTERCEPT
30
20
10
1/V1
1/V1
5
10
15
20
25
30
-1/K1
-1/K2
SLOPE
1·2
1·0
0·8
0·6
0·4
KA/V1
KA/V1
0
5
10
15
20
25
30
-KA/KiA KB
-KA/KiA KB
FORMATE-1 mM-1

Figure 18. Replots of slope and intercept values against the reciprocal of the respective NAD^+ concentrations for the "low" formate segment in Fig. 16.

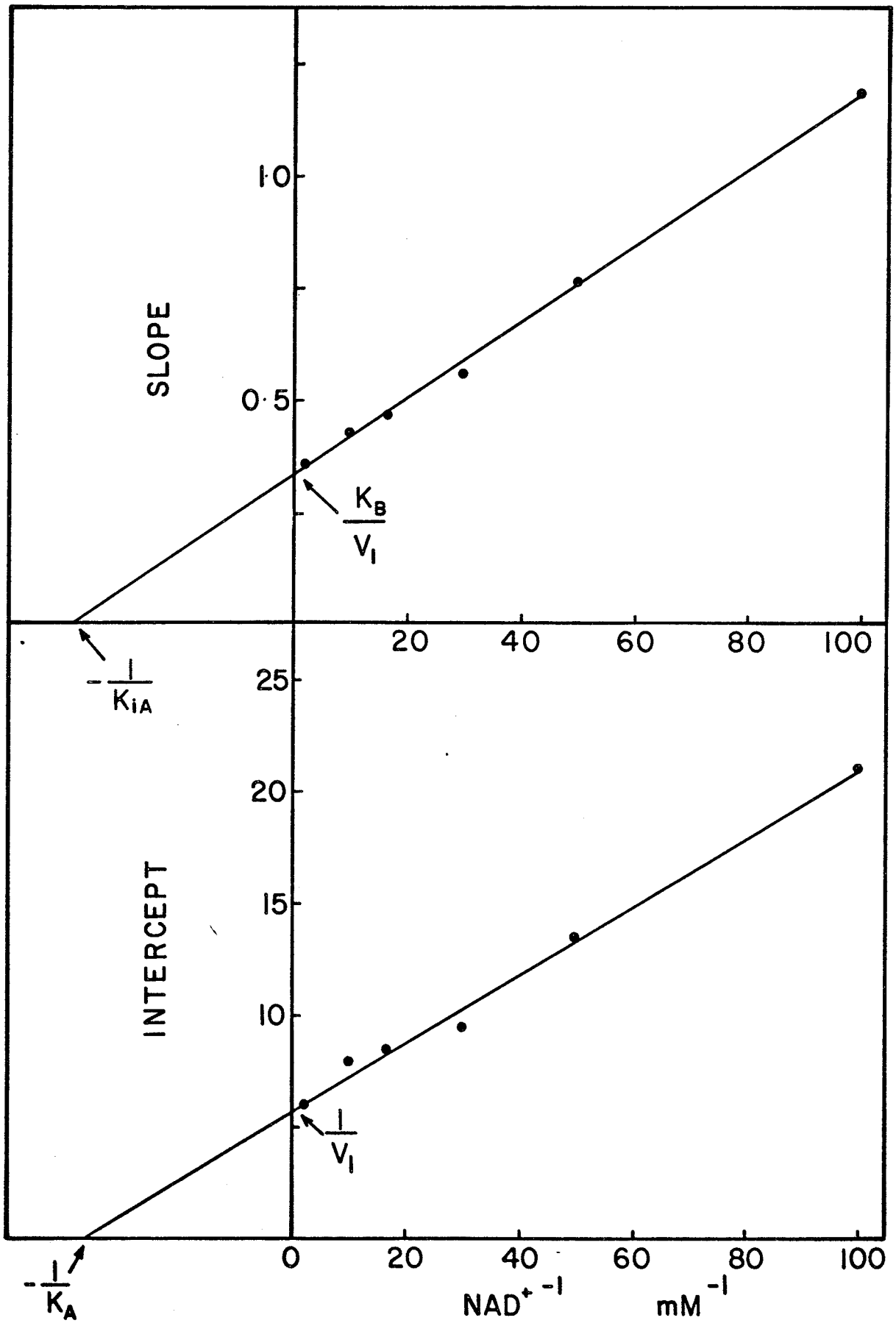
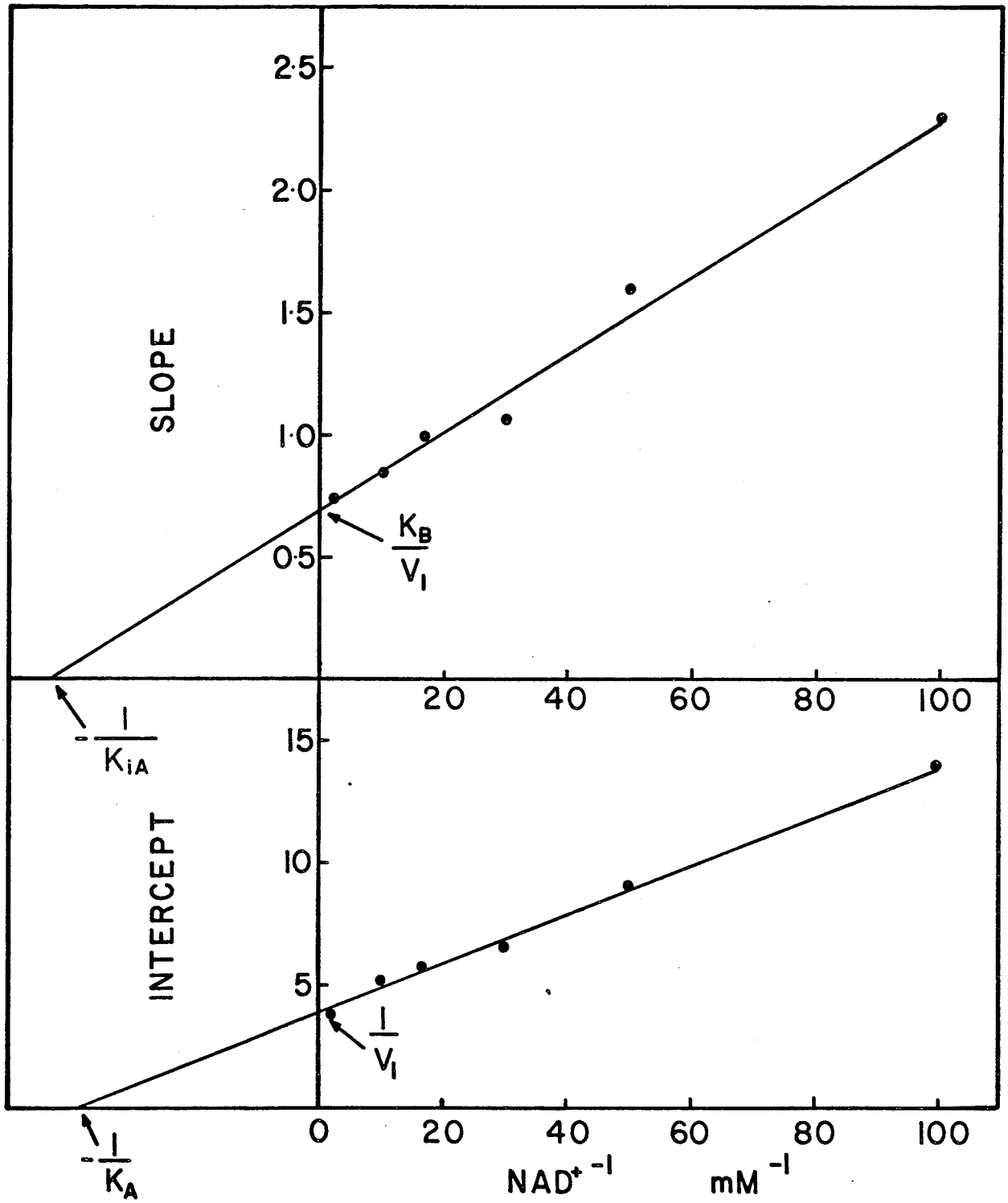

SLOPE
1·0
0·5
$\frac{K_B}{V_1}$
$-\frac{1}{K_{iA}}$
20
40
60
80
100
25
20
15
10
INTERCEPT
$\frac{1}{V_1}$
$-\frac{1}{K_A}$
0
20
40
60
80
100
NAD^+ $^{-1}$
mM^{-1}

Figure 19. Replots of slope and intercept values against the reciprocal of the respective NAD^+ concentrations for the "high" formate segment in Fig. 16.


SLOPE
2·5
2·0
1·5
1·0
0·5
$\frac{K_B}{V_1}$
$-\frac{1}{K_{iA}}$
20
40
60
80
100
INTERCEPT
15
10
5
$\frac{1}{V_1}$
$-\frac{1}{K_A}$
0
20
40
60
80
100
$NAD^+{}^{-1}$
mM^{-1}

for the change in reaction velocity at higher formate concentrations. In the same figure, the slopes replot again resulted in two values for $\frac{V_1}{K_a}$ and $\frac{K_{ia}K_b}{K_a}$ (A = NAD^+, B = formate). For $\frac{V_1}{K_a}$, the values were 4.8 and 3.1 and for $\frac{K_{ia}K_b}{K_a}$ they were 0.29 mM and 0.083 mM formate.

Replots of Fig. 16 are illustrated in Figs. 18 & 19. For the "low formate" segment, K_a = 0.027 mM NAD^+, V_1 = 0.18, $\frac{V}{K_b}$ = 3.0 and K_{ia} = 0.026 mM NAD^+. For the "high formate" segment, K_a = 0.025 mM NAD^+, V_1 = 0.255, $\frac{V}{K_b}$ = 1.4 and K_{ia} = 0.023 mM NAD^+. Thus both K_a and K_{ia} values agree with the apparent K_m value of 0.025 mM NAD^+ at either concentration of formate.

Product Inhibition Studies

In an effort to determine the mechanism of FDH activity; inhibition by the two products, reduced NAD^+ (NADH) and bicarbonate, was studied. Following Cleland's nomenclature, the inhibition will be called "competitive" if only the slope of the plots changes, "uncompetitive" if only the intercept changes and "non-competitive" if both the slope and intercept change. The inhibition constant as determined from replots of intercept against inhibitor concentration will be referred to as K_{iI} and that determined from replots of

slope against inhibitor concentration as K_{is}.

NADH : NAD^+

NADH inhibition was studied at varying concentrations of NAD^+. The double reciprocal plots are presented in Figs. 20 & 21. The inhibition pattern obtained was competitive in nature at both non-saturating (0.1 mM) and saturating (3 mM) formate concentrations. The significance of the broken line is discussed later. Both the replots of the slope values were linear (Fig. 22). The K_{is} for NADH was 0.009 mM with 0.1 mM formate and 0.02 mM with 3 mM formate.

NADH : Formate

Figs. 23 & 24 show the double reciprocal plots that were obtained for NADH inhibition with varying formate concentrations. In order to simplify matters, the two segments of the biphasic lines will be treated separately. At low concentrations of formate, the plots revealed a non-competitive inhibition when NAD^+ was non-saturating (0.03 mM). When NAD^+ was saturating (0.3 mM) the degree of inhibition was much reduced and the inhibition became uncompetitive. The intercept and slope replots were linear (Figs. 25 & 26). With 0.03 mM NAD^+, the K_{is} = 0.03 mM NADH and K_{iI} = 0.64 mM NADH. With 0.3 mM NAD^+, the K_{iI} was 0.17 mM NADH.

At high concentrations of formate, however, the plots obtained show a non-competitive inhibition pattern at both non-saturating and saturating NAD^+ concentrations. All intercept and slope replots obtained were again linear. With 0.03 mM NAD^+, K_{is} = 0.22 mM NADH and K_{iI} = 0.16 mM NADH. At saturating (0.3 mM) NAD^+, K_{is} = 0.20 mM NADH and K_{iI} = 0.22 mM NADH. Fig. 26 & 27.

Bicarbonate : NAD^+

A non-competitive double reciprocal plot was obtained when HCO_3^- inhibition was studied at varying NAD^+ concentrations with formate at non-saturating (0.1 mM) levels as shown in Fig. 28. The slope and intercept replots were both linear (Fig. 29). Values obtained were K_{is} = 0.06 M HCO_3^- and K_{iI} = 0.33 M HCO_3^-. However, at saturating levels of formate (3.3 mM), the double reciprocal plot obtained indicated uncompetitive inhibition (Fig. 30). The resulting intercept replot was linear with a value for K_{iI} = 0.20 M HCO_3^- (Fig. 31).

Bicarbonate : Formate

Again, as in the case of NADH : Formate, the double reciprocal plots yielded biphasic lines. However, in both segments (that is, high and low concentrations of formate), the inhibition pattern was non-

competitive for both non-saturating (0.03 mM) and saturating (0.3 mM) levels of NAD^+ (Figs. 32 and 33).

The intercept and slope replots obtained were all linear and are presented in Figs. 34, 35, 36 & 37. At non-saturating levels of NAD^+ (0.03 mM), with low concentrations of formate, the values obtained were K_{is} = 0.18 M HCO_3^- and K_{iI} = 0.43 M HCO_3^-. With high concentrations of formate, K_{is} = 0.22 M HCO_3^- and K_{iI} = 0.47 M HCO_3^-. At saturating (0.3 mM) levels of NAD^+; with low formate concentrations, K_{is} = 0.15 M HCO_3^- and K_{iI} = 0.64 M HCO_3^- and with high formate concentrations, the values were K_{is} = 0.52 M HCO_3^- and K_{iI} = 0.31 M HCO_3^-.

Initial velocity plots at pH 5.6

FDH purified at pH 7.5 was precipitated with saturated ammonium sulfate and resuspended in 0.05 M potassium phosphate buffer (pH 5.6). Initial velocity studies were done as at pH 7.5, except the reaction buffer used was pH 5.6 potassium phosphate (0.05 M). Fig. 38 shows the plot obtained with varying concentrations of NAD^+. The apparent K_m value (for pH 5.6) was 0.034 mM NAD^+, a value slightly higher than that at pH 7.5. With formate concentrations being varied however, the plots obtained were biphasic like those obtained at pH 7.5. Values obtained for the apparent K_ms

Figure 20. Double reciprocal plot showing product inhibition of FDH by NADH with varying concentrations of NAD^+ in the presence of non-saturating (0.1 mM) formate. Concentrations of NADH are given below:

A = 0.01 mM NADH

B = 0.02 mM NADH

C = 0.06 mM NADH

D = 0.1 mM NADH

The broken line indicates data in the absence of NADH.

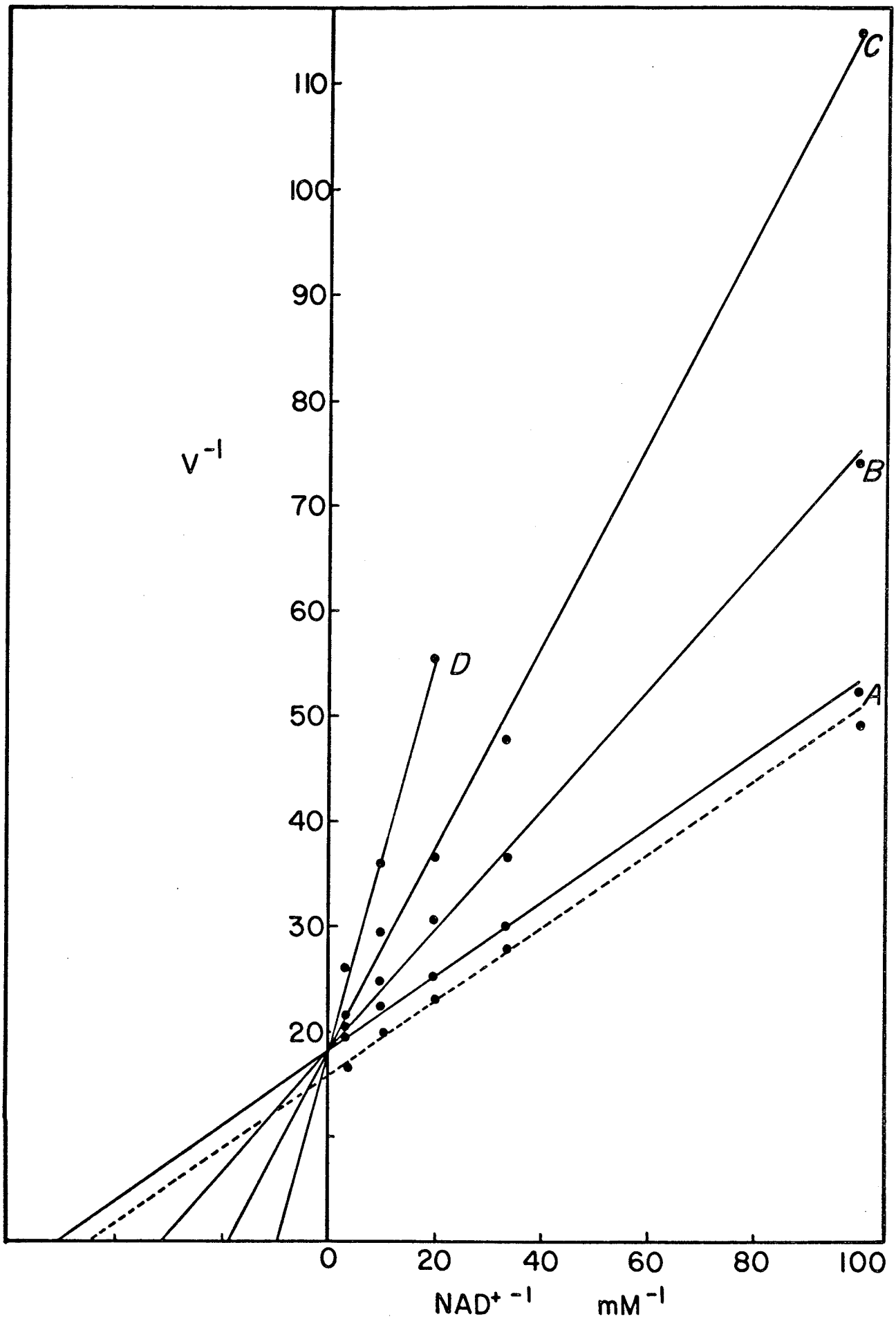

C
B
A
D
110
100
90
80
70
60
50
40
30
20
V⁻¹
0
20
40
60
80
100
NAD+ ⁻¹ mM⁻¹

Figure 21. Double reciprocal plot showing product inhibition of FDH by NADH with varying concentrations of NAD^+ in the presence of saturating (3 mM) formate.

A = 0.01 mM NADH

B = 0.02 mM NADH

C = 0.06 mM NADH

D = 0.10 mM NADH

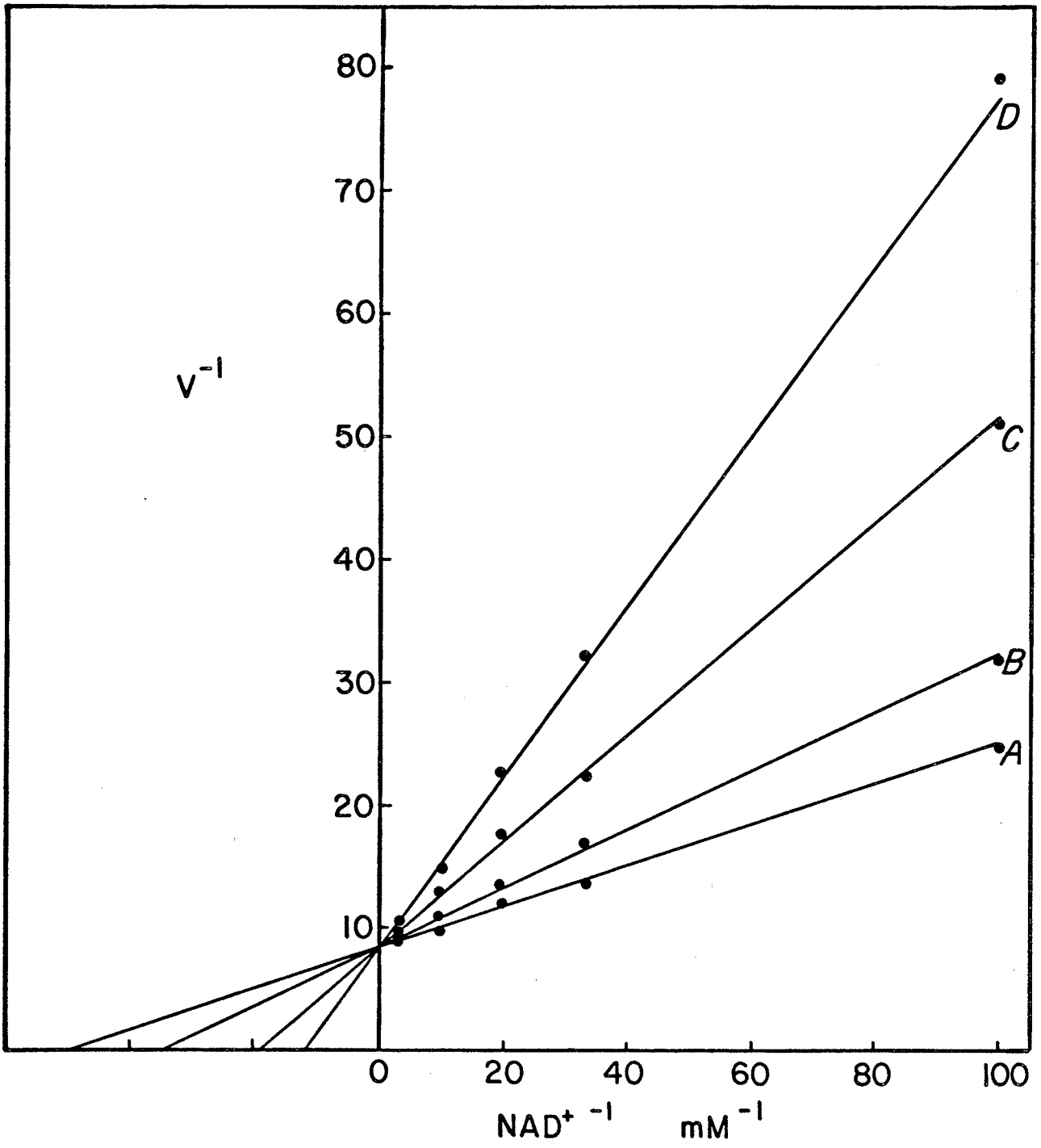

80
70
60
50
40
30
20
10
V⁻¹
D
C
B
A
0
20
40
60
80
100
NAD+ -1 mM -1

Figure 22. Replots of slope values from Fig. 20 and Fig. 21 against NADH concentration.

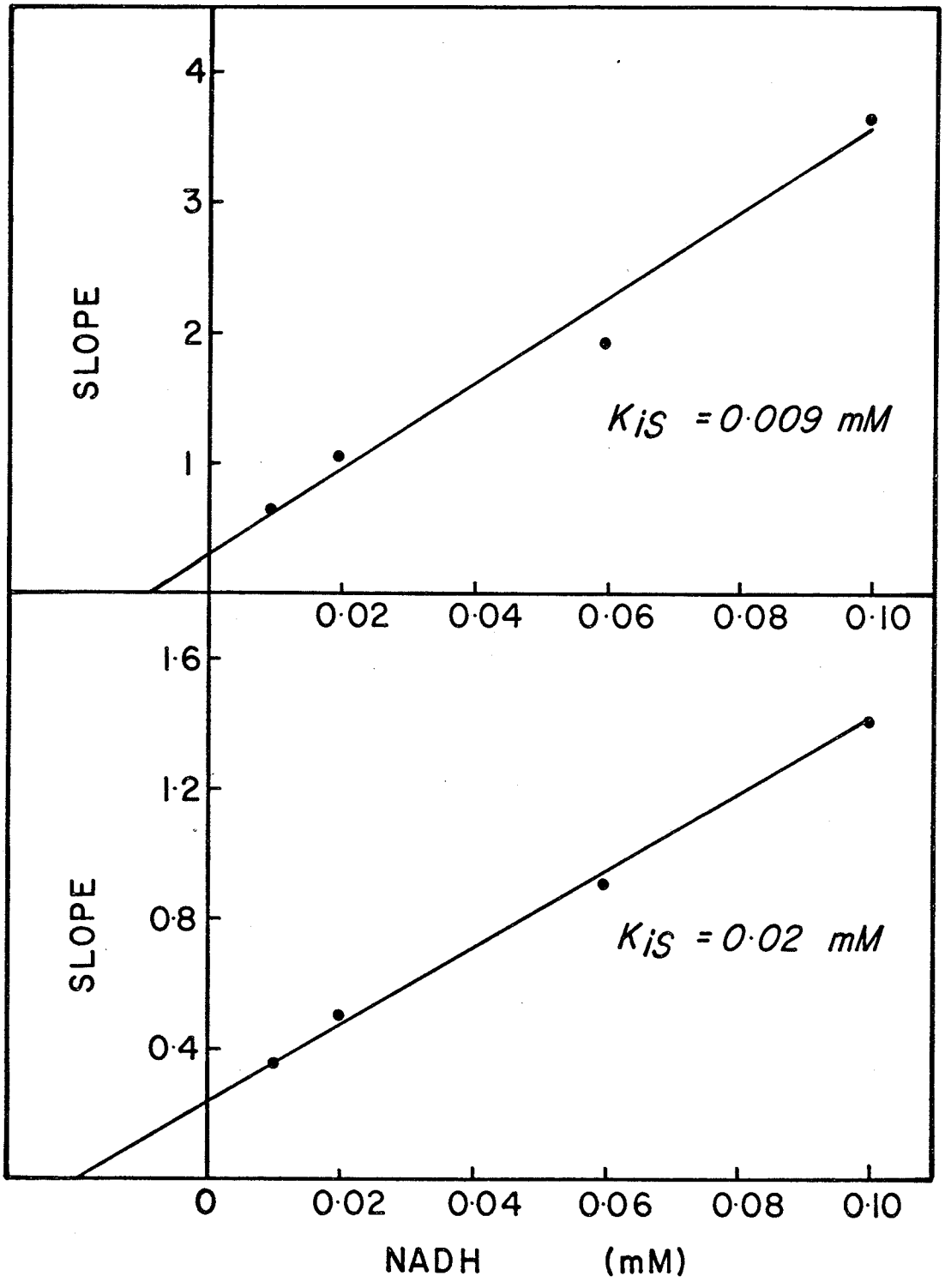

SLOPE
4
3
2
1
K_{is} = 0·009 mM
0·02
0·04
0·06
0·08
0·10
SLOPE
1·6
1·2
0·8
0·4
K_{is} = 0·02 mM
0
0·02
0·04
0·06
0·08
0·10
NADH (mM)

Figure 23. Double reciprocal plot showing product inhibition of FDH by NADH with varying concentrations of formate in the presence of non-saturating (0.03 mM) NAD^+.

A = no NADH

B = 0.01 mM NADH

C = 0.03 mM NADH

D = 0.06 mM NADH

E = 0.10 mM NADH

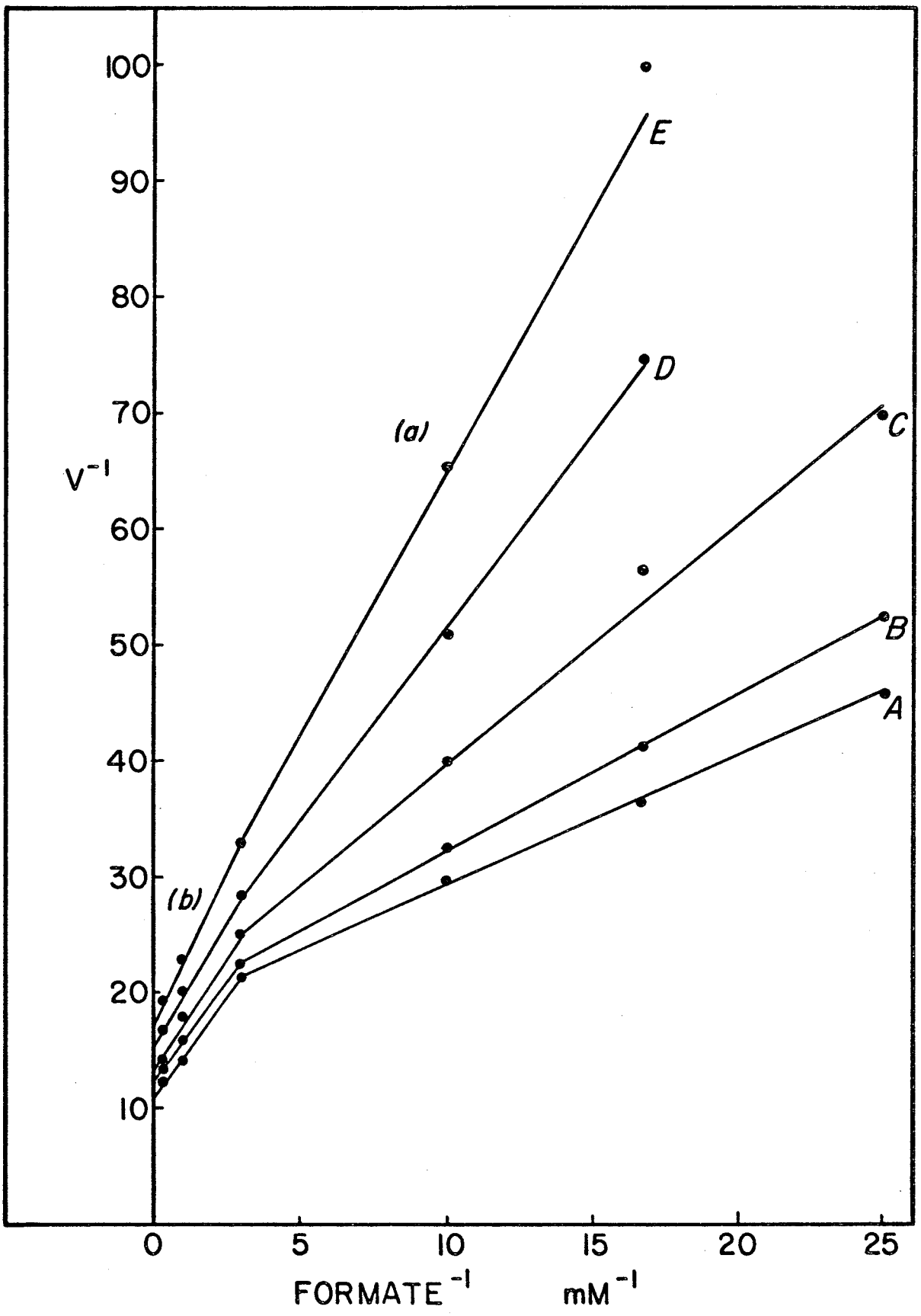

100
90
80
70
60
50
40
30
20
10
V⁻¹
(a)
(b)
E
D
C
B
A
0
5
10
15
20
25
FORMATE⁻¹ mM⁻¹

Figure 24. Double reciprocal plot with the same conditions as Fig. 23 but in the presence of saturating (0.3 mM) NAD^+.

A = no NADH

B = 0.03 mM NADH

C = 0.06 mM NADH

D = 0.10 mM NADH

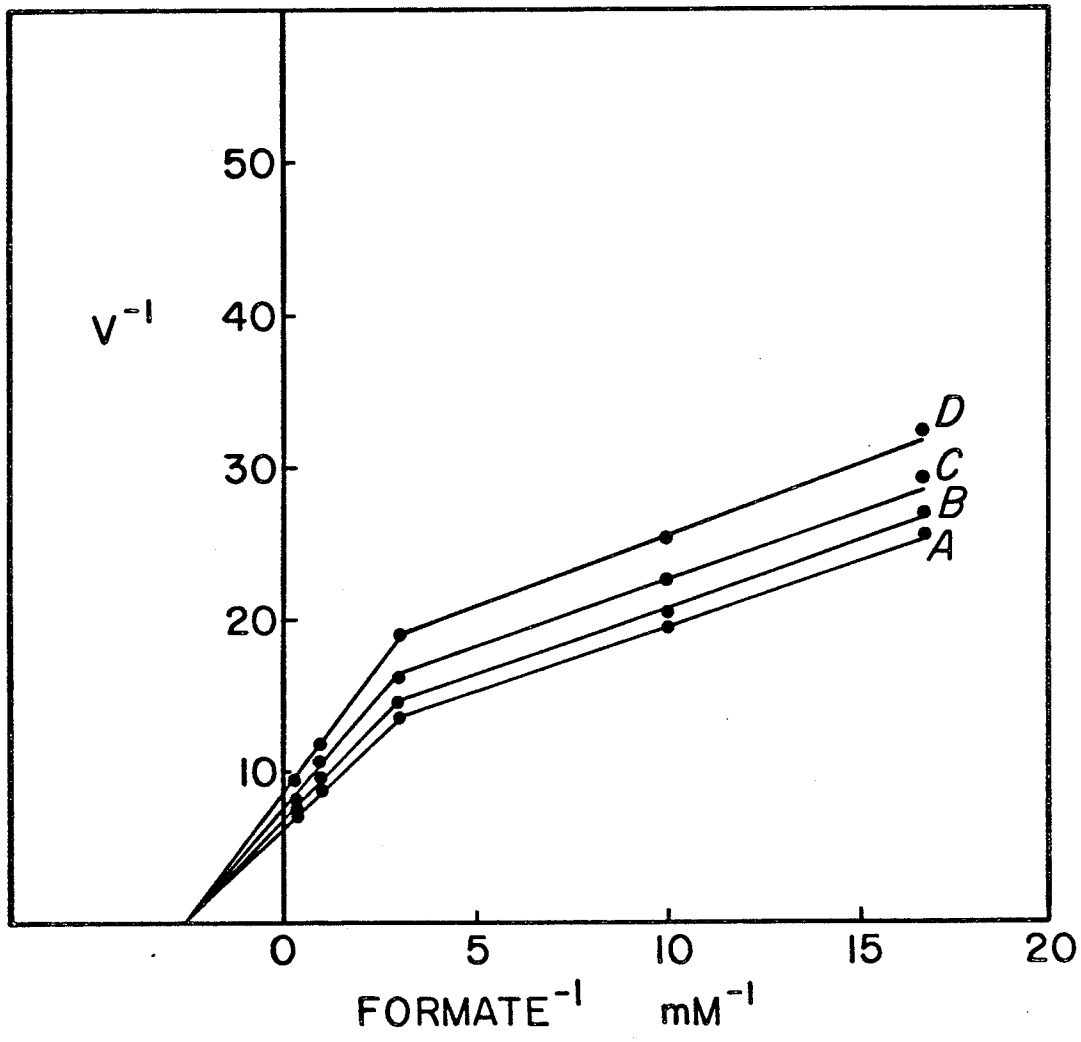

50
40
30
20
10
V^{-1}
D
C
B
A
0
5
10
15
20
FORMATE^{-1} mM^{-1}

Figure 25. Replots of intercept and slope values from the "low" formate segment in Fig. 23 against NADH concentration.

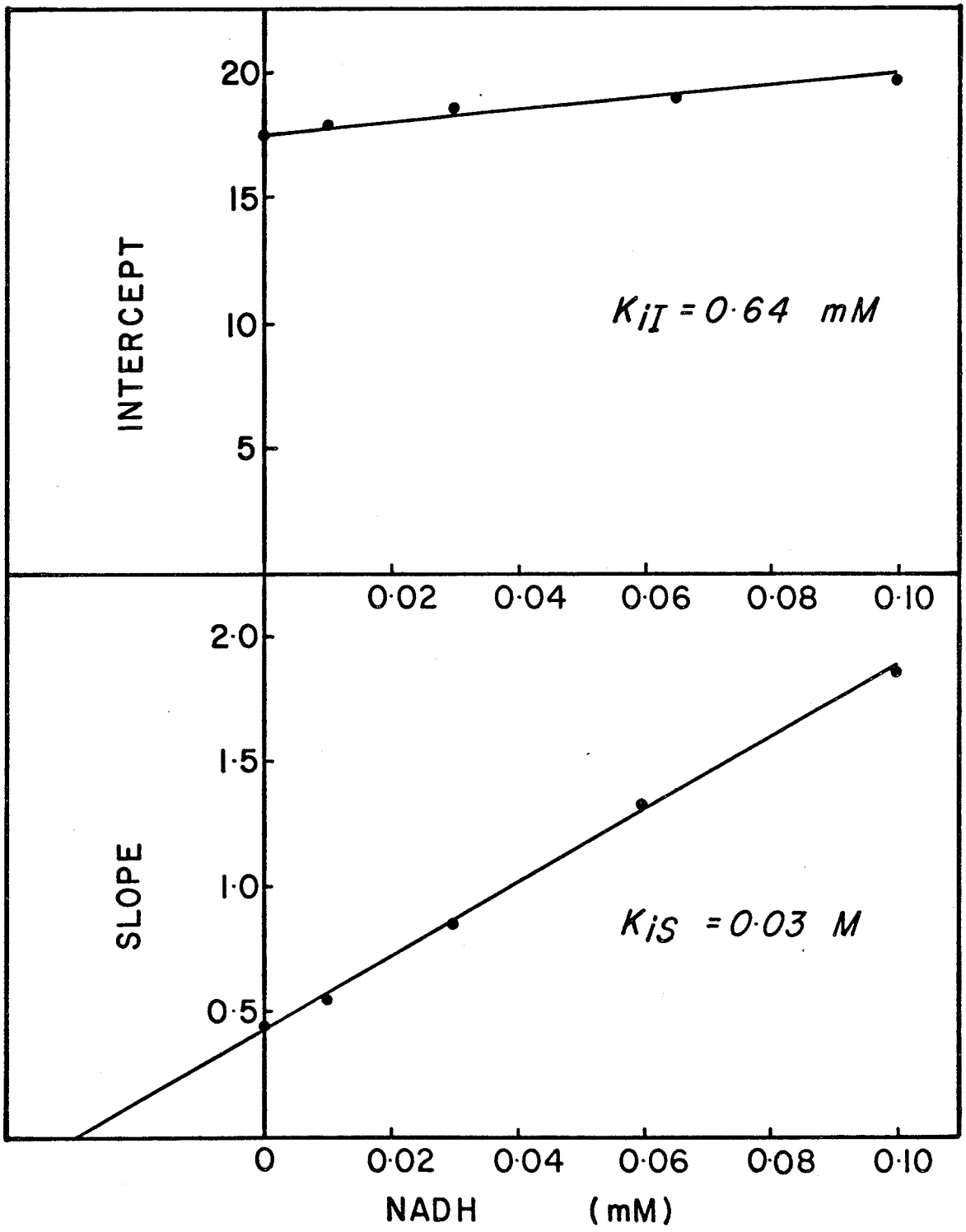

INTERCEPT
20
15
10
5
K_{iI} = 0·64 mM
0·02
0·04
0·06
0·08
0·10
SLOPE
2·0
1·5
1·0
0·5
K_{iS} = 0·03 M
0
0·02
0·04
0·06
0·08
0·10
NADH (mM)

Figure 26. Replots of intercept and slope values from the "high" formate segment in Fig. 23 against NADH concentration.

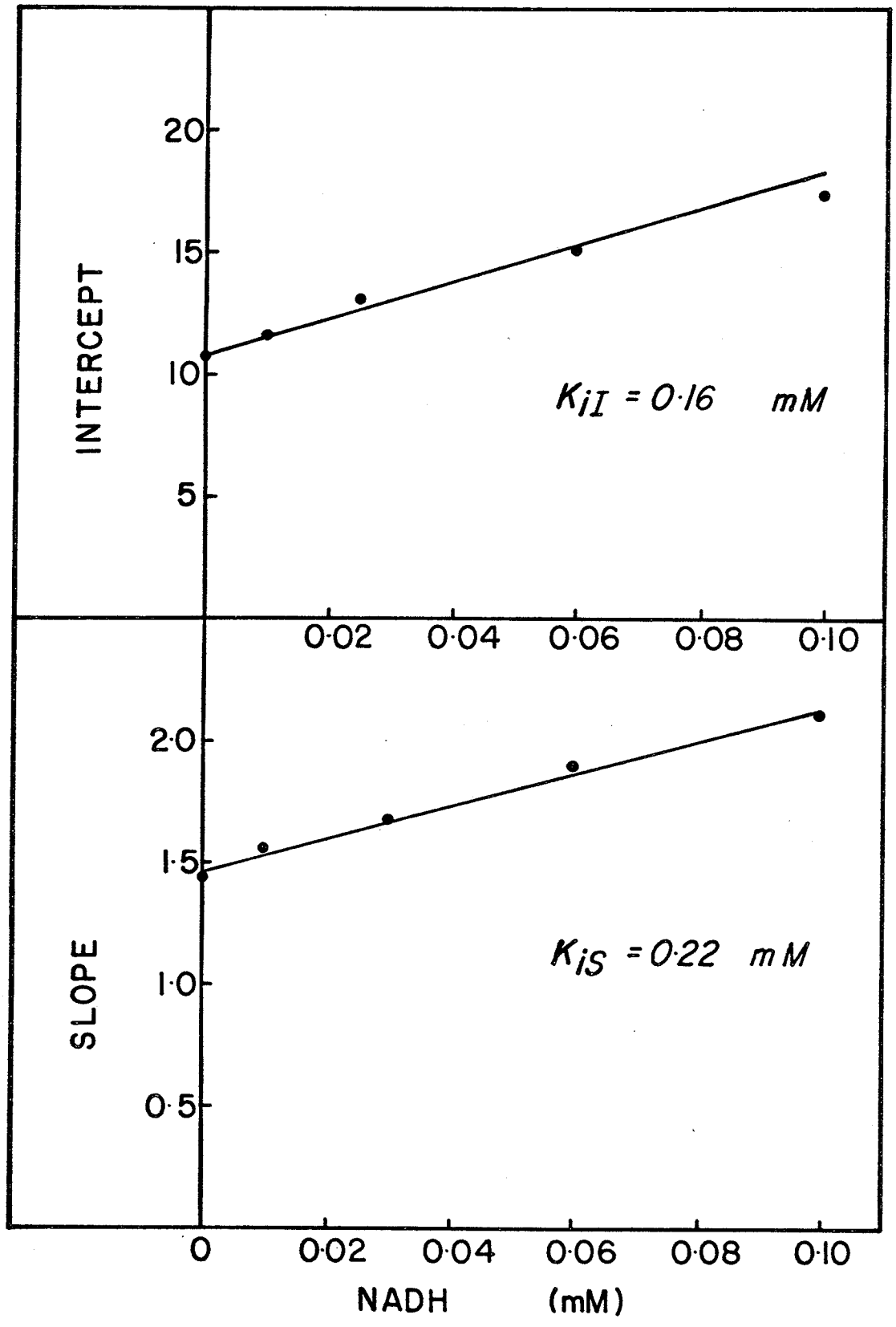

INTERCEPT
20
15
10
5
K_{iI} = 0·16 mM
0·02
0·04
0·06
0·08
0·10
SLOPE
2·0
1·5
1·0
0·5
K_{iS} = 0·22 mM
0
0·02
0·04
0·06
0·08
0·10
NADH (mM)

Figure 27. (i) Replot of intercept values from the "low" formate segment in Fig. 24 against NADH concentration.

(ii) and (iii) Replots of intercept and slope values from the "high" formate segment in Fig. 24 against NADH concentration.

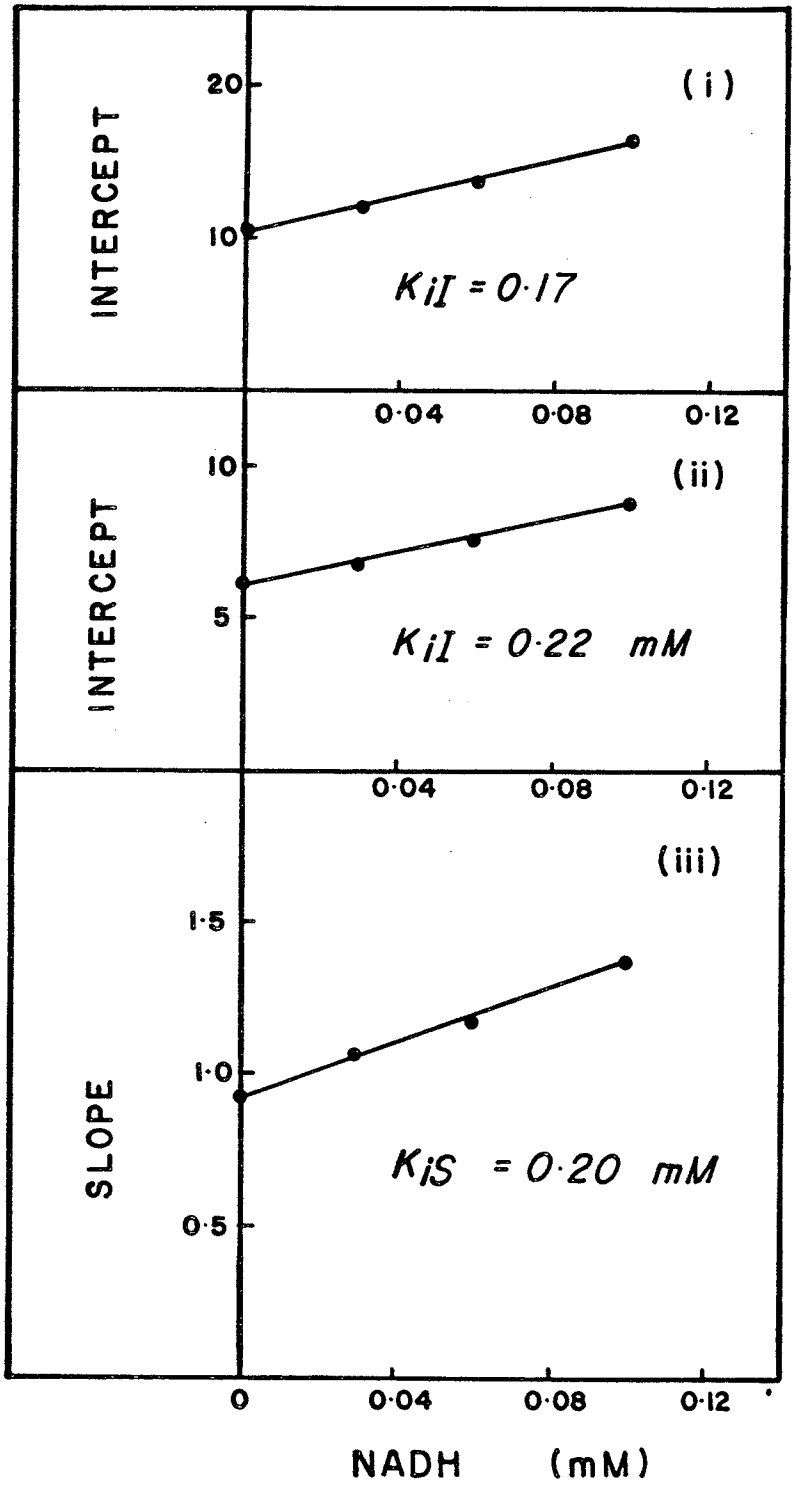

INTERCEPT
20
10
(i)
K_{iI} = 0·17
0·04
0·08
0·12
INTERCEPT
10
5
(ii)
K_{iI} = 0·22 mM
0·04
0·08
0·12
(iii)
SLOPE
1·5
1·0
0·5
K_{iS} = 0·20 mM
0
0·04
0·08
0·12
NADH (mM)

Figure 28. Double reciprocal plot showing product inhibition of FDH by bicarbonate with varying concentrations of NAD^+ in the presence of non-saturating (0.1 mM) levels of formate.

A = control (no added HCO_3^-)

B = 0.05 mM HCO_3^-

C = 0.1 M HCO_3^-

D = 0.2 M HCO_3^-

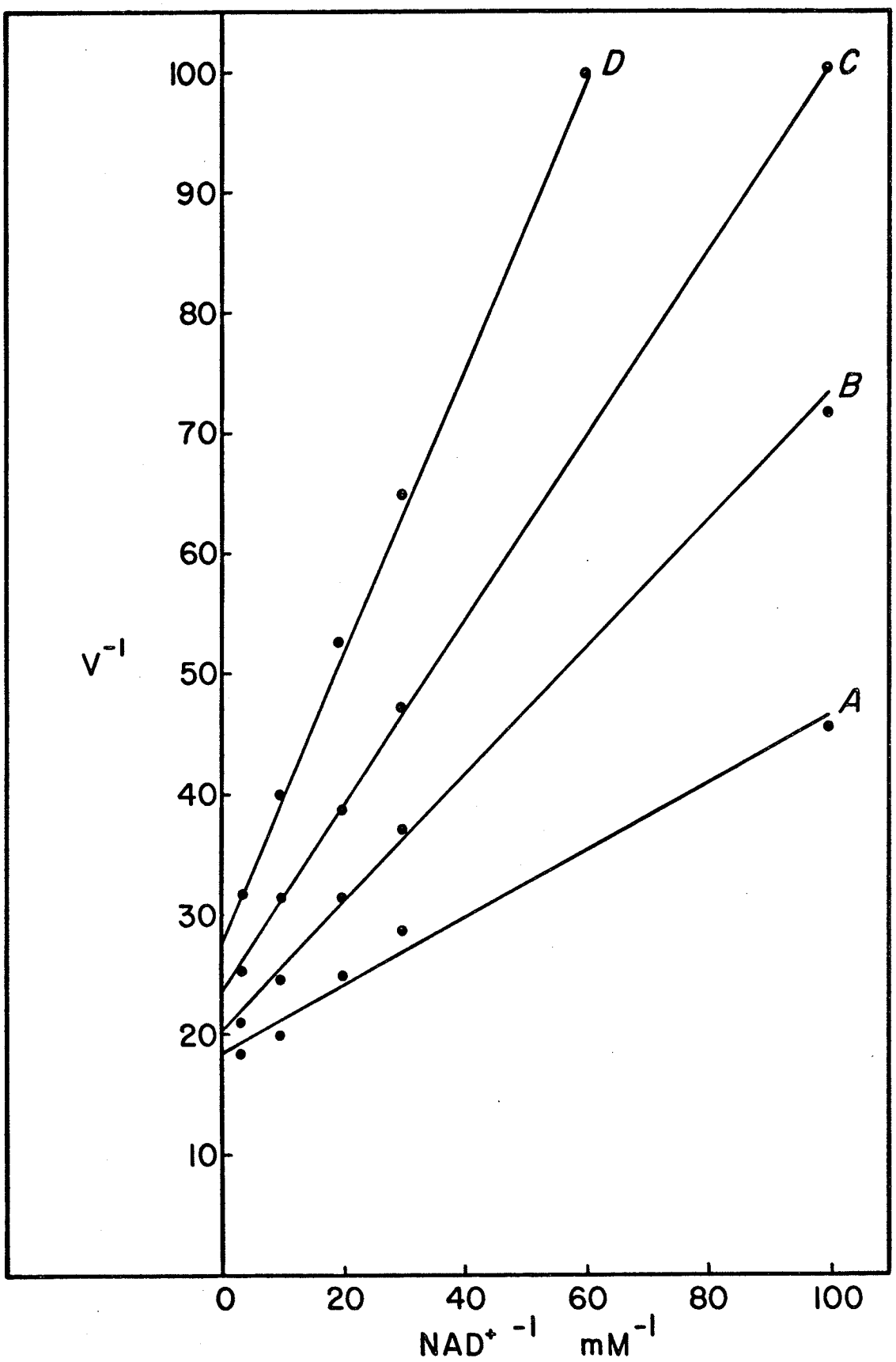

100
90
80
70
60
50
40
30
20
10
V⁻¹
0
20
40
60
80
100
NAD+ -1 mM-1
D
C
B
A

Figure 29. Replots of the slope and intercept values in Fig. 28 against bicarbonate concentrations.

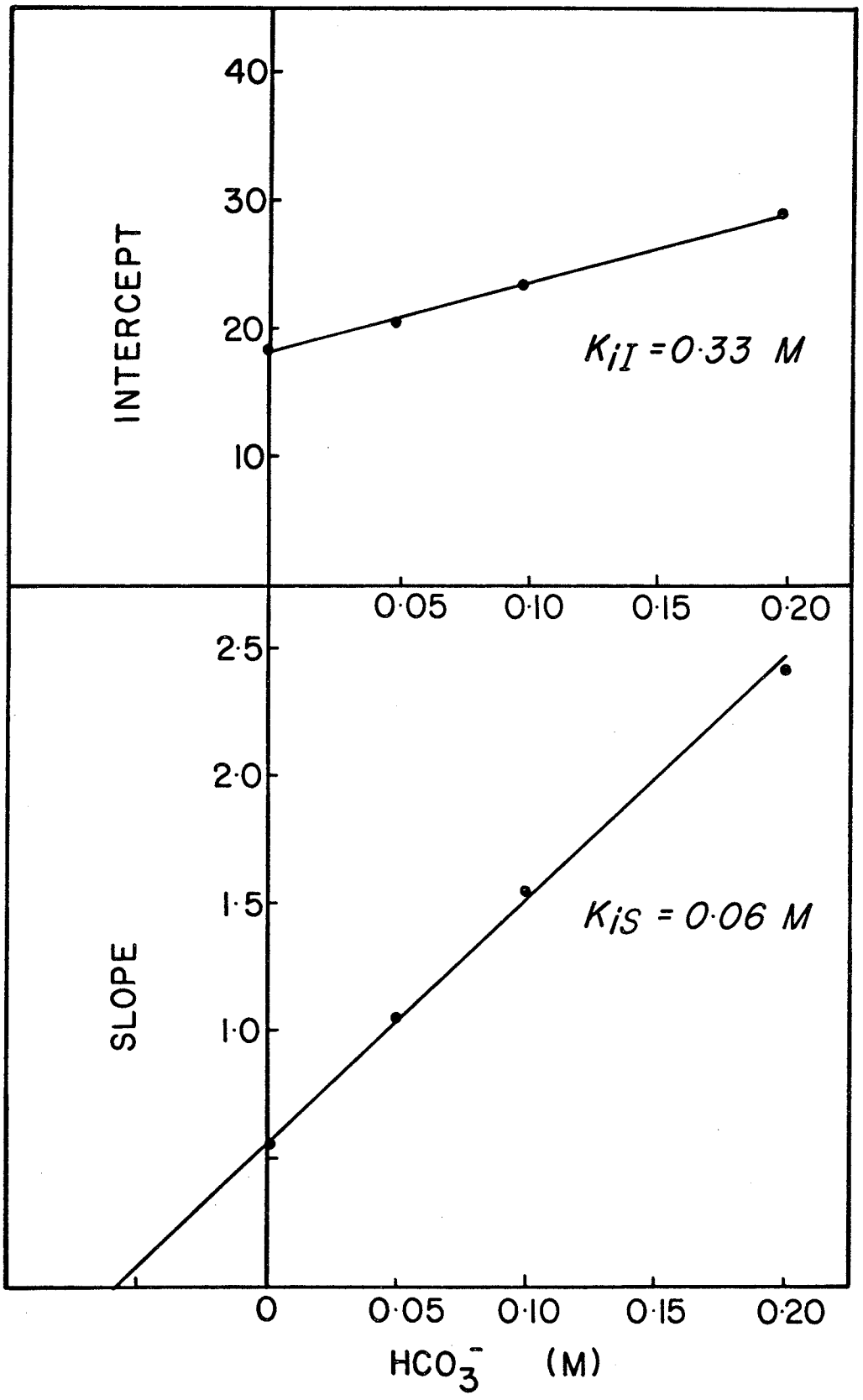

INTERCEPT
40
30
20
10
K_{iI} = 0·33 M
0·05
0·10
0·15
0·20
SLOPE
2·5
2·0
1·5
1·0
K_{iS} = 0·06 M
0
0·05
0·10
0·15
0·20
HCO_3^- (M)

Figure 30. Double reciprocal plot showing product inhibition of FDH by bicarbonate with varying concentrations of NAD^+ in the presence of saturating (3.3 mM) levels of formate.

A = control (no added HCO_3^-)

B = 0.05 M HCO_3^-

C = 0.1 M HCO_3^-

D = 0.2 M HCO_3^-

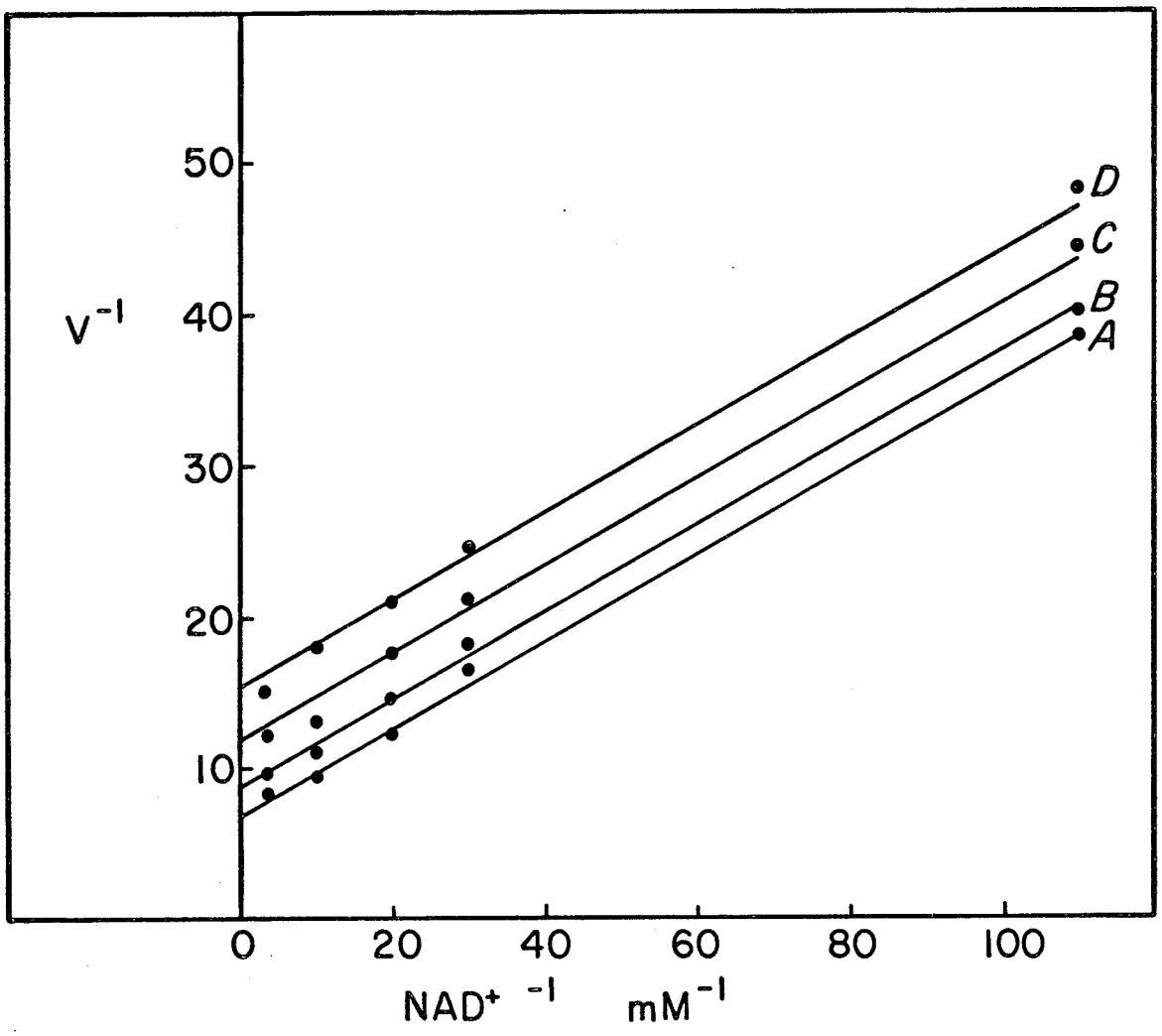

50
40
30
20
10
V⁻¹
D
C
B
A
0
20
40
60
80
100
NAD+ ⁻¹ mM⁻¹

Figure 31. Replot of intercept values from Fig. 30 against bicarbonate concentration.

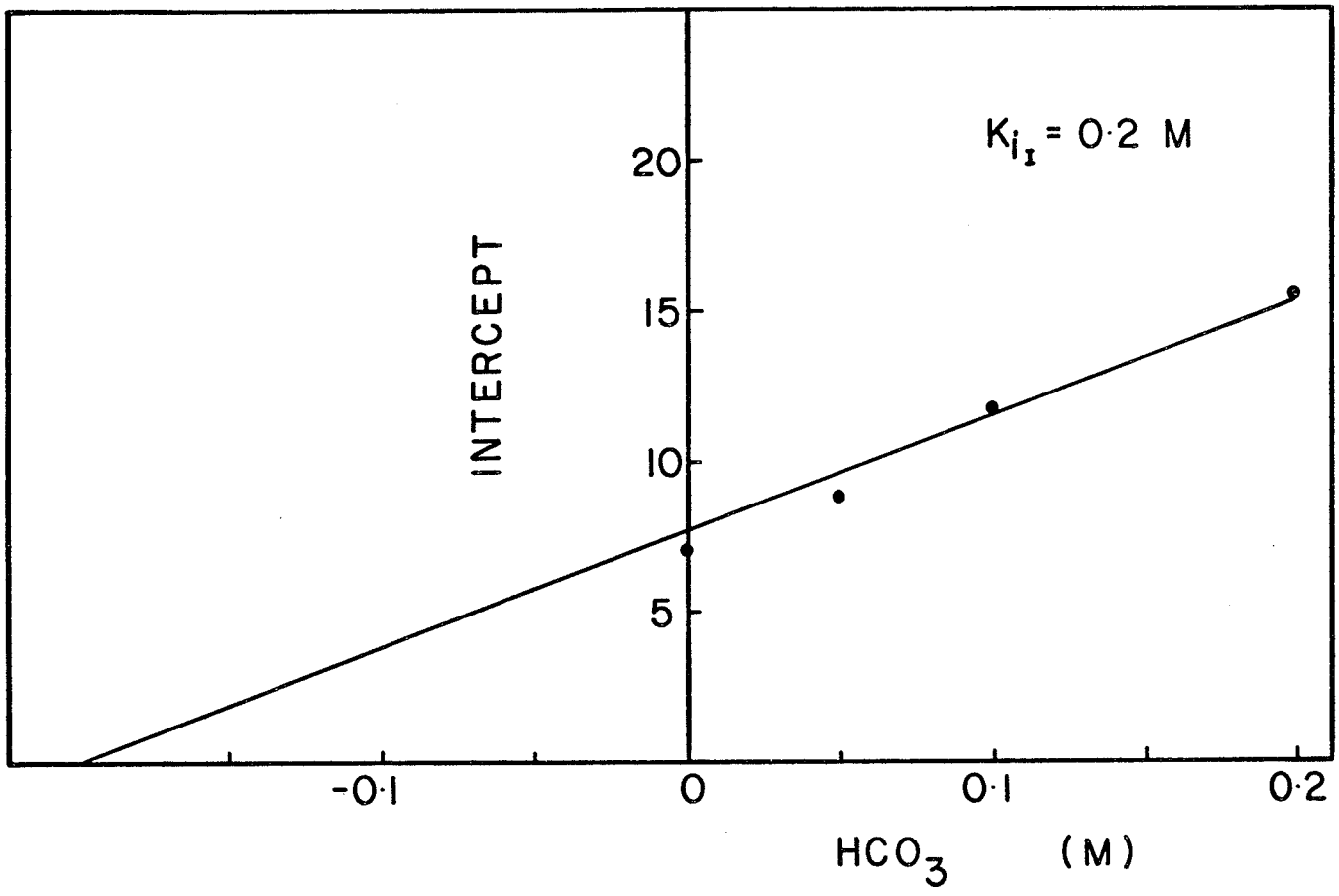

K_{i_I} = 0·2 M
INTERCEPT
20
15
10
5
-0·1
0
0·1
0·2
HCO_3 (M)

Figure 32. Double reciprocal plot of bicarbonate inhibition of FDH with varying concentrations of formate in the presence of non-saturating (0.03 mM) NAD^+.

A = control (no added HCO_3^-)

B = 0.05 M HCO_3^-

C = 0.1 M HCO_3^-

D = 0.2 M HCO_3^-

E = 0.3 M HCO_3^-

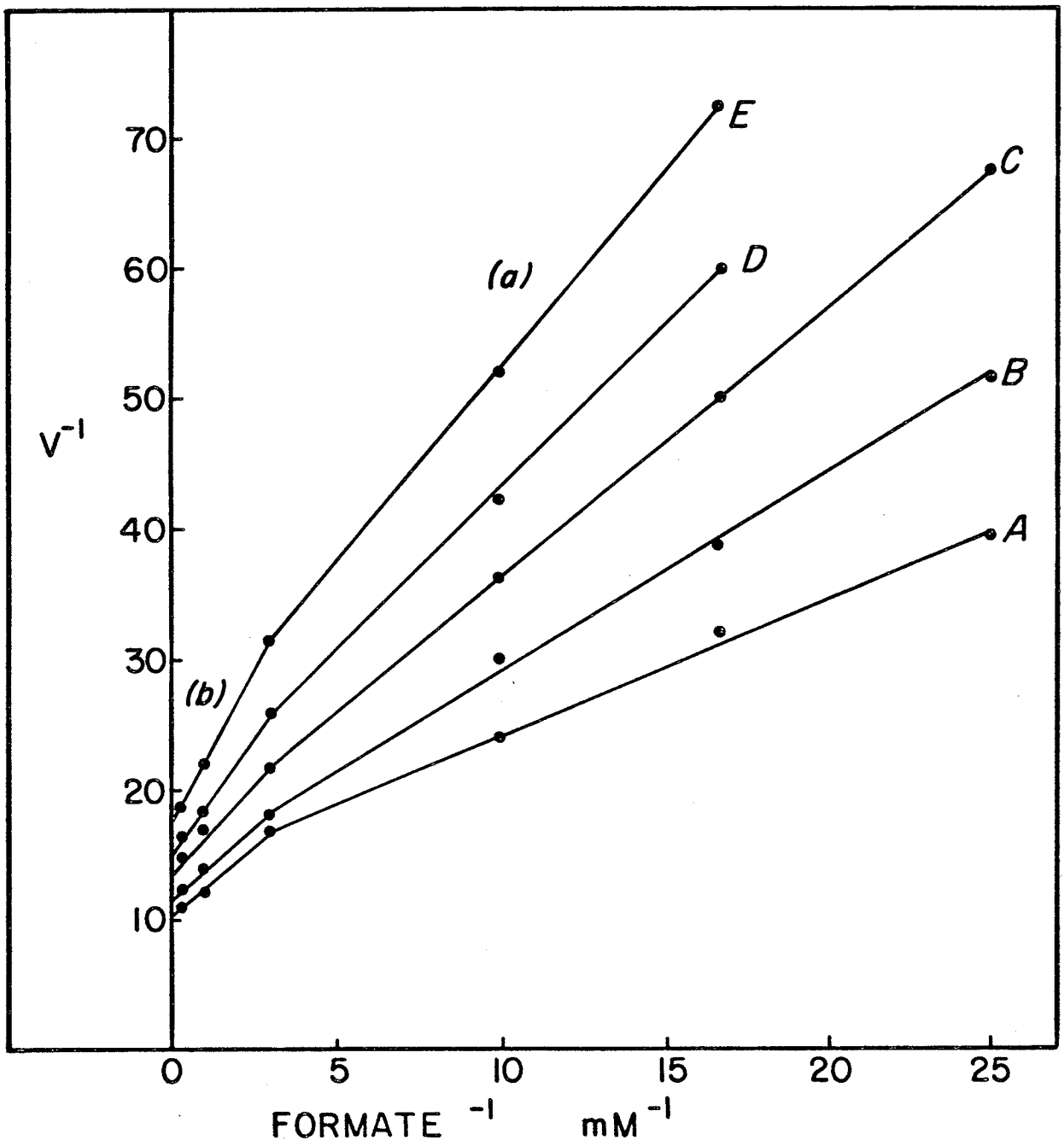

70
60
50
40
30
20
10
V⁻¹
(a)
(b)
E
D
C
B
A
0
5
10
15
20
25
FORMATE ⁻¹ mM⁻¹

Figure 33. Double reciprocal plot of bicarbonate inhibition of FDH with varying concentrations of formate in the presence of saturating (0.3 mM) NAD^+.

A = control (no added HCO_3^-)

B = 0.05 M HCO_3^-

C = 0.1 M HCO_3^-

D = 0.2 M HCO_3^-

E = 0.3 M HCO_3^-

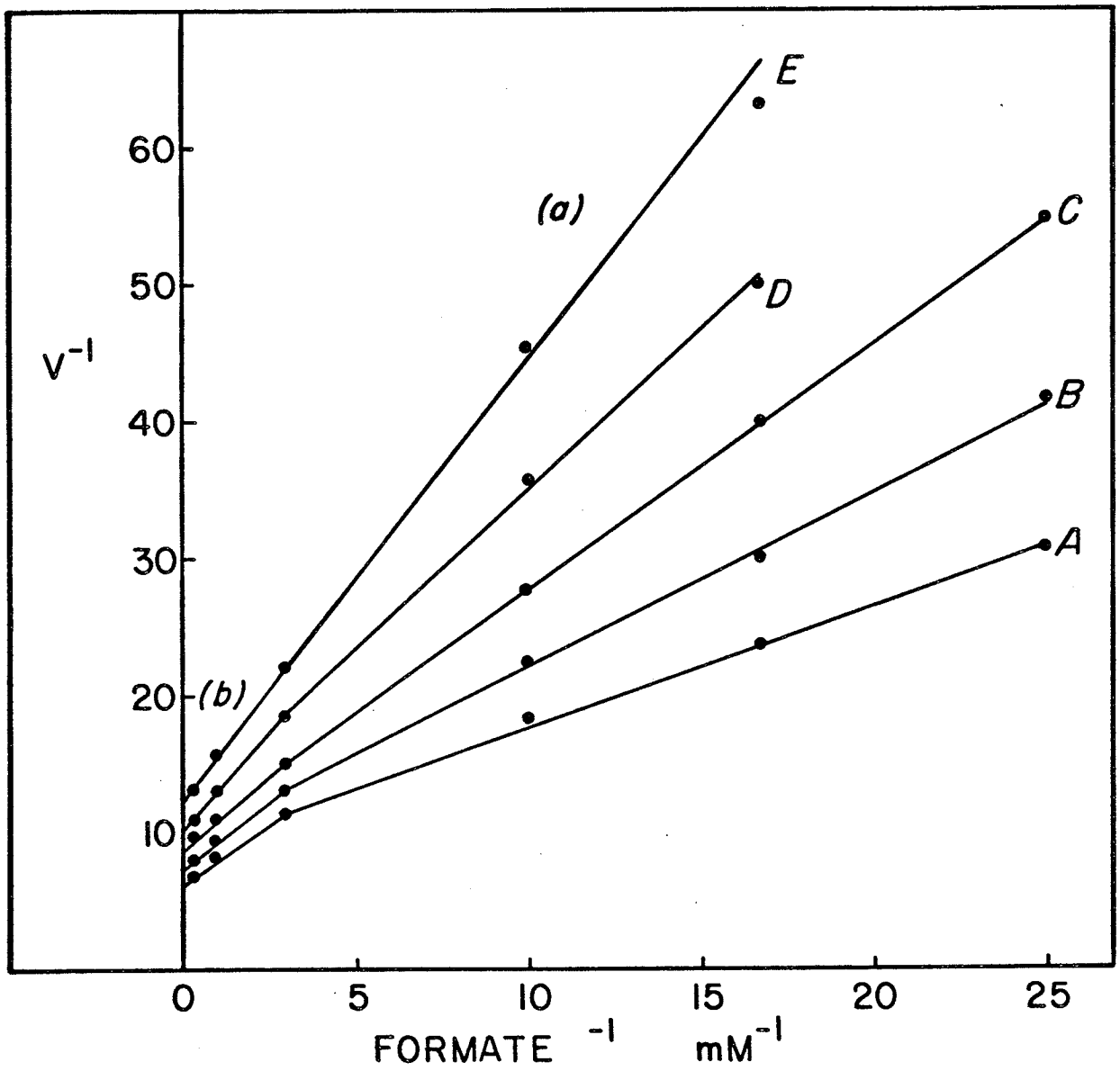

E
C
D
B
A
(a)
(b)
60
50
40
30
20
10
V⁻¹
0
5
10
15
20
25
FORMATE ⁻¹ mM⁻¹

Figure 34. Replots of intercept and slope values in the "low" formate segment (a) of Fig. 32. against bicarbonate concentration.

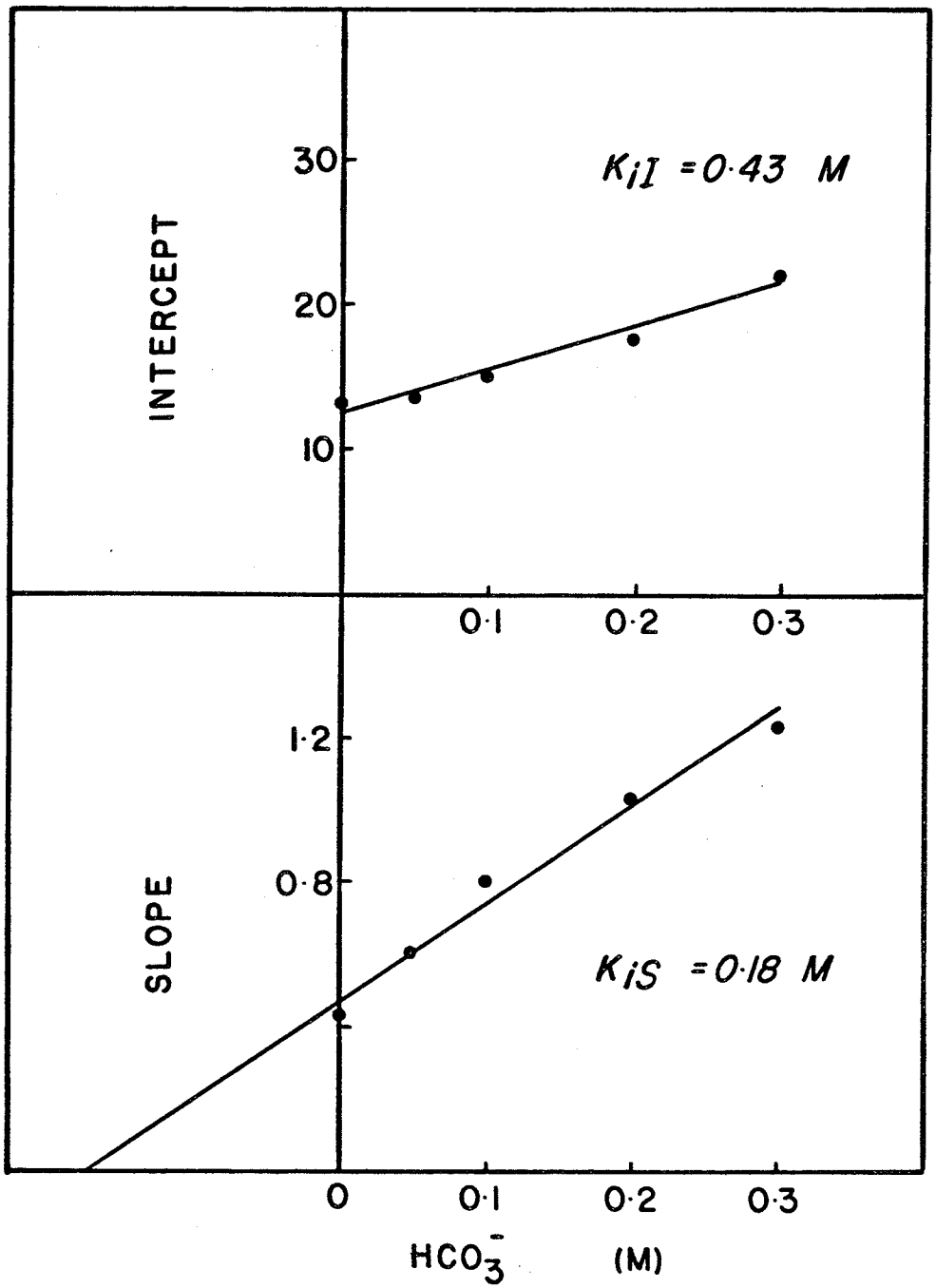

INTERCEPT
30
20
10
K_{iI} = 0·43 M
0·1
0·2
0·3
SLOPE
1·2
0·8
K_{iS} = 0·18 M
0
0·1
0·2
0·3
HCO_3^- (M)

Figure 35. Replots of intercept and slope values in the "high" formate segment (b) of Fig. 32 against bicarbonate concentration.

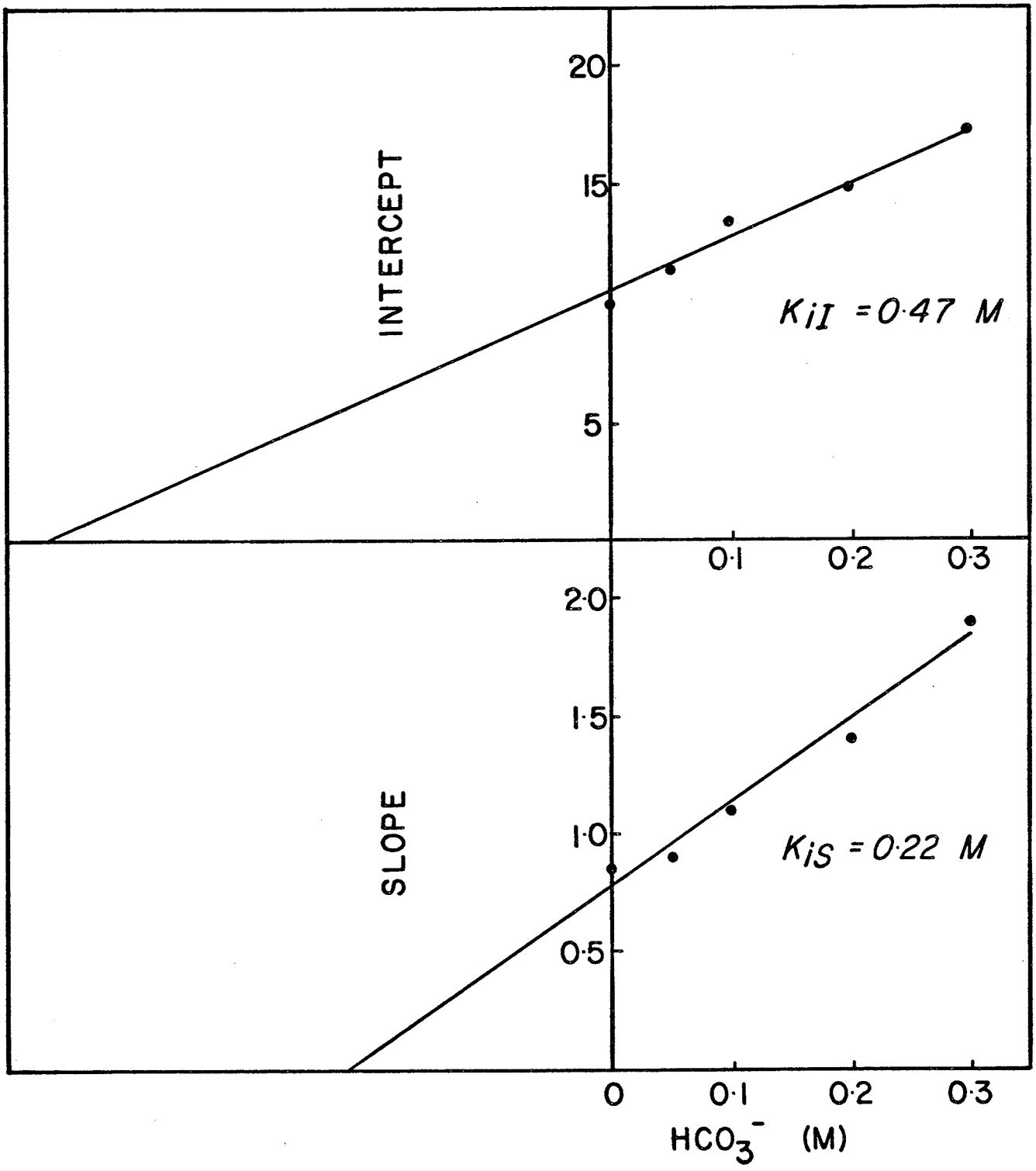

INTERCEPT
20
15
5
K_{iI} = 0·47 M
0·1
0·2
0·3
SLOPE
2·0
1·5
1·0
0·5
K_{iS} = 0·22 M
0
0·1
0·2
0·3
HCO_3^- (M)

Figure 36. Replots of intercept and slope values in the "low" formate segment (a) of Fig. 33 against bicarbonate concentration.

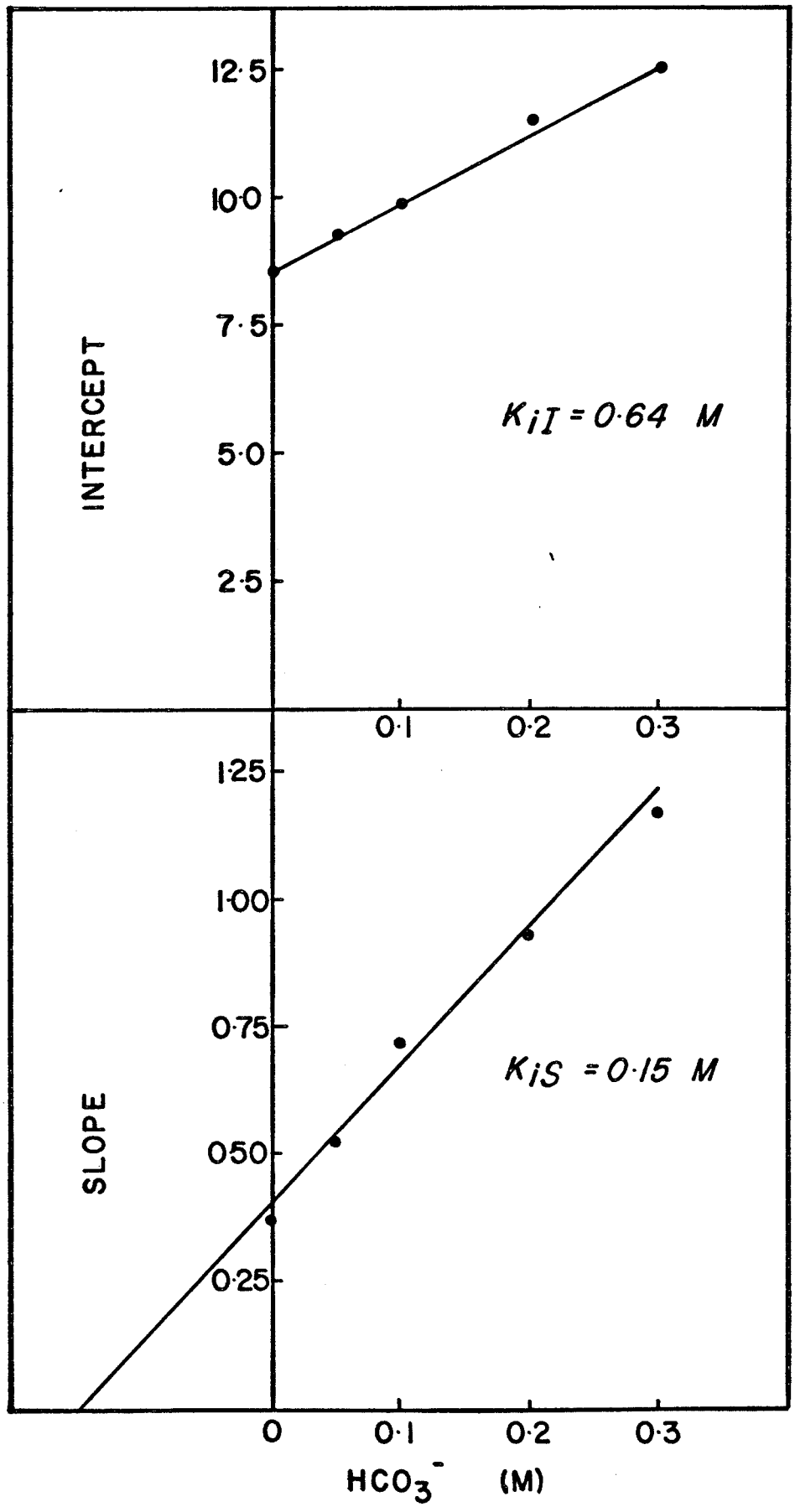

12·5
10·0
7·5
5·0
2·5
INTERCEPT
K_{iI} = 0·64 M
0·1
0·2
0·3
1·25
1·00
0·75
0·50
0·25
SLOPE
K_{iS} = 0·15 M
0
0·1
0·2
0·3
HCO_3^- (M)

Figure 37. Replots of intercept and slope values in the "high" formate segment (b) of Fig. 33 against bicarbonate concentration.

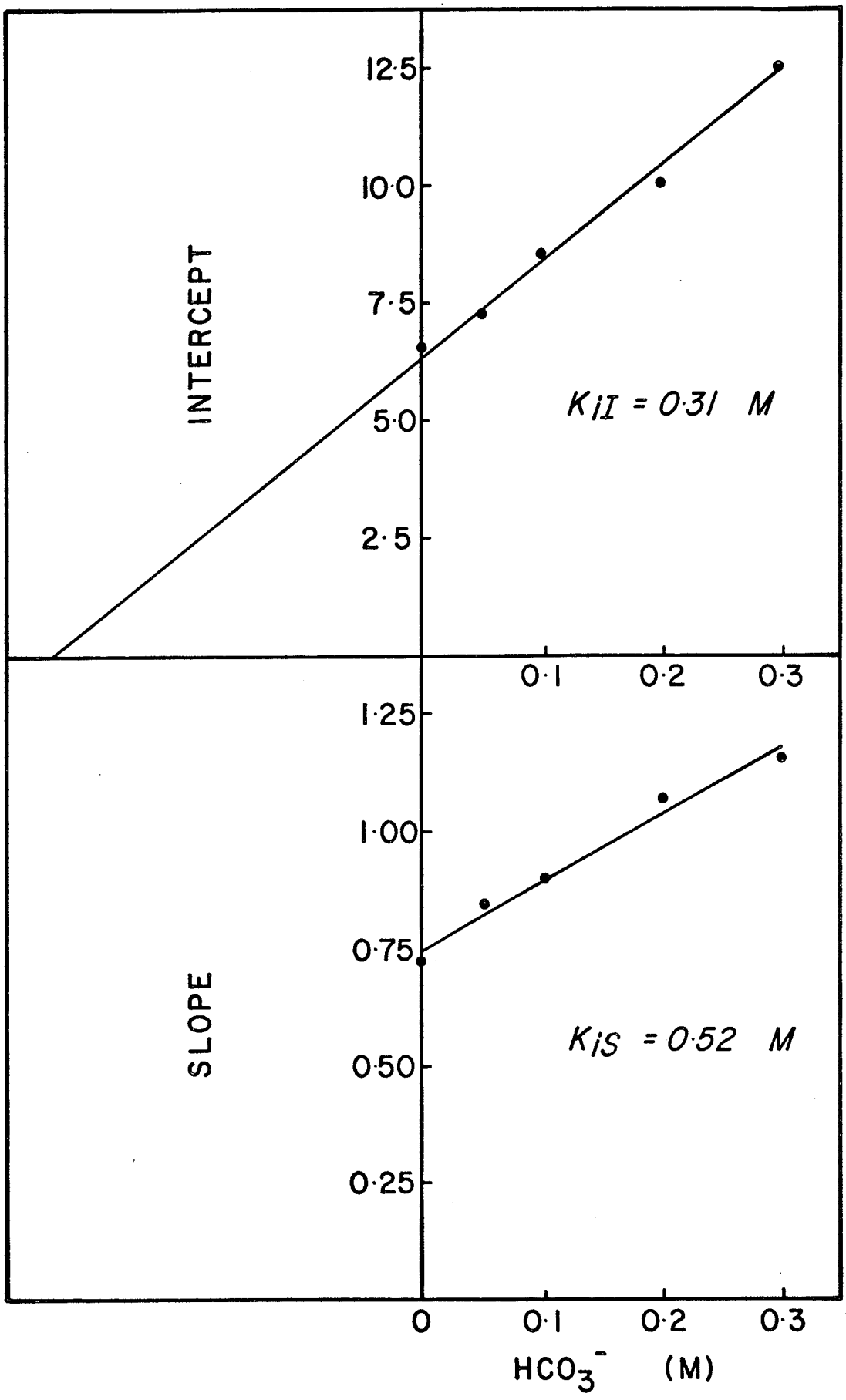

12·5
10·0
7·5
5·0
2·5
INTERCEPT
K_{iI} = 0·31 M
0·1
0·2
0·3
1·25
1·00
0·75
0·50
0·25
SLOPE
K_{iS} = 0·52 M
0
0·1
0·2
0·3
HCO_3^- (M)

Figure 38. Double reciprocal plots of initial velocities with fixed concentrations of formate against varying concentrations of NAD^+ at pH 5.6.

A = 0.05 mM formate

B = 0.2 mM formate

C = 0.6 mM formate

D = 1.0 mM formate

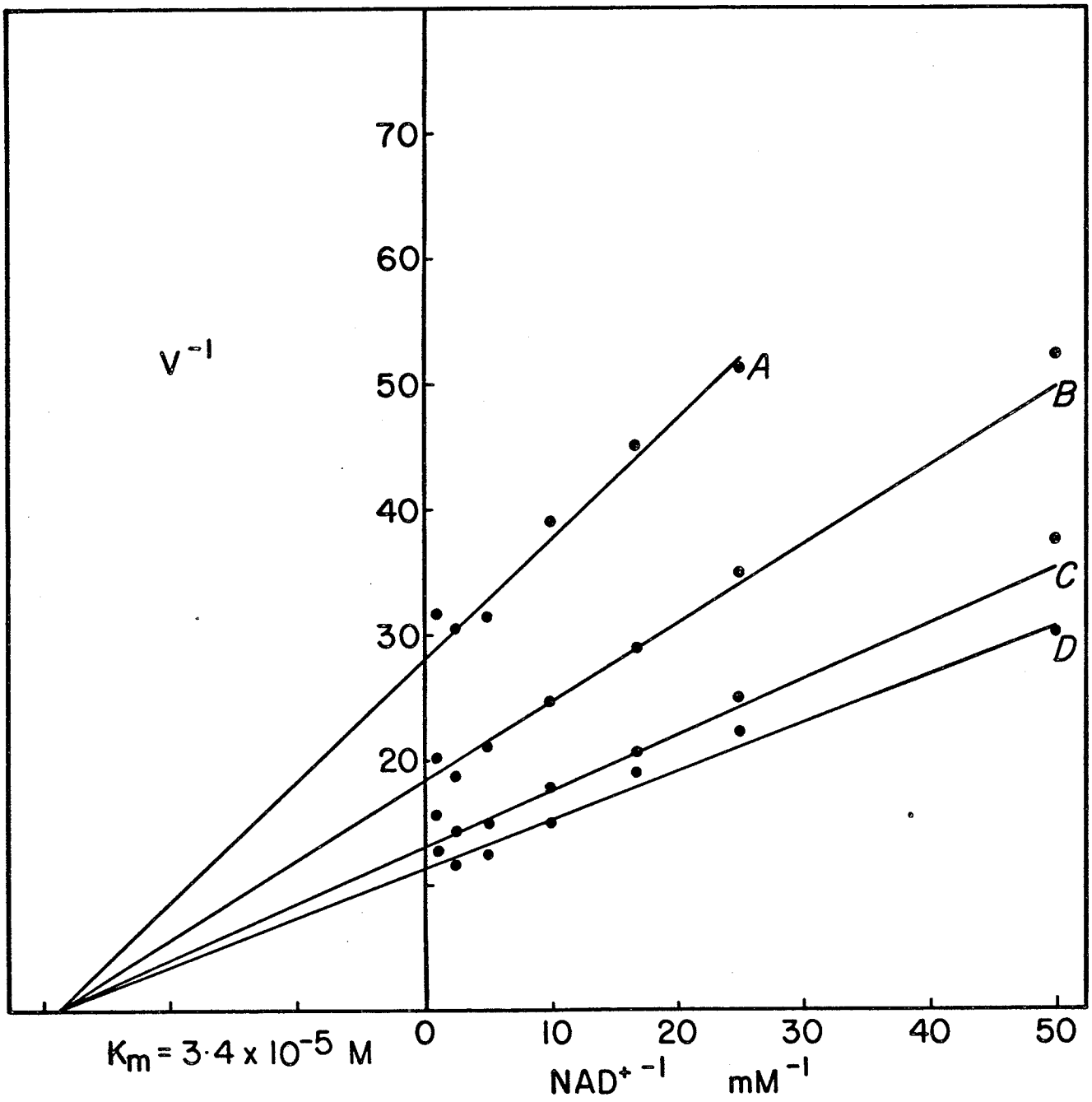

70
60
V^{-1}
50
A
B
40
C
30
D
20
0
10
20
30
40
50
$K_m = 3.4 \times 10^{-5}$ M
$NAD^+{}^{-1}$ mM^{-1}

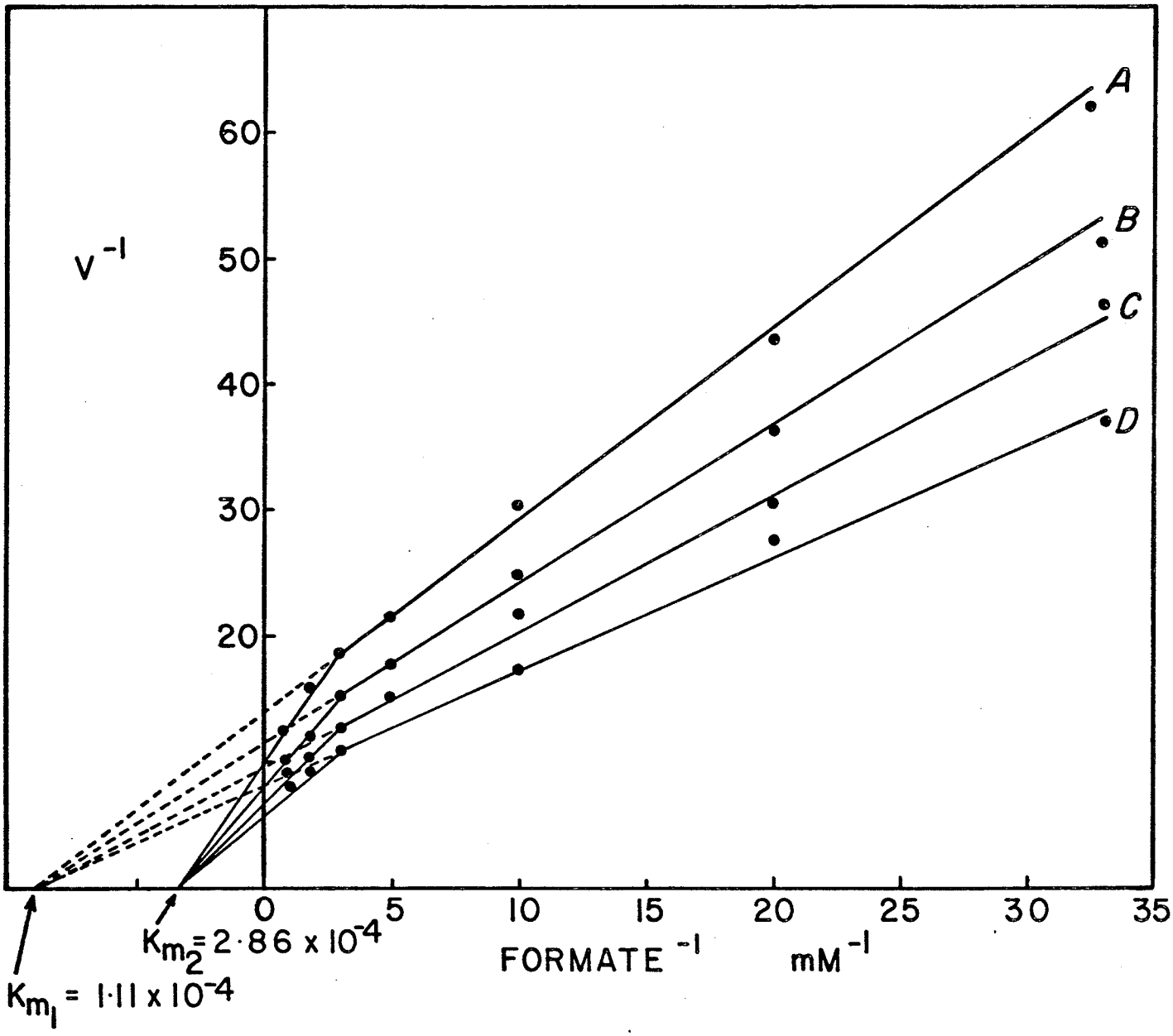
Figure 39. Double reciprocal plots of initial velocities with fixed concentrations of NAD^+ against varying concentrations of formate at pH 5.6.

A = 0.06 mM NAD^+

B = 0.10 mM NAD^+

C = 0.20 mM NAD^+

D = 0.60 mM NAD^+


A
B
C
D
60
50
40
30
20
V^{-1}
0
5
10
15
20
25
30
35
$K_{m_2} = 2 \cdot 86 \times 10^{-4}$
$K_{m_1} = 1 \cdot 11 \times 10^{-4}$
FORMATE $^{-1}$ mM $^{-1}$

were K_{m_1} = 0.11 mM formate and K_{m_2} = 0.29 mM formate. This is illustrated in Fig. 39. These K_m values were close to those obtained at pH 7.5. The replots of intercepts and slopes of Figs. 38 & 39 against the reciprocal of substrate concentration produced patterns (not shown) identical to those obtained at pH 7.5.

Inhibition by heavy metals

FDH was inhibited strongly by the divalent metal ions Hg^{++} and Cu^{++}, and to a lesser extend by Zn^{++} and Mn^{++} as shown in Table II. Ca^{++}, Mg^{++} and Cu^{+} did not inhibit the enzyme. Fig. 40 illustrates the Cu^{++} inhibition at both pH 7.5 and pH 5.6 and its reversal by DTT. FDH appears to possess essential thiol groups which are very readily attacked by divalent heavy metals since 0.03 mM Hg^{++} effected an inhibition of 80%. DTT at 1mM was sufficient to reverse the Cu^{++} (0.1 mM) inhibition but Hg^{++} (0.1 mM) inhibition required a concentration of 10 mM DTT. Other sulfhydryl inhibitors used were PCMB and iodoacetate. PCMB inhibited the enzyme activity completely at 0.1 mM concentrations but the inhibition by iodoacetate at the same concentration was only 28%. Both inhibitions were reversed by incubation with DTT. No titrations were undertaken using these inhibitors as the quantity of purified enzyme available at any given time was quite small.

Figure 40. Inhibition by Cupric ions and its reversal by DTT. FDH activity was measured spectrophotometrically as described in "Methods".

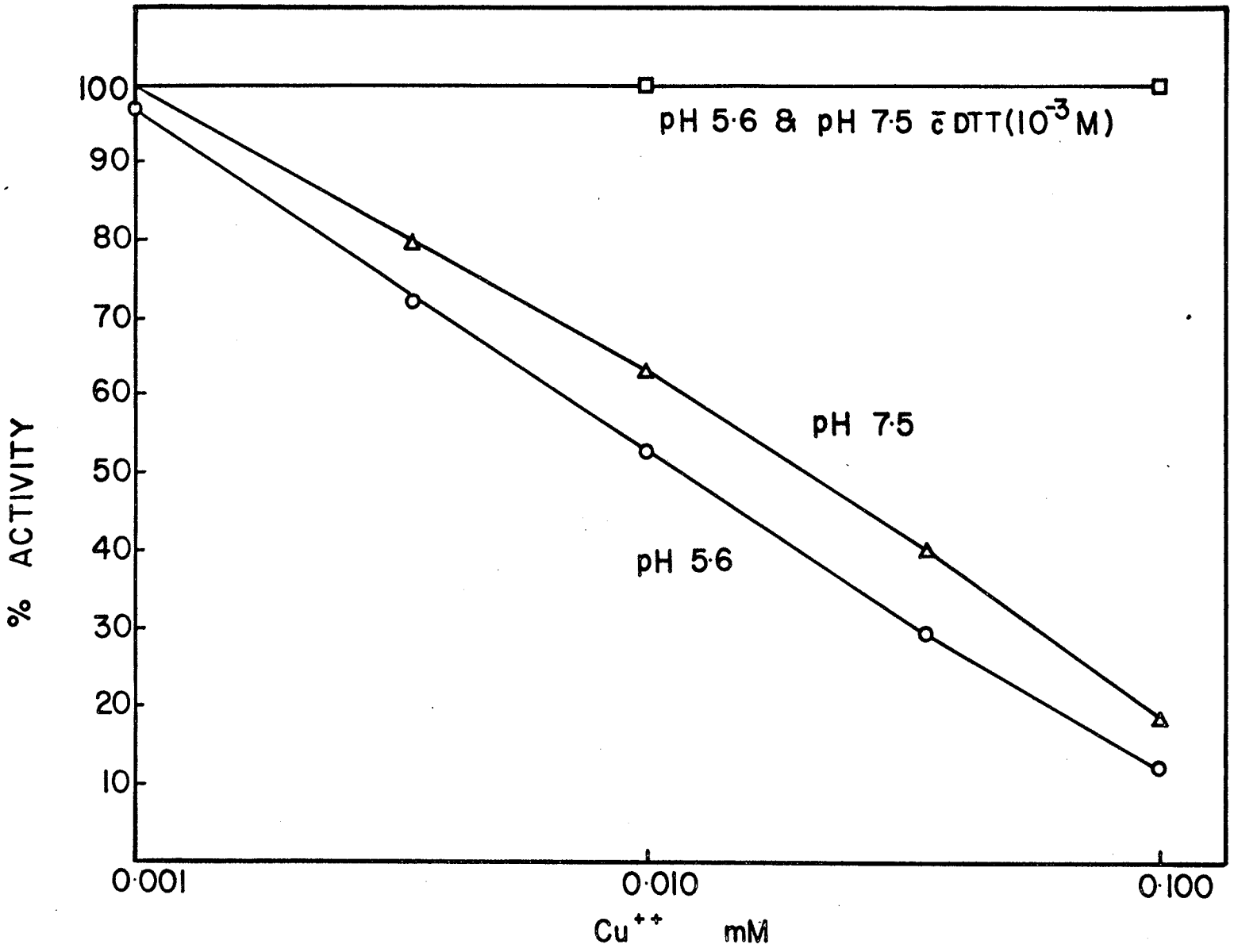

100
90
80
70
60
50
40
30
20
10
% ACTIVITY
pH 5·6 & pH 7·5 ē DTT(10^{-3} M)
pH 7·5
pH 5·6
0·001
0·010
0·100
Cu^{++} mM

TABLE II

Effect of divalent metal ions on FDH activity

Metal	Concentration	Activity (% of control)
Cu^{++} ($SO_4^{=}$)	0.002 mM	96
	0.005 mM	74
	0.03 mM	28
	0.3 mM	0
Hg^{++} (Cl^{-})	0.003 mM	80
	0.03 mM	20
	0.3 mM	0
Mn^{++} ($SO_4^{=}$)	3 mM	43
Zn^{++} ($SO_4^{=}$)	3 mM	40
Ca^{++} (Cl^{-})	3 mM	100
Mg^{++} ($SO_4^{=}$)	3 mM	100
Monovalent copper and sulfhydryl inhibitors		
Cu^{+} (Cl^{-})	0.1 mM	100
PCMB	0.1 mM	0
Iodoacetetic acid	0.1 mM	72

TABLE III

Effect of cyanide and other inhibitors on FDH activity

Inhibitor	NAD^+	Activity (% of control)
Potassium cyanide,		
0.03 mM	-	86
0.1 mM	-	65
0.1 mM	0.5 mM	52
1.0 mM	-	12
1.0 mM	0.5 mM	3
Sodium azide,		
0.1 mM	-	92
1.0 mM	-	55
1.0 mM	0.5 mM	63
10 mM	-	10
10 mM	0.5 mM	15
Bathocuproine sulfonate,		
0.1 mM	-	90
0.1 mM	0.5 mM	98
1.0 mM	-	48
1.0 mM	0.5 mM	60
Sodium sulfite,		
0.01 mM	-	100
0.1 mM	-	80
0.1 mM	0.5 mM	85
1.0 mM	-	76
1.0 mM	0.5 mM	83

Table III Continued.

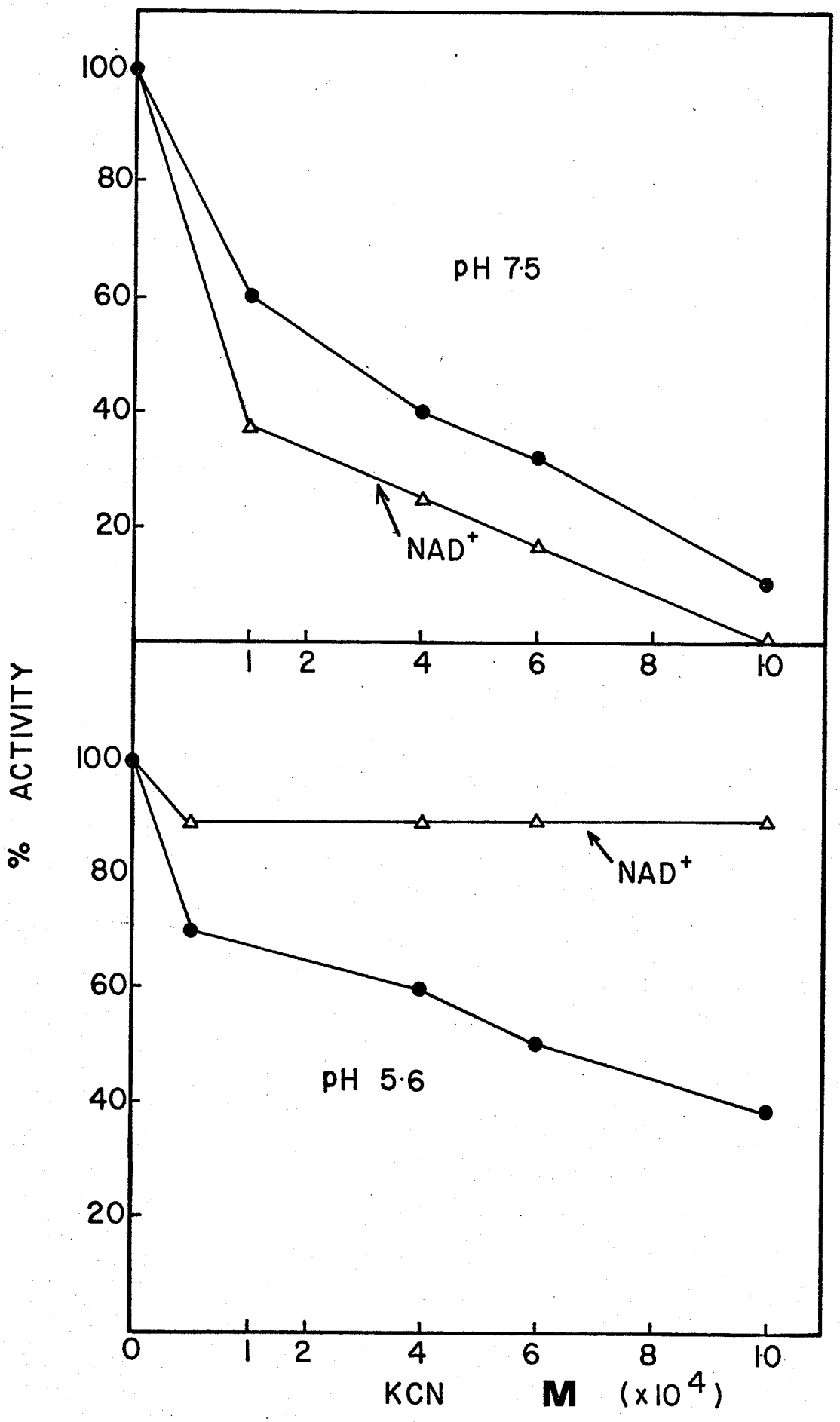
Inhibitor	NAD^+	Activity (% of control)
O-phenanthroline,		
0.1 mM	-	91
0.1 mM	0.5 mM	98
1.0 mM	-	85
1.0 mM	0.5 mM	92
Sodium diethyldithiocarbamate,		
1.0 mM	-	89
1.0 mM	0.5 mM	92
α,α' dipyridyl,		
1.0 mM	-	0
Sodium sulfide,		
0.3 mM	-	98
0.3 mM	0.5 mM	98
3.0 mM	-	97
Allyl thiourea,		
0.1 mM	-	93
0.1 mM	0.5 mM	93
1.0 mM	-	89
EDTA,		
1 mM	-	96
10 mM	-	89

Inhibitors were preincubated with the enzyme (50 µg protein) in the reaction cuvette at 25°C in the presence and absence of NAD^+ before addition of substrates to start the reaction (see "Methods").

Figure 41. Inhibition of FDH by cyanide and the effect of NAD^+, at pH 7.5 and pH 5.6.

FDH assayed after 2 minute pre-incubation with KCN.

NAD^+ (0.5 mM) added before pre-incubation with KCN.


100
80
60
40
20
pH 7·5
NAD+
1
2
4
6
8
10
% ACTIVITY
100
80
60
40
20
NAD+
pH 5·6
0
1
2
4
6
8
10
KCN M (x10^4)

Inhibition by cyanide and other inhibitors

The effect of cyanide and various other inhibitors on FDH was studied and the results are shown in Table III.

FDH activity was not affected much by the chelators of divalent metals, that is, α,α´ dipyridyl, sodium diethyldithiocarbamate, EDTA, o-phenanthroline and allyl thiourea, losing only less than 10% of activity with 1 mM inhibitor. However, bathocuproine sulfonate, a monovalent copper chelator, exerted somewhat stronger inhibition on FDH activity. These results seem to indicate that the enzyme does not possess essential divalent metals necessary for activity. A monovalent metal, like Cu^{+}, may be present however.

Sodium azide and particularly potassium cyanide were strong inhibitors of FDH. The inhibition by cyanide seemed worthy of further interest as the inclusion of NAD^{+} during preincubation of the enzyme with cyanide in the reaction cuvette enhanced, rather than diminished the inhibition. Also, incubation of FDH with 10 mM cyanide did not produce any inhibition of FDH if the enzyme was kept at its original concentration of approximately 4-5 mg per ml protein. The enzyme needed to be diluted from that concentration (1:100 in the experiments) and then preincubated in the presence of cyanide.

Cyanide and azide are the standard inhibitors of cytochrome oxidase. Sodium sulfide, an inhibitor of cytochrome oxidase, did not inhibit FDH at 3 mM. Carbon monoxide, when bubbled into diluted FDH in a cuvette for 45 seconds, reduced the activity by 30%. Since the same treatment of an undiluted FDH preparation also reduced the activity by 30%, the effect is unlike that of cyanide.

The effect of sodium sulfite was tested to see whether sulfite would form a complex with NAD^+, resulting in FDH inhibition. The result in Table III suggests that sulfite does not replace cyanide in its inhibitory effect.

The role of NAD^+ was investigated further by observing its effect on cyanide inhibition at pH 7.5 and 5.6. The data obtained are shown in Fig. 41. Whilst the presence of NAD^+ increased the inhibition by cyanide at pH 7.5, its presence at pH 5.6 produced a marked protection against cyanide inhibition.

Effect of Guanidine hydrochloride and Ureas

The effect of dissociating the enzyme FDH with guanidine hydrochloride and urea was investigated. Fig. 42 illustrates the effect of concentration of these two agents on enzyme activity. Guanidine hydrochloride was more effective than urea. At 3 M concentration, it caused total loss of activity whereas at

the same concentration of urea, slightly more than half the activity of the enzyme remained. The effect of time of exposure to guanidine hydrochloride is shown in Fig. 43 . With 1 M guanidine hydrochloride, 5 mM NAD^+ conferred some protection against the inactivation (presumably by dissociation) but at 3 M guanidine hydrochloride, it did not offer any protection. Two plots are shown in the figure for inactivation by 1 M guanidine hydrochloride. This is because a lag developed in the activity of FDH after 5 minutes exposure to the agent. The unbroken line measures the velocity achieved after the lag period. At 5 minutes there was a short lag of 30 seconds, this increased with exposure time. After 10 minutes exposure, the lag was 3.5 minutes, at 20 minutes exposure, it was 4 minutes and at 120 minutes, it took 11.5 minutes after the addition of the substrates to start the reaction before the velocity measured was achieved. However, after 15 minutes exposure, the maximum rate achieved after the lag remained undiminished up to 120 minutes of exposure. The broken line in the figure shows the activity measured immediately after addition of the substrates to start the enzyme reaction.

The effect of exposure to 6 M urea is illustrated in Figs. 44 & 45 . They also show the influence of NAD^+, formate and NADH on inactivation by urea. NAD^+

conferred increasing protection with increasing concentration. Formate caused an apparent increase in urea inactivation with increasing concentration. However this is due to the inactivation of FDH by formate itself, as mentioned earlier and shown in Fig. 9 . With NADH, some degree of protection is conferred. The lower concentration of 0.05 mM being more effective than the higher concentration of 0.5 mM.

Fig. 42. Loss of FDH activity by preincubation with 1 M Urea and Guanidine hydrochloride.

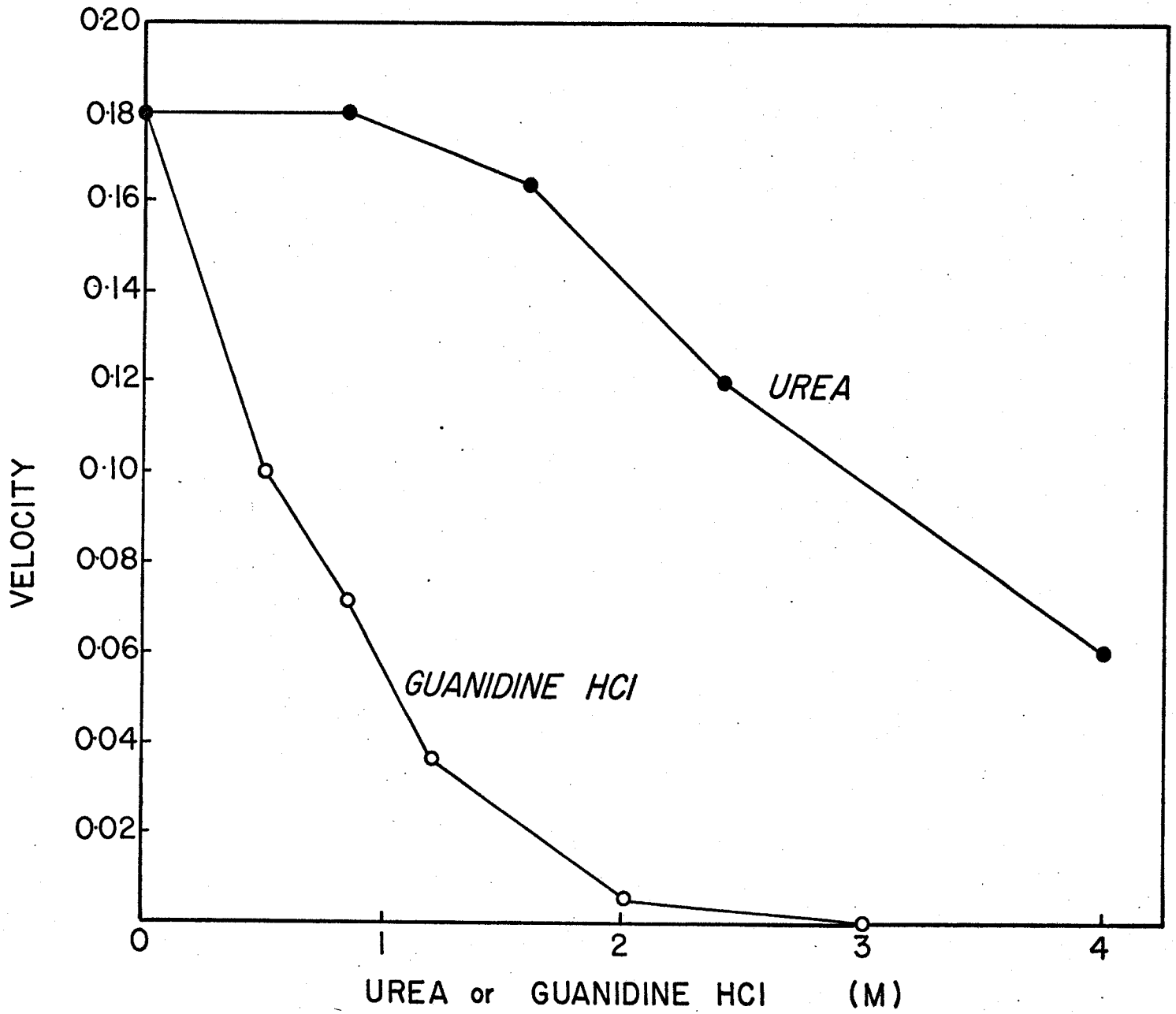

0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
VELOCITY
UREA
GUANIDINE HCl
0
1
2
3
4
UREA or GUANIDINE HCl (M)

Figure 43. Effect of NAD^+ on guanidine hydrochloride inactivation of FDH.

(——Δ——) FDH activity measured immediately after addition of substrates.

(—Δ—) FDH activity measured after end of lag period following addition of substrates. (See "Results")

(O) 5 mM NAD^+ added to FDH before incubation with 1 M guanidine HCl. Activity measured immediately after addition of substrates.

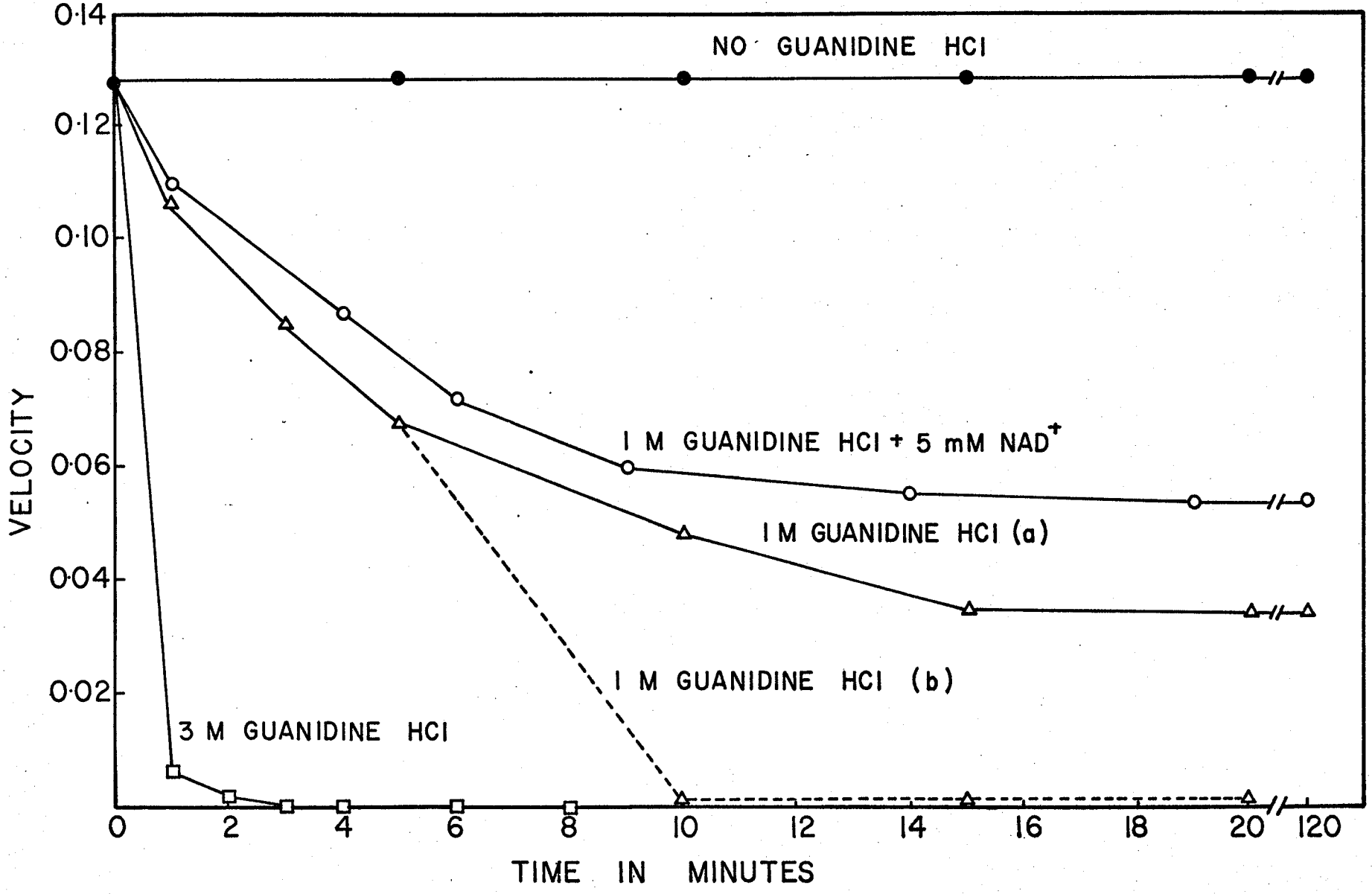

0·14
0·12
0·10
0·08
0·06
0·04
0·02
VELOCITY
NO GUANIDINE HCl
1 M GUANIDINE HCl + 5 mM NAD+
1 M GUANIDINE HCl (a)
1 M GUANIDINE HCl (b)
3 M GUANIDINE HCl
0
2
4
6
8
10
12
14
16
18
20
120
TIME IN MINUTES

Figure 44. Effect of NAD^+ and formate on inhibition of FDH by 6 M urea.

(■) 0.05 mM NAD^+ added to enzyme solution before incubation with urea.

(□) 0.5 mM NAD^+ added to enzyme solution before incubation with urea.

(○) 5 mM NAD^+ added to enzyme solution before incubation with urea.

(▲) 0.5 mM formate added to enzyme solution before incubation with urea.

(●) 5 mM formate added to enzyme solution before incubation with urea.

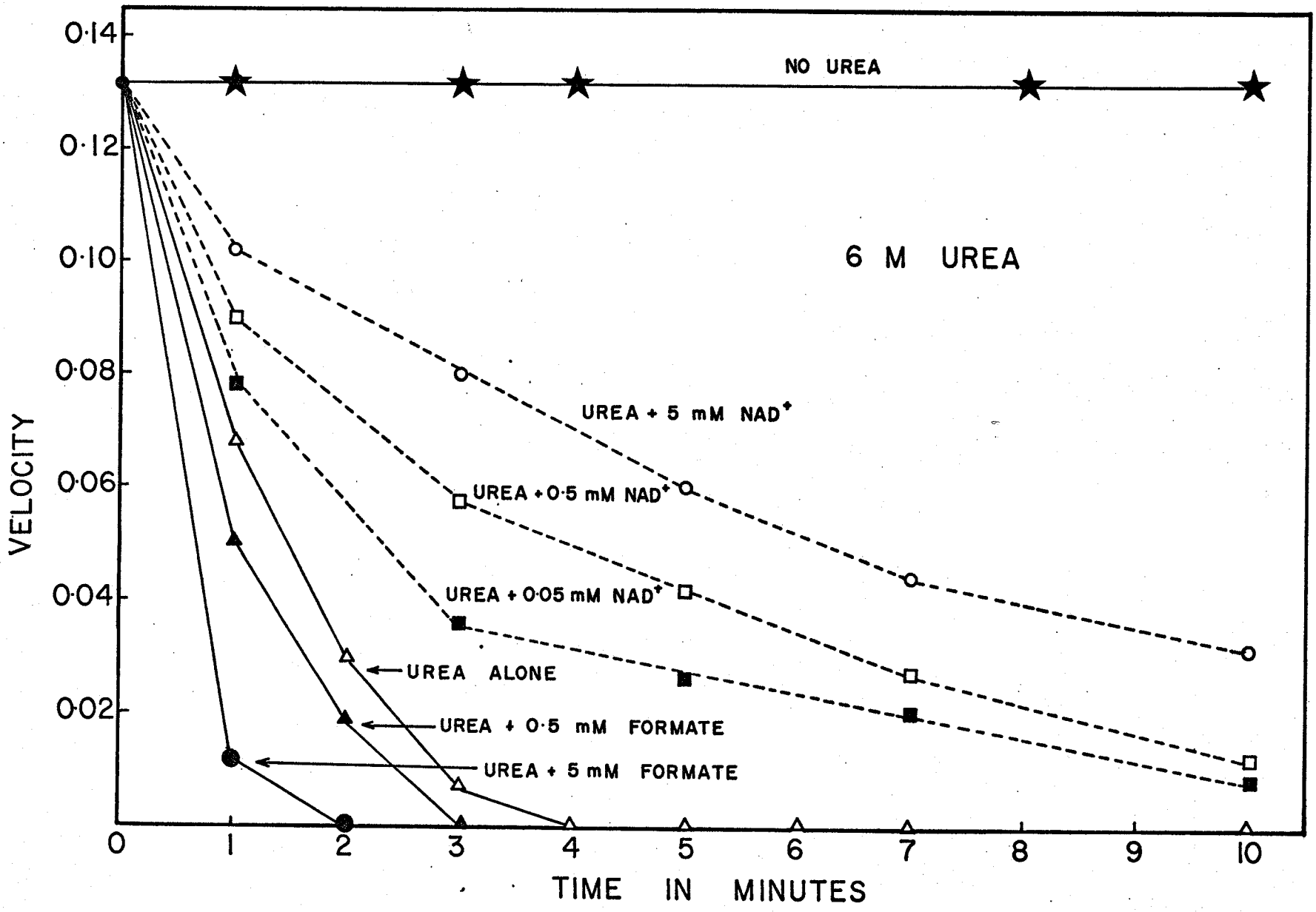
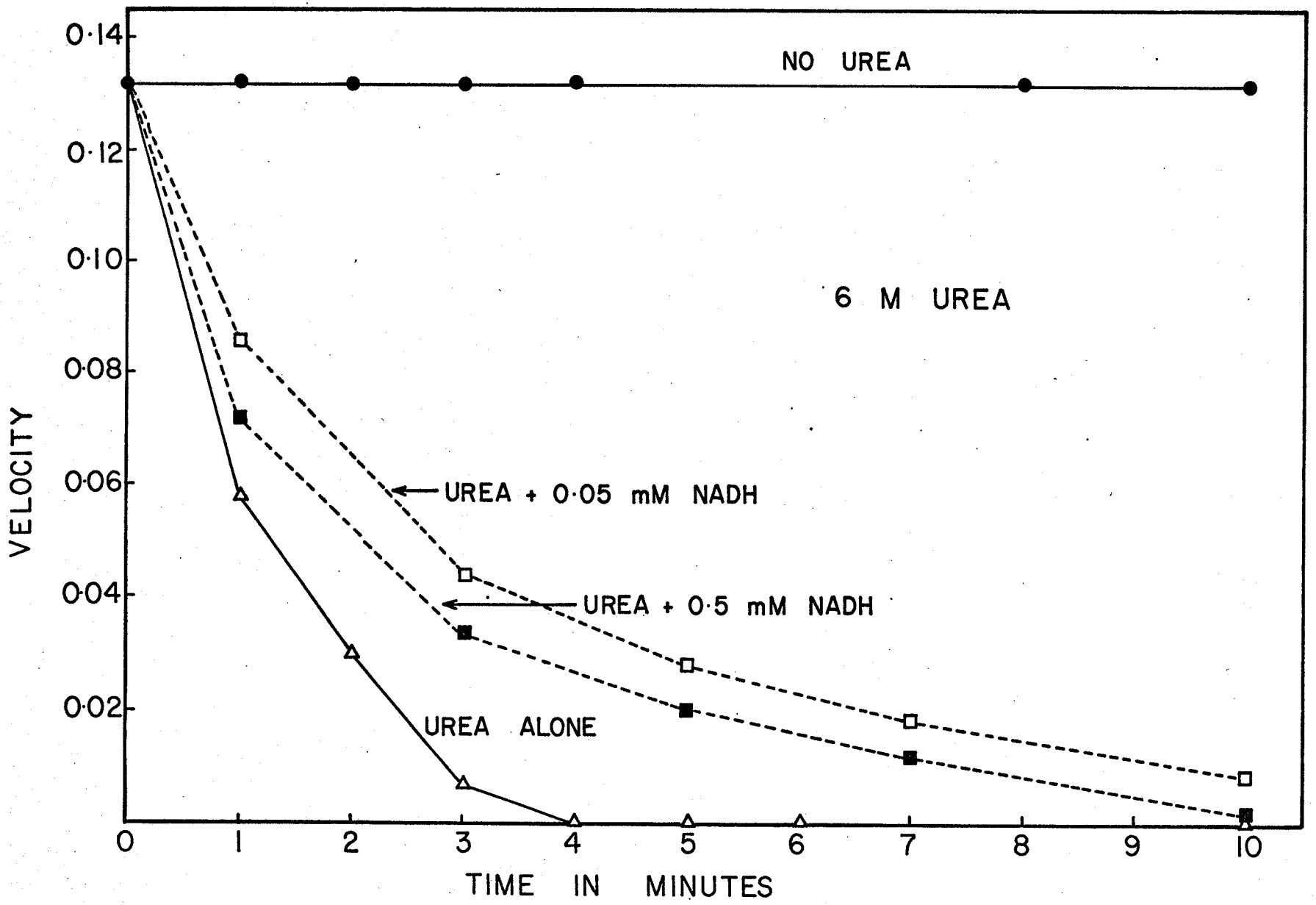

0·14
0·12
0·10
0·08
0·06
0·04
0·02
VELOCITY
NO UREA
6 M UREA
UREA + 5 mM NAD+
UREA + 0·5 mM NAD+
UREA + 0·05 mM NAD+
UREA ALONE
UREA + 0·5 mM FORMATE
UREA + 5 mM FORMATE
0
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES

Figure 45. Effect of NADH on the inhibition of FDH by 6 M urea.

(□) 0.05 mM NADH added to enzyme solution before incubation with urea.

(■) 0.5 mM NADH added to enzyme solution before incubation with urea.


0·14
0·12
0·10
0·08
0·06
0·04
0·02
VELOCITY
NO UREA
6 M UREA
UREA + 0·05 mM NADH
UREA + 0·5 mM NADH
UREA ALONE
0
1
2
3
4
5
6
7
8
9
10
TIME IN MINUTES

Adaptation of Glutamate-grown cells to Formate

Whole cells grown on liquid minimal glutamate medium were collected and washed as described in "Methods". They were used as inoculum for growth on liquid minimal formate medium. Aliquots were taken at various times and the whole cells collected and tested for ability to oxidize glutamate and formate. The Clark oxygen electrode and Gilson Oxygraph were utilized to monitor the specific activity of the cells (see "Methods"). Specific activity of the cells for the two substrates were plotted against time and is illustrated in Fig. 46 . The rate at which whole cells could oxidize formate showed a sharp rise at between 14 to 16 hours after exposure to formate. This was quite consistent and repeatable provided the glutamate grown cells were active and undamaged in the washing process. The cells were concomittantly checked after treatment with toluene (see "Methods"). As shown in Fig. 47, the treatment did not seem to significantly affect the specific activity of the cells for the oxidation of formate. Rise in specific activity of the treated and untreated cells closely paralleled each other. Glutamate activity however was affected more by toluene treatment although the activities of untreated and toluenized cells still followed a similar pattern of change.

Figure 46. Oxidation of an external source of formate by cells growing on glutamate as measured with the Clark oxygen electrode.

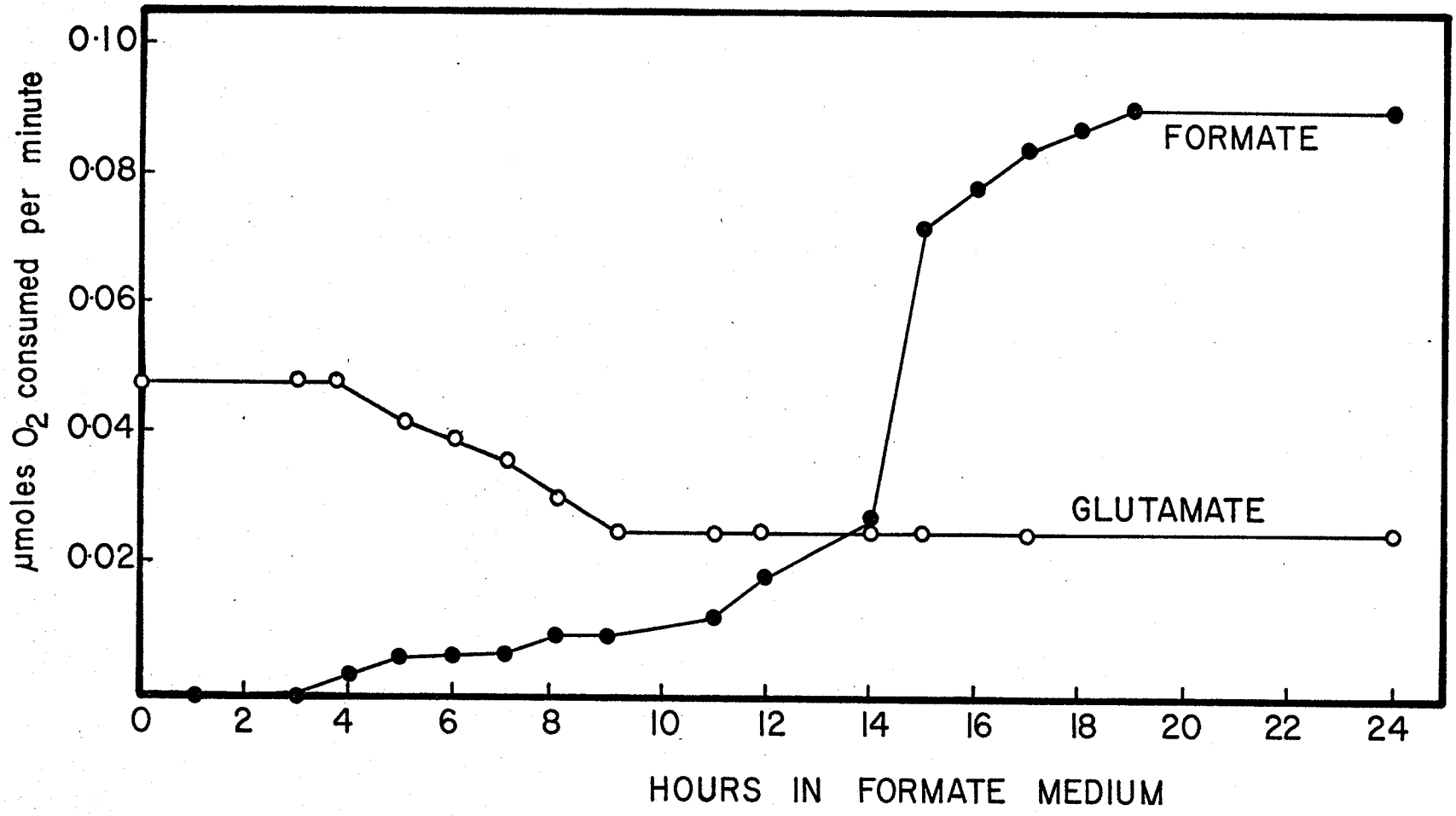

0·10
0·08
0·06
0·04
0·02
μmoles O_2 consumed per minute
FORMATE
GLUTAMATE
0
2
4
6
8
10
12
14
16
18
20
22
24
HOURS IN FORMATE MEDIUM

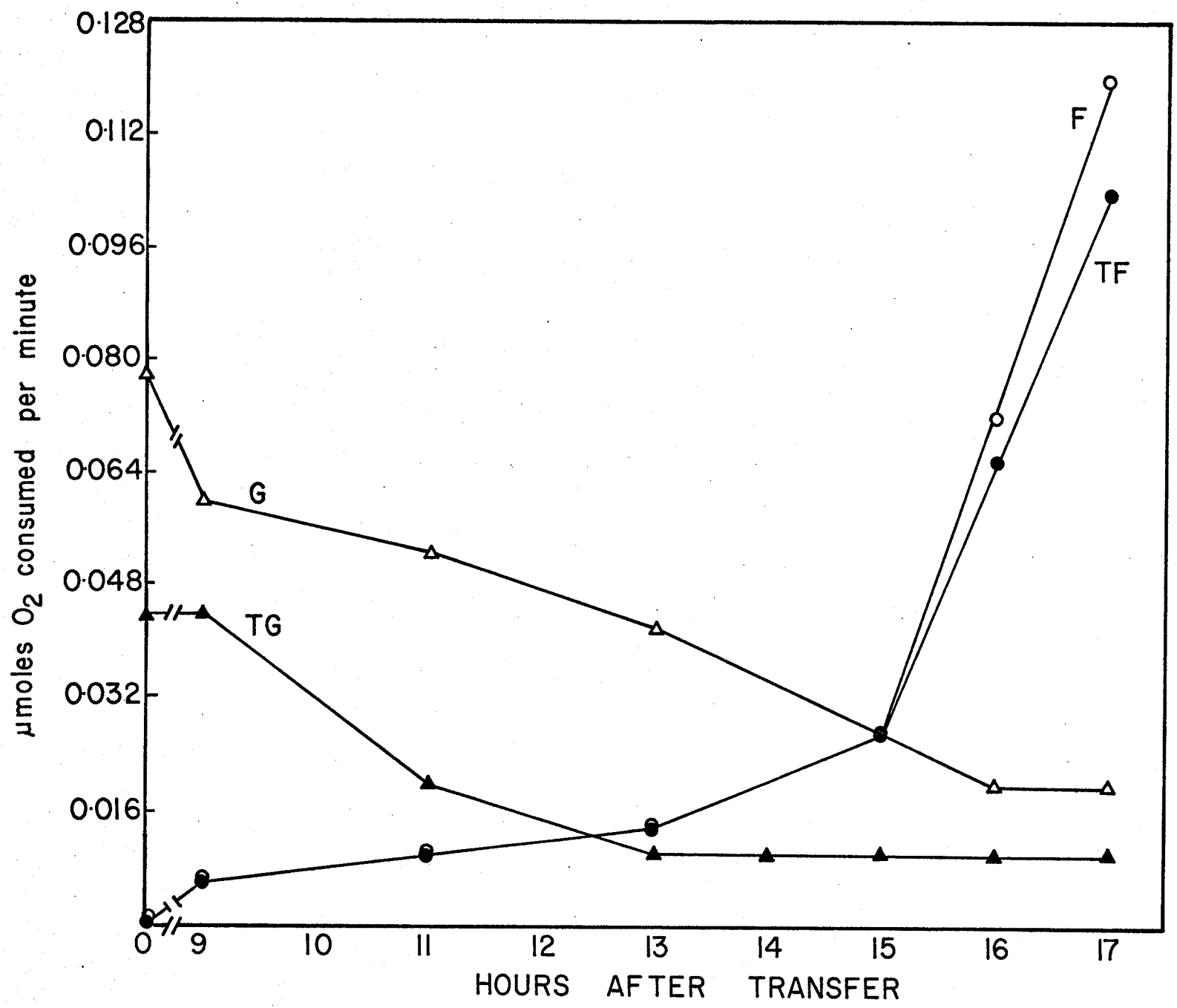
Figure 47. Comparison of glutamate-and formate-oxidizing activities of toluene-treated and untreated whole cells of P. oxalaticus growing on glutamate.

G = Glutamate oxidation by untreated cells.

TG = Glutamate oxidation by toluenized cells.

F = Formate oxidation by untreated cells.

TF = Formate oxidation by toluenized cells.


0·128
0·112
0·096
0·080
0·064
0·048
0·032
0·016
µmoles O_2 consumed per minute
F
TF
G
TG
0
9
10
11
12
13
14
15
16
17
HOURS AFTER TRANSFER

Formate oxidation by Oxalate-grown cells

During the course of this study it was noted that when Pseudomonas oxalaticus grew on liquid minimal oxalate medium, the whole cells were not always competent in oxidizing formate when so challenged (using the Gilson Oxygraph and Clark electrode). This situation occurred with cells obtained from early stages of growth. Fig. 48 shows the relative specific activities of the rate of oxidation of oxalate and formate by cells growing on oxalate as sole carbon source. The time period before the rise in specific activity for formate oxidation was variable and was influenced by the size and relative age of the inoculum (early oxalate growing cells). It was decided to investigate this phenomenon since the FDH activity in oxalate-grown cells during the early stages of the growth cycle was always high. The FDH activity was only low in the extracts of oxalate growing cells which were obtained during the late logarithmic or stationary phase of growth. The method of toluenizing cells (Kornberg and Reeves, 1972) was again employed in order to eliminate any permeability problem.

Aliquots of oxalate growing cells were taken at time intervals and with and without the toluene treatment were concomittantly examined for their oxalate- and formate-oxidizing activities in the Oxygraph with a Clark Oxygen electrode. As can be seen in Fig. 49 , formate was oxidized

readily by the toluenized whole cells at times well before the intact untreated cells were able to do so. The toluenized cells oxidized oxalate at less than half the rate of untreated cells and the added presence of CoA, ATP, NAD^+, $NADP^+$ had negligible effect on the rate. French Press extracts of these untreated oxalate-grown cells that were not able to oxidize formate possessed a reasonably high FDH activity (approximately 60% of the specific activity of mid-logarithmic cells).

In order to examine the possibility that the accumulation of formate in the oxalate medium was responsible for the appearance of cells competent in oxidizing formate when challenged with an external formate source, the level of formate in the medium was followed during the growth of cells in oxalate. After the collection of cells by centrifugation, the supernatant of each aliquot was frozen and saved for determining the concentration of formate. Formate was determined as described in "Methods". Fig. 50 shows that formate does accumulate, although at a very low concentration, prior to the appearance of formate oxidizing activity of intact whole cells.

The effect of the uncouplers carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP) and dinitrophenol (DNP) on the oxalate and formate oxidizing capability of oxalate-growing cells was investigated. Fig. 51 shows the results

Figure 48. Specific activities of formate-and oxalate-oxidation by cells growing on oxalate.

(○) Specific activity of formate oxidation

(●) Specific activity of oxalate oxidation

(Δ) Cell density (log Klett units)

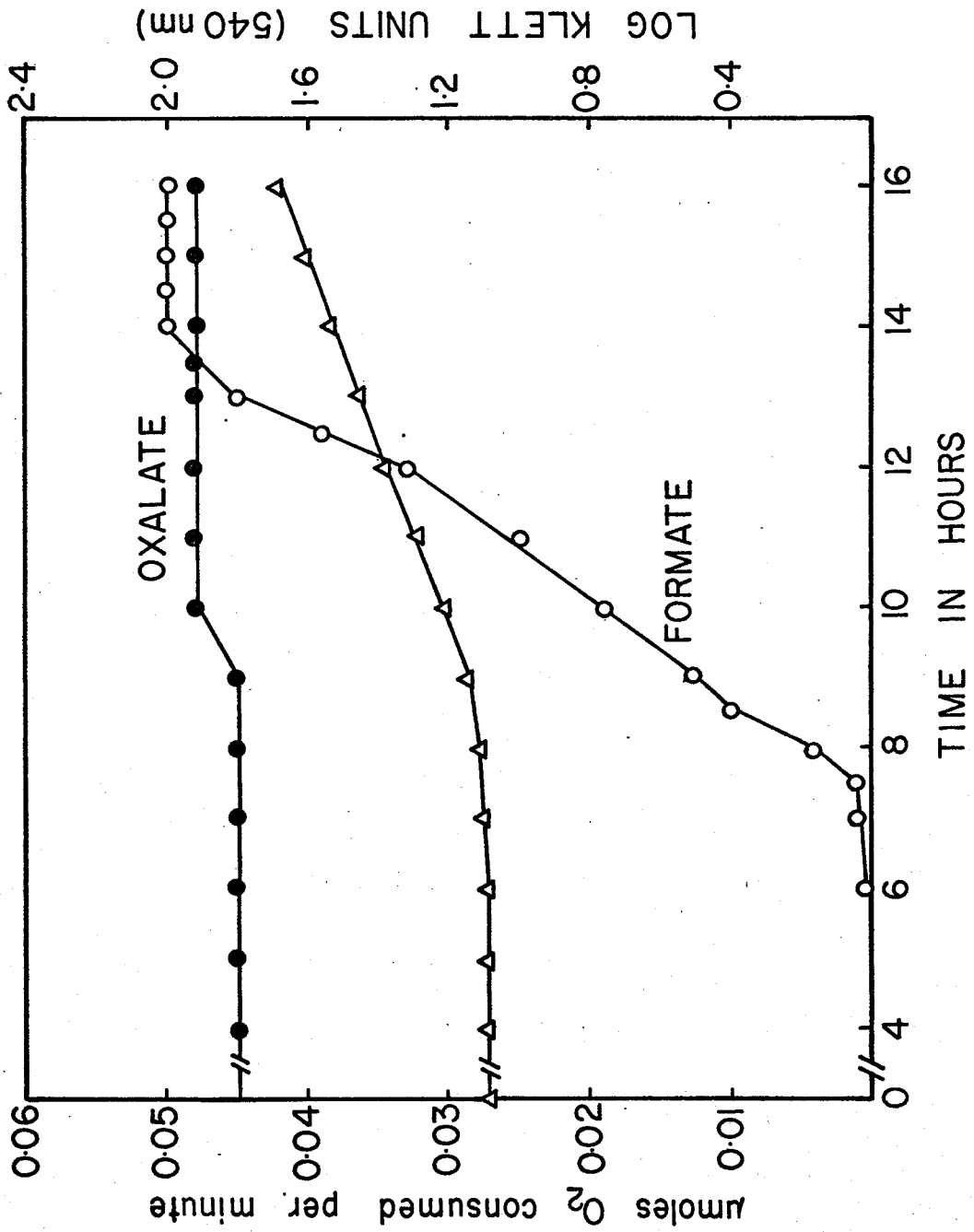

LOG KLETT UNITS (540 nm)
2·4
2·0
1·6
1·2
0·8
0·4
μmoles O_2 consumed per minute
0·06
0·05
0·04
0·03
0·02
0·01
0
4
6
8
10
12
14
16
TIME IN HOURS
OXALATE
FORMATE

Figure 49. Comparison of the formate-and oxalate oxidizing activities of toluene-treated and untreated cells growing on oxalate.

0 = Oxalate-oxidation by untreated cells.

T0 = Oxalate-oxidation by toluenized cells.

F = Formate-oxidation by untreated cells.

TF = Formate-oxidation by toluenized cells.

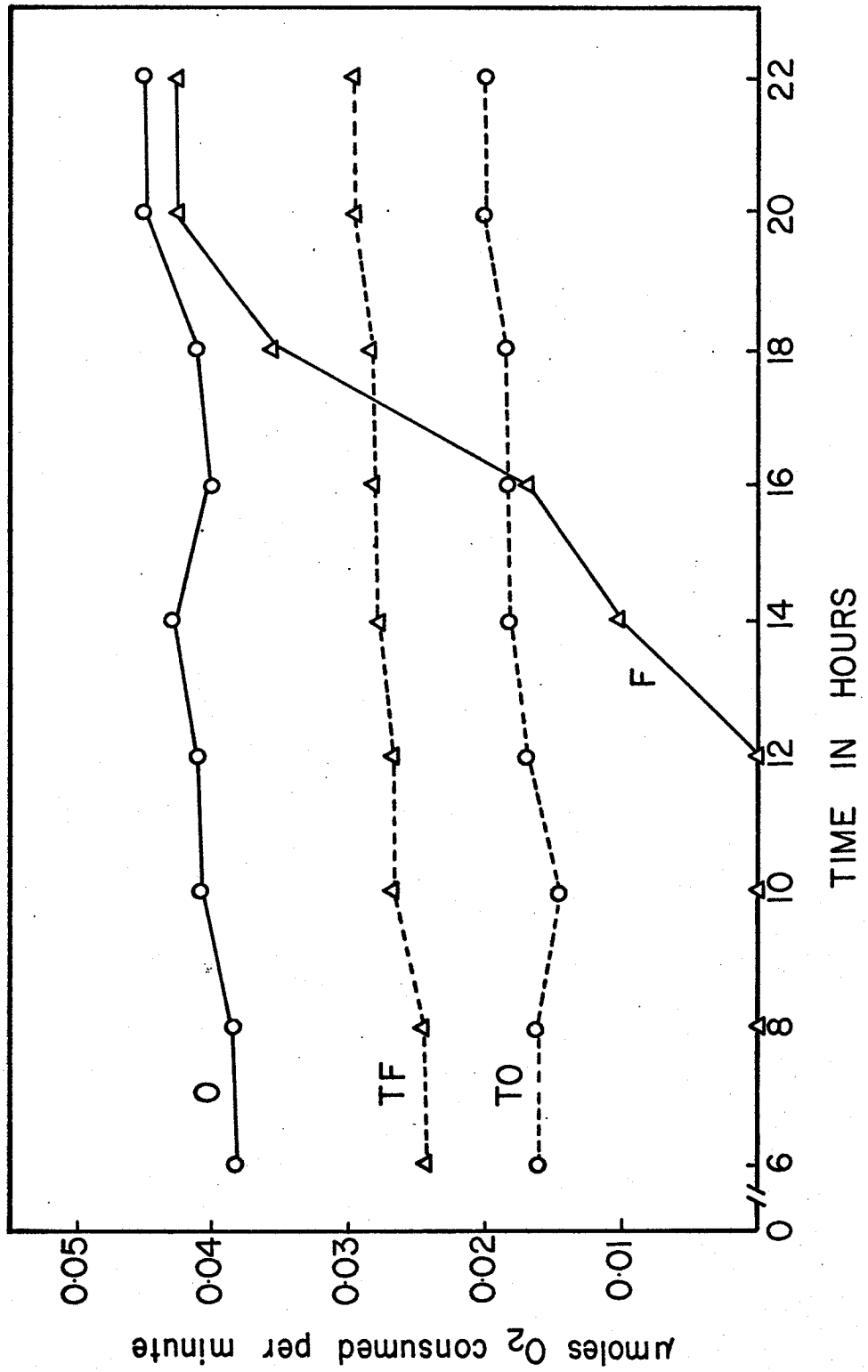

µmoles O2 consumed per minute
0·05
0·04
0·03
0·02
0·01
0
6
8
10
12
14
16
18
20
22
TIME IN HOURS
O
TF
TO
F

Figure 50. Efflux of formate into the medium and the competence of oxalate-growing cells in the oxidationof an external source of formate.

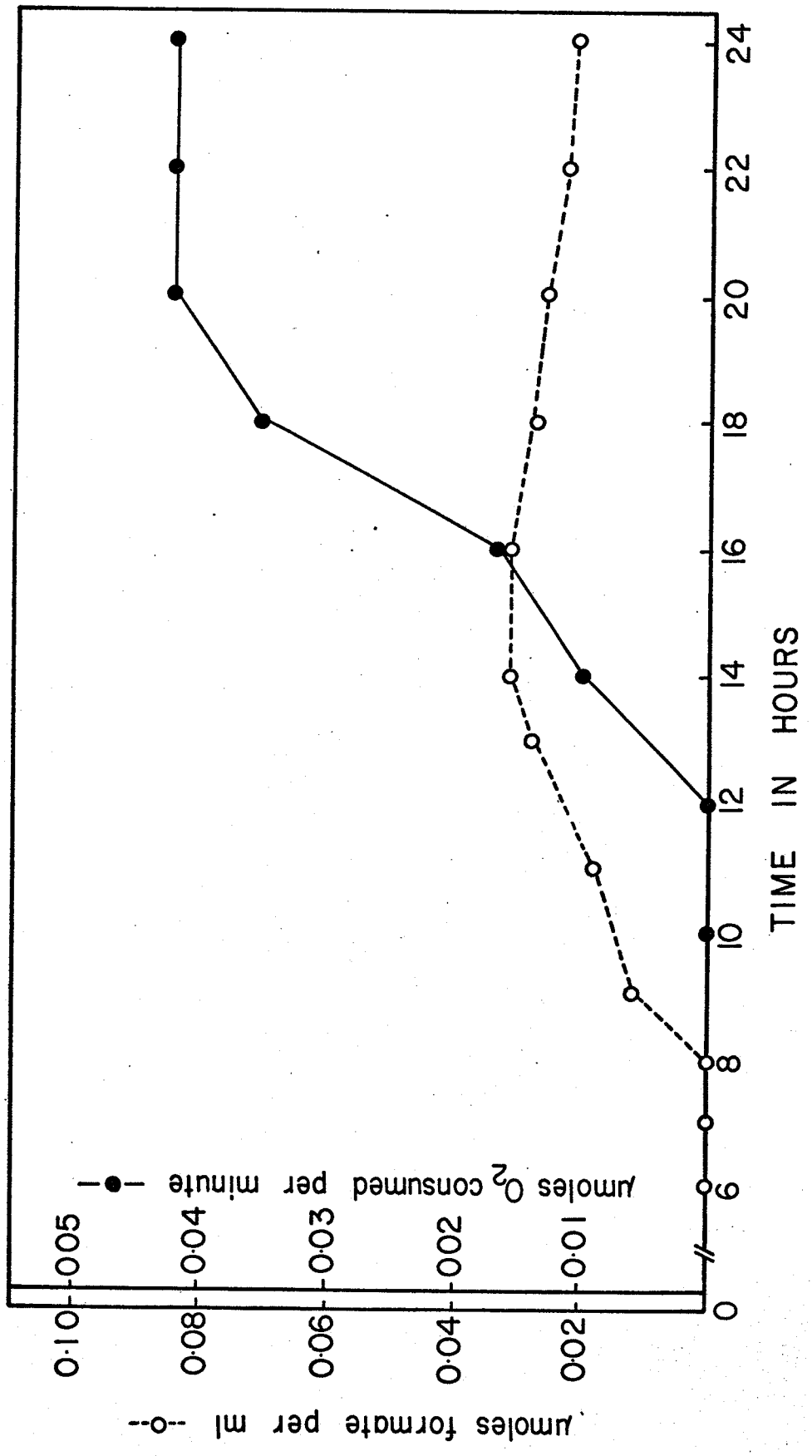

µmoles formate per ml --o--
µmoles O_2 consumed per minute —●—
0·10
0·08
0·06
0·04
0·02
0·05
0·04
0·03
002
0·01
0
6
8
10
12
14
16
18
20
22
24
TIME IN HOURS

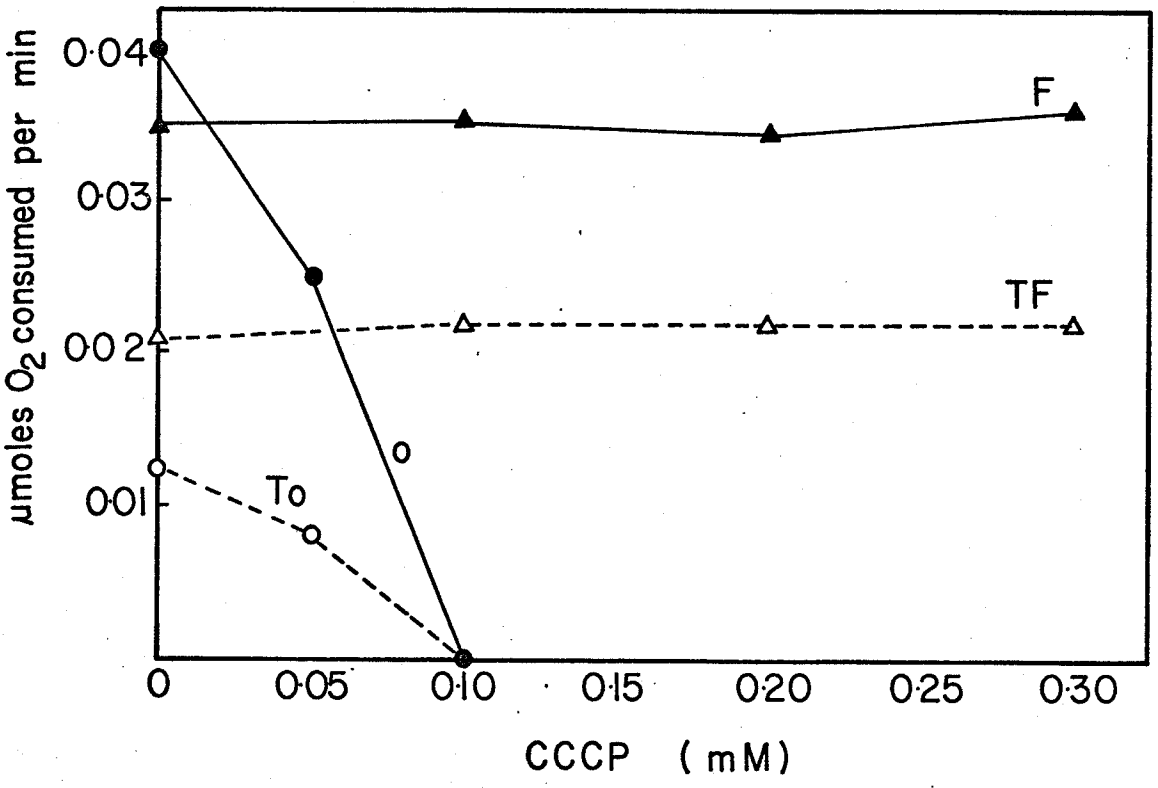
Figure 51. The effects of the uncouplers CCCP and DNP on the formate- and oxalate-oxidizing activities of oxalate-grown cells: Comparison between toluenized and non-toluenized cells.

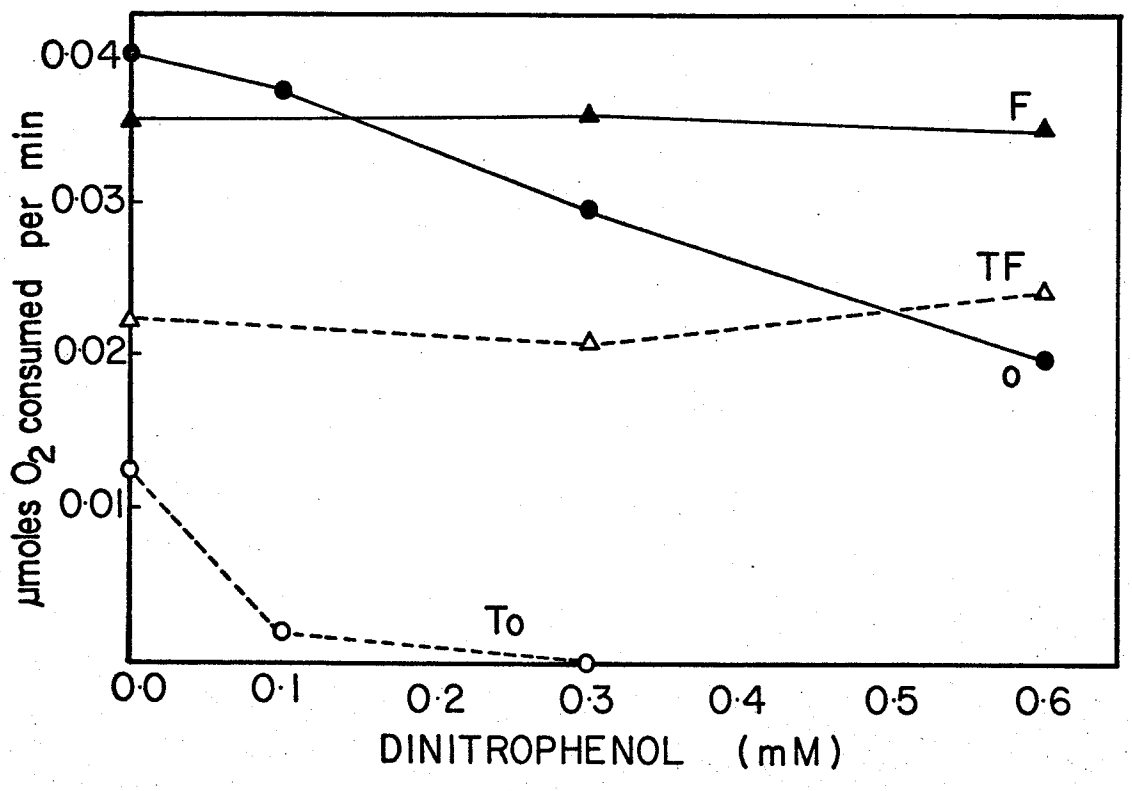
0 = oxalate-oxidation by untoluenized cells

TO = Oxalate-oxidation by toluenized cells

F = Formate-oxidation by untoluenized cells

TF = Formate-oxidation by toluenized cells


0·04
0·03
0·02
0·01
µmoles O_2 consumed per min
F
TF
o
To
0
0·05
0·10
0·15
0·20
0·25
0·30
CCCP (mM)


0·04
0·03
0·02
0·01
µmoles O_2 consumed per min
F
TF
o
To
0·0
0·1
0·2
0·3
0·4
0·5
0·6
DINITROPHENOL (mM)

obtained. The formate oxidizing activity of oxalate-grown cells was unaffected by both uncouplers, while the oxalate oxidation was inhibited. CCCP was a more potent inhibitor of the oxalate oxidizing capability of the cells than DNP. Toluenized cells were included in the experiment as a check on the permeability of the cells to the uncouplers.

Growth of Glutamate-grown Cells on Solid Formate Medium.

When Pseudomonas oxalaticus growing on minimal glutamate agar or on nutrient agar was streaked or spread directly on minimal salts formate agar plates, very few colonies usually resulted. Counts of serial dilutions were erratic. High dilutions (low cell numbers) produced no colonies on the plates whereas low dilutions (high cell numbers) resulted in some plates with many colonies and others with only a few (plates that were seeded with approximately the same numbers of cells each!). The same problem was encountered with minimal oxalate agar plates. Experiments were therefore attempted to determine the possible cause. The possibility that the majority of the cells in a population of glutamate grown cells did not have the potential to utilize formate or oxalate as sole carbon and energy source was all but eliminated by the following experiment. A suspension of the glutamate-grown cells was diluted in 0.1 M potassium phosphate buffer (pH

Leaf blank to correct numbering.

7.0) until each tube in a set statistically contained 0.1 to 0.2 cells; that is, one tube out of five or ten should have had a single bacterium. One out of five or ten tubes showed growth after incubation.

As a further check, glutamate agar plates with suitable counts were replica plated (Lederberg and Lederberg, 1952) on formate agar plates. Of just over 3,000 colonies screened, 21 failed to produce colonies on the formate plates via the velveteen. However, when these colonies were subsequently picked off the glutamate agar with a sterile needle and transferred onto formate agar thus, they were all able to grow on the formate agar. It therefore seemed that all the cells had the potential ability to grow on formate as sole carbon and energy source. Conditions and factors which might enable the glutamate cells to grow on oxalate and formate agar were therefore investigated. Cells grown on glutamate were washed once with 0.1 M potassium phosphate buffer (pH 7.0) and suspended in formate medium. Aliquots were removed at various times, serially diluted with the buffer (supplemented with 10 mM sodium formate) and plated out on a series of glutamate and formate plates in quintuplicate. The same experiment was carried out using oxalate instead of formate. Since the results for formate and oxalate experiments closely paralleled each other, only the data for formate experiments will be cited. Fig. 52 shows the data

obtained using the phosphate buffer alone for washing and diluting the cells. In Fig. 53 , the graph shows the counts obtained using the same phosphate buffer supplemented with 10 mM sodium formate for washing and diluting the cells. From the data on Fig. 52 , it was clear that exposure to formate was important. At about 4 hours exposure, the counts obtained on formate agar was roughly the same as those on glutamate agar. Using 10 mM sodium formate in the buffer (Fig. 53) reduced the required time of exposure to formate to achieve a "full" count. Although a significant part of the time of exposure to formate can be accounted for in the time taken to wash and dilute the cells; nevertheless the inclusion of formate in the buffer does account for some reduction of the time necessary to get equivalent-to-glutamate counts.

In order to eliminate the need to suspend the glutamate cells in formate medium before plating on formate plates; 0.01% yeast extract, an autoclaved formate cell suspension and formate cell extracts were incorporated into separate sets of minimal formate plates. In all three cases the counts and required time of exposure to formate remained unaffected. It was then decided to test the effect of pyruvate which had been reported to be essential for growth of the organism on formate (Hoepner and Trautwein, 1971). Concentrations of 1 mM and 3 mM sodium pyruvate were incorporated

in two separate sets of formate agar plates. The data are presented in Fig. 53 and represented by the broken lines. As can be seen, the time of exposure to formate was all but eliminated in achieving closely equivalent counts to glutamate plates in the formate agar plates supplemented with 3 mM pyruvate.

Figure 52. Effect of exposure to liquid formate, before plating, on the counts of glutamate-grown cells plated on formate agar.

(●) Plate counts on glutamate agar.

(○) Plate counts on formate agar.

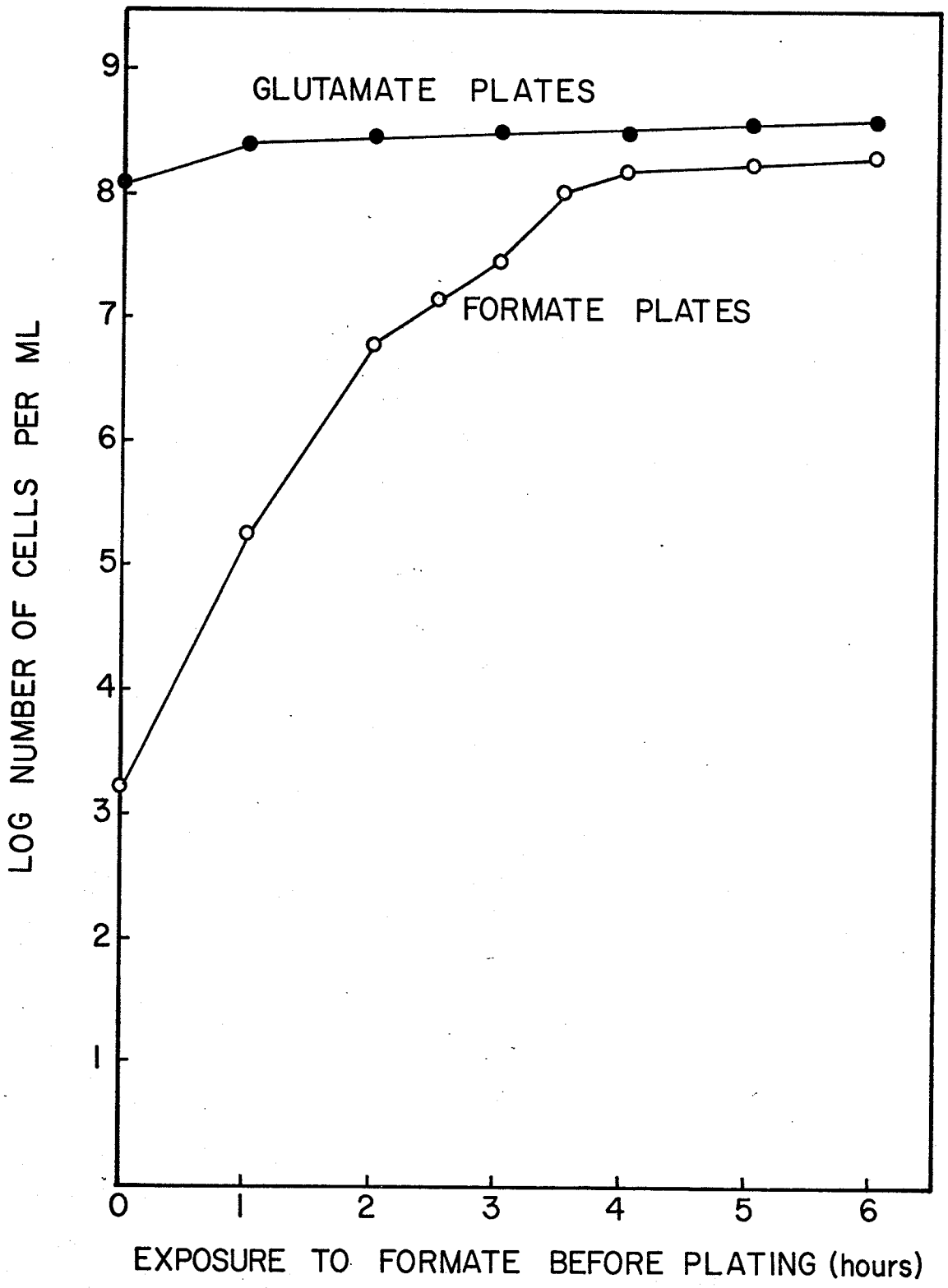
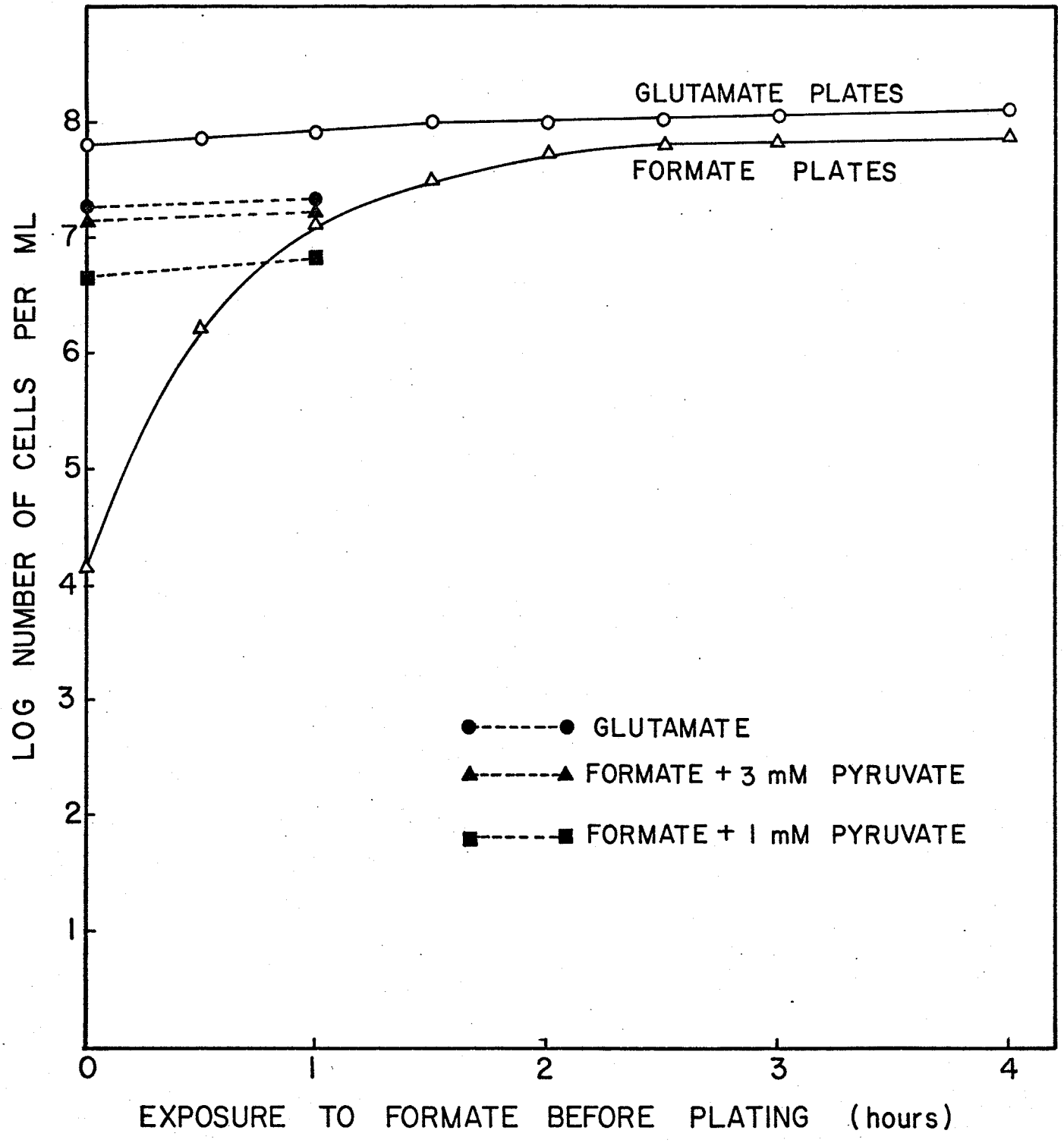

GLUTAMATE PLATES
FORMATE PLATES
LOG NUMBER OF CELLS PER ML
EXPOSURE TO FORMATE BEFORE PLATING (hours)
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6

Figure 53. Effect of the presence of 10 mM formate in the washing and dilution buffer of the experiment shown in Fig. 52 (solid lines), and the effect of incorporation of 1 mM and 3 mM pyruvate in the formate agar (broken lines) on the counts of glutamate-grown cells plated on formate agar.


GLUTAMATE PLATES
FORMATE PLATES
LOG NUMBER OF CELLS PER ML
8
7
6
5
4
3
2
1
0
1
2
3
4
EXPOSURE TO FORMATE BEFORE PLATING (hours)
GLUTAMATE
FORMATE + 3 mM PYRUVATE
FORMATE + 1 mM PYRUVATE

D I S C U S S I O N

DISCUSSION

General comparison of the Pseudomonas oxalaticus FDH with other FDH's.

From the published literature surveyed in the "Historical" section, it appears that the FDH obtained from Pseudomonas oxalaticus (OX 1) is very similar in many respects to the enzyme obtained from plants (peas and beans). Both are NAD^+-linked enzymes and are affected to approximately the same degree and in a like manner by the same inhibitors, the most notable ones being cyanide and azide. Earlier papers on the plant FDH gave an impression of a rather low affinity for formate; in addition the specific activities of the enzyme preparations were quite low compared to the P. oxalaticus preparation. Johnson et al (1964 b) alluded to this fact in their paper on the micro-estimation of formate using the P. oxalaticus FDH. This view has since needed to be rectified as the enzyme preparation of Poiger et al (1969) from peas was also suitable for use in the micro-estimation of formate. Ohyama and Yamazaki's (1974) homogenous preparation of FDH, also from peas, had a specific activity as high as that of the P. oxalaticus enzyme.

The FDHs found in Escherichia coli, Desulfovibrio spp. and the Clostridia, are rather different, both in nature and function, from the P. oxalaticus enzyme. In E. coli and Desulfovibrio spp., FDH is cytochrome-linked (cytochrome b_1 for E. coli and cytochrome c_3 for Desulfovibrio spp.), possibly with ubiquinone as intermediate electron-carrier. The enzyme is part of the formate-hydrogenlyase system (Gest and Peck, 1955; Peck and Gest, 1957) and seems to be associated with the nitrate-reductase system (Itagaki, 1964) in E. coli. There is no evidence, thus far, of reduction of either NAD^+ or $NADP^+$ with these FDH's.

The enzyme found in some Clostridia, under in vivo conditions, works in the direction of carbon dioxide reduction; that is, the formation of formate from carbon dioxide. This is the first reaction in the sequence leading from carbon dioxide to the subsequent formation of acetate. As mentioned in "Historical", the equilibrium of the reaction is strongly in favour of the oxidation of formate to carbon dioxide. To overcome this and reverse the thermodynamically favoured reaction, the FDH is linked to formyl tetrahydrofolate synthetase. This FDH is $NADP^+$-linked (Li et al, 1966; Andreesen et al, 1971). All these FDH's that are cytochrome-linked are in the class EC 1.2.2. -.

The FDH from Rhodospeudomonas palustris (Yoch and

Lindstrom, 1969), however, does resemble the P. oxalaticus enzyme in many respects. Both the plant and R. plaustris enzymes will be discussed in more detail in comparison with the P. oxalaticus FDH in a later portion of this "Discussion".

Growth properties of Pseudomonas oxalaticus.

Interest has been generated in the FDH from P. oxalaticus ever since Quayle and his various colleagues published their work on the organism. As a consequence, other groups have subsequently been investigating the organism and the enzyme itself further. Hoepner and Trautwein (1971) came to the conclusion that their P. oxalaticus culture was unable to grow on formate alone and that the presence of a small amount of cosubstrate, like pyruvate, was necessary for growth on formate. The culture used by this writer, however, showed no such requirement. Although reluctance to grow on minimal oxalate broth was encountered at times when the organism was transferred directly from a nutrient agar plate, this could be overcome by transferring a colony to minimal formate broth instead (where no difficulty was ever encountered) and then transferring a colony subsequently obtained on formate agar to the oxalate broth. Quayle himself did note earlier (1966) that cultures maintained on nutrient agar, even when supplemented with 5 mM formate or oxalate, became sluggish

(with time) to growth in minimal formate or oxalate.

In the present work, the organism was never subcultured more than once or twice on nutrient agar without subsequently exposing it to formate or oxalate. It was maintained on minimal salt (see "Methods") slants with 10 mM formate or oxalate as sole carbon source. Under sterile paraffin oil at $2^{o}C$, the cultures remained viable up to 3 months. Since this writer encountered reluctance of the culture from nutrient agar to grow on oxalate, it does seem that this ability to grow on oxalate (or formate) as sole carbon source needs to be encouraged often. This could be a reason for Hoepner's strain of cosubstrate-requiring P. oxalaticus since that culture was maintained on 0.8% yeast extract slants. As a further note, our own culture, used in this present work, is unable to grow on glucose alone as sole carbon source although it originally was able to do so.

The effect of pyruvate on the ability of glutamate-grown cells to develop colonies in the formate agar plates (Fig. 53) suggests that pyruvate somehow helps the organism during the metabolic transition to formate; but once adapted to formate, the organism does not require pyruvate for growth.

It was noticed quite early in this work that colonies on nutrient or T-soy agar always had rough edges whereas those on minimal salts oxalate, formate or glutamate agar had smooth, entire edges although the colonies were much smaller. However, it was not until late in this work, when electronmicrographs were taken of ultra-sections of the organism grown on the various media, that this point came up again. Sections of cells from nutrient and T-soy broth always revealed cells with cell-walls that did not withstand the rigours of fixation and dehydration well, resulting in a wavy, wrinkled appearance. Since the cells grown on minimal salts with glutamate showed normal, smooth cell-walls in the electronmicrographs the difference seemed to parallel the difference in the colony appearance. It seemed likely that a lack of sufficient quantity of salts in the environment might affect the integrity of the cell-wall. This was confirmed when a typical nutrient agar colony was streaked onto LB agar (1% NaCl) or nutrient and T-soy agar made up with 0.1 M sodium-potassium phosphate buffer (pH 7.0) in place of distilled water. The resulting colonies had smooth edges and the electronmicrographs showed normal, smooth cell-walls.

The mean generation times of P. oxalaticus, growing on oxalate and formate as sole carbon source, obtained in this work agrees closely with those obtained by Blackmore

and Quayle (1968 b). The mean generation time obtained for minimal glutamate was as fast as that for T-soy broth, indicating that an external supply of cofactors and growth factors does not seem to increase the growth rate when the carbon source itself is not the limiting factor.

Enzyme Purity

As mentioned in the results, the P. oxalaticus FDH purified in this study, is thought to be close to homogeneity in spite of the presence of minor protein bands in the polyacrylamide gel electrophoretogram. This is because the enzyme purified by Hoepner and Trautwin (1972) also showed more than one protein band (2 major and 3 minor) after gel electrophoresis and was composed of two molecular species. Sucrose density gradients exhibited one distinct peak after ultracentrifugation in the presence of high NAD^+ concentration (0.01 M) and Sephadex gel sieving also produced a distinct protein peak. When the purified FDH was electrophoresized in polyacrylamide disc gels at pH 9.3 ("standard" gels), a multitude of bands resulted, showing instability of the enzyme under the conditions. It is therefore plausible that one or two of the minor protein bands obtained at pH 8.0 were due to the enzyme protein itself.

TABLE IV

SUMMARY OF FORMATE DEHYDROGENASE KINETIC DATA

A. Initial velocity studies

Varying substrate	Double reciprocal plots	Slope replot	Intercept replot
NAD^+	linear-intersecting	biphasic, linear	biphasic, linear
Formate (low)	linear-intersecting	linear	linear
Formate (high)	linear-intersecting	linear	linear

Biphasic

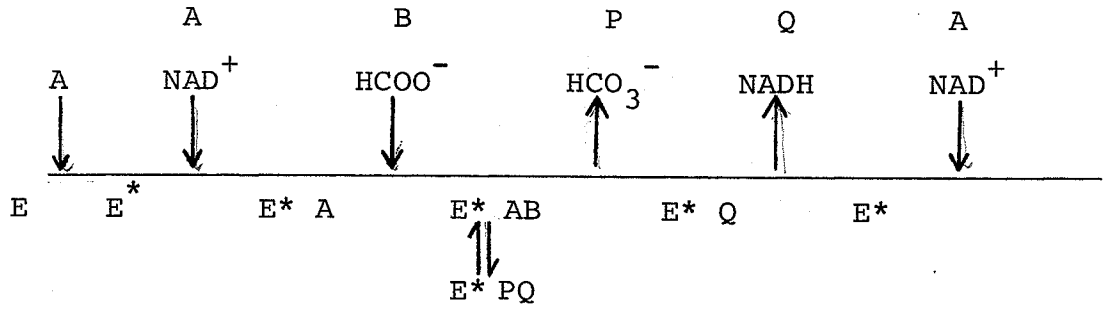
B. Product inhibition studies

Product	Varying substrate	Double reciprocal plots	Slope replot	Intercept replot	Inhibition
NADH	NAD^+ (non-sat. Formate)	linear-intersecting	linear	n.a.	C
	NAD^+ (sat. Formate)	linear-intersecting	linear	n.a.	C
NADH	low Formate (non-sat.NAD^+)	linear-intersecting	linear	linear	NC
	low Formate (sat. NAD^+)	linear-parallel	n.a.	linear	UC
NADH	high Formate (non.sat.NAD^+)	linear-intersecting	linear	linear	NC
	high Formate (sat. NAD^+)	linear-intersecting	linear	linear	NC
Bicarbonate	NAD^+ (non-sat. Formate)	linear-intersecting	linear	linear	NC
	NAD^+ (sat. Formate)	linear-parallel	n.a.	linear	UC
Bicarbonate	low Formate (non-sat.NAD^+)	linear-intersecting	linear	linear	NC
	low Formate (sat.$NAD^\pm$)	linear-intersecting	linear	linear	NC
Bicarbonate	high Formate (non-sat.NAD^+)	linear-intersecting	linear	linear	NC
	high Formate (sat. NAD^+)	linear-intersecting	linear	linear	NC

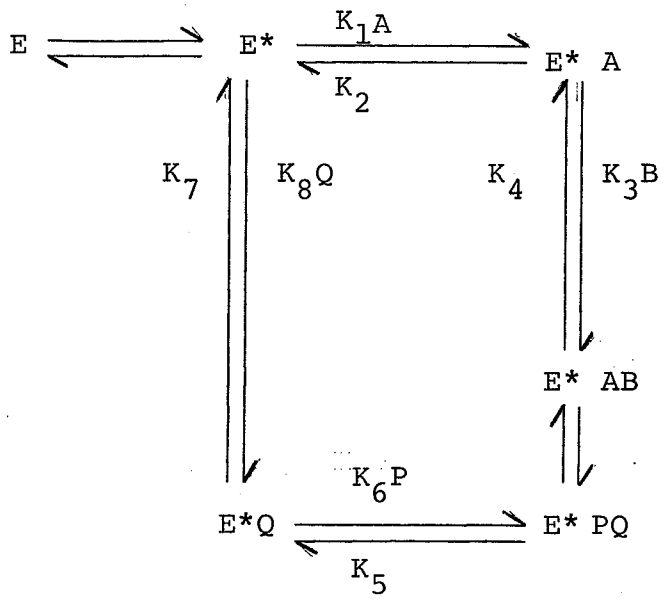
n.a. = not applicable, C = competitive, NC = non-competitive, UC = uncompetitive, sat. = saturating

Kinetics of the Enzyme

Table IV shows a summary of the kinetic results obtained for FDH. From these results the following model and mechanism is proposed for FDH according to the graphical shorthand representation of Cleland (1963 a):



The reaction sequence is termed an Ordered Bi Bi, where the substrates A and B enter in sequence followed by the products P and Q leaving, again in sequence. The reason for a superscript asterisk to the enzyme "E" is that catalytic quantities (0.005 to 0.01 mM) of NADH or NAD^+ must be present to prevent a lag (of several minutes duration, see "Results"). A schematic representation of the reaction would be:



From this scheme, the usual procedure is to employ the method of King and Altman (1956) to derive the rate equation. The rate equation for an Ordered Bi Bi reaction is well known and is given below, according to Cleland (1963 a):

$$v = \frac{V_1V_2\ (AB - \frac{PQ}{K_{eq}})}{K_{ia}K_bV_2 + K_bV_2A + K_aV_2B + V_2AB + \frac{K_qV_1Q}{K_{eq}} + \frac{K_pV_1Q}{K_{eq}}}$$

$$+ \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}} + \frac{K_aV_2BQ}{K_{iq}} + \frac{V_2ABP}{K_{ip}} + \frac{V_1BPQ}{K_{ib}K_{eq}}$$

..... Eqn. (1)

$$K_{eq} = \frac{V_iK_pK_{iq}}{V_2K_bK_{ib}} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_{ip}K_q}{K_{ib}Ka}$$

where A and B are the substrates, P and Q the products. In this discussion it is assumed that the enzyme was already in the E* (activated) state. It is a reasonable assumption since FDH was purified and stored in the presence of 0.01 mM NADH or NAD^+.

In the absence of product and in the double reciprocal form the equation (1) becomes:

$$\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) + \frac{K_a}{V_1}\left(\frac{1}{A}\right)\left(1 + \frac{K_{ia}K_b}{K_a B}\right) \quad \text{..... Eqn. (2)}$$

$$\text{or } \frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right) + \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(\frac{1}{B}\right) \quad \text{..... Eqn. (2')}$$

From this equation we can easily see the effect of changing substrate concentration on intercept or slope of the double reciprocal lines. Double reciprocal plots of velocity against varying concentrations of A or B should yield families of linear, intersecting lines.

For the product inhibition studies, equation (1) may be rearranged. When P or Q = 0, numerator = V_1V_2AB.

$$\frac{1}{v} = \frac{K_{ia}K_b}{V_1AB} + \frac{K_b}{V_1B} + \frac{K_a}{V_1A} + \frac{1}{V_1} + \frac{K_qP}{V_2K_{eq}AB} + \frac{K_pQ}{V_2K_{eq}AB} + \frac{PQ}{V_2K_{eq}AB}$$

$$+ \frac{K_qP}{V_2K_{eq}K_{ia}B} + \frac{K_aQ}{V_2K_{iq}A} + \frac{P}{V_1K_{ip}} + \frac{PQ}{V_2K_{eq}K_{ib}A}$$

Letting $K_1 = \frac{K_{ia}K_b}{V_1}$, $K_2 = \frac{K_b}{V_1}$, $K_3 = \frac{K_a}{V_1}$, $K_4 = \frac{1}{V_1}$,

$$K_5 = \frac{K_q}{V_2K_{eq}}, \quad K_6 = \frac{K_p}{V_2K_{eq}}, \quad K_7 = \frac{1}{V_2K_{eq}}, \quad K_8 = \frac{K_q}{V_2K_{eq}K_{ia}},$$

$$K_9 = \frac{K_a}{V_1K_{iq}}, \quad K_{10} = \frac{1}{V_1K_{ip}}, \quad K_{11} = \frac{1}{V_2K_{eq}K_{ib}}$$

the equation becomes,

$$\frac{1}{v} = \frac{K_1}{AB} + \frac{K_2}{B} + \frac{K_3}{A} + K_4 + \frac{K_5P}{AB} + \frac{K_6Q}{AB} + \frac{K_7PQ}{AB} + \frac{K_8P}{B} + \frac{K_9Q}{A} + K_{10}P + \frac{K_{11}PQ}{A} \quad \text{..... Eqn. (3)}$$

As with the experimental data presented in "Results", the two phases of the double reciprocal plots for formate are designated "low" and "high" levels of formate and will be treated separately with the assumption of activation at "high" formate concentration. As mentioned in "Results" and shown in Figs. 10 and 11, FDH required the presence of small amounts (0.01 mM or less) of NADH or NAD^+ for stability and activity. Otherwise, one encountered a long lag of several minutes before the reaction proceeded. This is in the nature of a "Hysteretic Enzyme" (Frieden, 1970) where an enzyme responds only slowly (usually in terms of the relative rate of reaction) to change in ligand, either substrate or modifier, concentration and results in a lag in the enzyme response to changes in the ligand level.

This aspect of the FDH property will be discussed further after presentation of a kinetic model.

The apparent activation at high formate levels during the assay of FDH activity, as evidenced by the biphasic double reciprocal plot in Fig. 16, is separate and distinct from the initial activation of the enzyme (elimination of the lag period) by low NAD^+ or NADH. An identical type of formate plot was obtained by Ohyama and Yamazaki (1975) with the pea (Pisum sativum) FDH, except that their two K_m values for formate were higher than those obtained here for Pseudomonas oxalaticus FDH.

"Low" B (formate) plots

The data concerned with the "low" formate concentration segment of the graphs will be discussed first. From the form of equation (2), we can see that both slope and intercept change with varying concentrations of the substrates. This is confirmed by the families of linear intersecting lines obtained in the plots for initial velocities in Figs. 15 and 16. Since the lines intersect on the X-axis for both substrate plots, the apparent K_m's are equal to the true K_m's, i.e. K_a and K_b, and $K_a = K_{ia}$.

Bicarbonate inhibition

To study P (HCO_3^-) inhibition, Q (NADH) is set as 0, then equation (3) becomes:

$$\frac{1}{v} = \frac{K_1}{AB} + \frac{K_2}{B} + \frac{K_3}{A} + K_4 + \frac{K_5P}{AB} + \frac{K_8P}{B} + K_{10}P \quad \text{..... Eqn. (4)}$$

With concentrations of A(NAD^+) being varied and at a non-saturating level of B (formate),

$$\frac{1}{v} = \left(K_4 + \frac{K_2}{B}\right) + \left(K_{10} + \frac{K_8}{B}\right) P + \left(\left(K_3 + \frac{K_1}{B}\right) + \frac{K_5P}{B}\right)\left(\frac{1}{A}\right) \text{....Eqn. (5)}$$

Intercept Slope

Since both intercept and slope functions contain P terms, both values will change when P changes. This indicates non-competitive inhibition, and was observed experimentally as illustrated by Fig. 28. At a saturating level of B (formate), we get:

$$\frac{1}{v} = (K_4 + K_{10}P) + K_3\left(\frac{1}{A}\right) \quad \text{..... Eqn. (6)}$$

Intercept Slope

Only the intercept value changes according to P, therefore the inhibition should be uncompetitive. This is as seen in Fig. 30, and is clear from the graphical shorthand representation; as saturation with B would break the reversible connection between A(E*A) and P(E* P).

When concentrations of B (formate) are varied ("low" level) at a non-saturating concentration of A (NAD^+),

$$\frac{1}{v} = (K_4 + \frac{K_3}{A} + K_{10}P) + \left[(K_2 + \frac{K_1}{A}) + (K_8 + \frac{K_5}{A})P\right](\frac{1}{B}) \text{...Eqn. (7)}$$

Both functions contain a $P(HCO_3^-)$ term, so the inhibition should be non-competitive, as illustrated in Fig. 32.

At a saturating level of A (NAD^+) we get,

$$\frac{1}{v} = (K_4 + K_{1p}P) + (K_2 + K_8P)(\frac{1}{B}) \text{ ..... Eqn. (8)}$$

The inhibition remains non-competitive, as shown by Fig. 33. As can be seen in the graphical shorthand representation, saturation of A does not affect the reversible connection between B (formate) and P (bicarbonate).

NADH inhibition

Setting P (HCO_3^-) as 0 eliminates the P terms of equation (3) and we get,

$$\frac{1}{v} = \frac{K_1}{AB} + \frac{K_2}{B} + \frac{K_3}{A} + K_4 + \frac{K_6Q}{AB} + \frac{K_9Q}{A} \text{ ..... Eqn. (9)}$$

When the concentration of A (NAD^+) is varied and B (formate) is not saturating the Q term only occurs in the slope function, so the inhibition should be competitive. This was observed experimentally and is illustrated by Fig. 20. At saturating B (formate), the inhibition remains competitive, as seen in Fig. 21. Looking at the graphical

shorthand scheme, it is apparent that the reversible connection between Q(NADH) and A(NAD^+) is unaffected by saturation with B (formate).

When the concentration ("low" level) of B (formate) is varied at non-saturating A(NAD^+), both intercept and slope functions change with Q(NADH) and the inhibition should therefore be non-competitive. This is seen in Fig. 23 (a). At saturating A(NAD^+), however, the Q(NADH) terms disappear from equation (9) and there should be no inhibition by Q. Going back to the graphical shorthand representation of the reaction sequence, we see that all E* will have been bound by saturating A(NAD^+) and therefore unavailable to Q. Hence Q(NADH) should not inhibit. In fact, the inhibition observed in Fig. 24 is very much smaller than that observed in Fig. 23, confirming the effect of saturating NAD^+ on NADH inhibition. The remaining inhibition, however, was uncompetitive.

Although the mechanism of this small inhibition is unclear, it might be related to the "activating" process. That is, E + a (small amount of NAD^+ or NADH) $\rightleftharpoons$ E*. Since this "activation" of FDH to eliminate the lengthy lag requires only 0.01 mM NADH (or NAD^+) and concentrations greater than 0.01 mM NADH begin to inhibit and result in a lowering of the maximal velocity of the reaction, even at saturating concentrations of substrates; any additional Q

(NADH) used in these experiments could result in inhibition of E ⟶ E*. This also applies when NAD^+ is non-saturating, but the degree of inhibition would be too small to be detected in the presence of a stronger product inhibition. As shown by the broken line in Fig. 20 (NADH inhibition with NAD^+ as variable substrate), the activity plot in the absence of NADH was parallel and very close to the line with 0.01 mM NADH, suggesting the occurrence of NADH inhibition not eliminated by NAD^+ saturation.

"High" B (formate) plots

The segments of the biphasic plots lying in what has been designated "high" formate (see "Results") concentrations will now be examined. An assumption is made for a plausible model, that is, that at a "high" formate level the enzyme form is distinct from E* (the "normal" activated form). This form, arbitrarily designated E^{Δ}, has a higher turnover number (maximal velocity) than E* as evidenced by the faster reaction rate obtained experimentally. E^{Δ} also has a K_m value for formate just over two-fold that of E* (see Fig. 16). Apparently a "high" formate (> 0.3 mM) concentration brings about the resulting steric change. The reaction sequence remains as Ordered Bi Bi. After the formation of E^{Δ}, the addition and removal sequence of substrates and products is the same as that for "low"

formate. The initial velocity patterns were identical to those obtained at "low" formate, intersecting at the X-axis for both substrate plots.

The product inhibition patterns were also the same except that NADH was found to be a non-competitive rather than an uncompetitive inhibitor with respect to formate at a saturating concentration of NAD^+.

Initial velocity plots at pH 5.6

Except for lower maximal velocity, V_1, (as compared to pH 7.5) the enzyme at pH 5.6 seems to be kinetically quite similar to that at pH 7.5. The K_m's were reasonably close in their values at both pH's. The initial velocity double reciprocal plots with both NAD^+ and formate (see Figs. 38 and 39) were also the same as those at pH 7.5.

Inhibition Studies

As stated in "Results", the evidence from the various metal inhibitors and chelators point to non-involvement of a divalent metal in the active site(s) of the FDH preparation. Only bathocuproine sulfonate inhibited the FDH to significant degree, so the possibility that a monovalent metal e.g. Cu^+, might be involved in the active site(s) cannot be ruled out.

The inhibition of FDH by cyanide and azide has been well-known from the early days of the pea enzyme (Mathews

and Vennesland, 1950; Davison, 1951). Recently, Ohyama and Yamazaki (1975) have done extensive work on the mechanism of the inhibition by both cyanide and azide. NAD^+-inhibitor complexes (Colowick et al, 1951; Kaplan and Ciotti, 1954; Van Eys et al, 1958) were formed in the presence of the enzyme and these complexes inhibited the enzyme reaction by binding to the active site(s). From the preliminary studies in "Results", it is reasonably clear that the situation is the same with P. oxalaticus FDH.

The presence of NAD^+ enhanced the inactivation by cyanide at pH 7.5. Ohyama and Yamazaki (1975) came to the conclusion that there were two NAD^+ sites but were surprised that the NAD^+-CN complex appeared to bind only one of the sites, causing the inhibition. The model for NADH-NAD^+ activation of FDH which is presented elsewhere in this "Discussion" could reasonably accomodate this result. The NAD^+-CN complex is assumed to bind only to the "activation" (high NAD^+ affinity) site resulting in an inactive enzyme and not to the substrate NAD^+ site. The complex probably has a higher affinity to the "activation" site than NAD^+, the natural activator. In this work, a rather unusual situation developed on lowering the pH of the assay conditions to pH 5.6. The presence of NAD^+ then protected the enzyme from inactivation by cyanide. Apart from the fact that the formation of the NAD^+-CN complex would be more unfavourable at an acid pH (Colowick et al, 1951) it is also plausible

that at pH 5.6, the affinity of the NAD^+-CN complex for the "activation" site might be lowered so that NAD^+ would bind to the site preferentially, resulting in the protection of the enzyme. It is also possible that there is another mechanism of cyanide inactivation of FDH that does not involve the NAD^+-CN complex and that is prevented when NAD^+ binds to the enzyme.

The inhibition by azide and the protective effect of NAD^+ might be similarly accounted for by assuming that the NAD^+-azide complex, if formed, has a lower affinity for the "activation" site than NAD^+ alone, or alternatively, that the NAD^+ binding protects the enzyme from azide inactivation without involving the complex.

Unfortunately, the inhibition studies done by this writer were only preliminary and no titrations or binding studies were attempted. This was due mainly to the difficulty in obtaining large amounts of the pure enzyme which would have been required.

A hypothetical model for FDH

From these results and other results presented in this thesis, as well as those reported for the pea enzyme (Ohyama and Yamazaki, 1974 and 1975), the following hypothetical model is proposed. (Fig. 54)

The FDH of Pseudomonas oxalaticus consists of a dimer* unit (*see note at end), each monomer having binding sites for both substrates, NAD^+ and formate. The NAD^+ site of one subunit (high affinity site) has to be occupied by

NAD^+ or NADH for the stability of active dimer units, preventing their dissociation and subsequent inactivation.

The active dimer unit catalyses the formate dehydrogenase reaction with one subunit (NAD^+ site occupied) acting as a regulatory subunit and the other subunit acting as catalytic subunit (low affinity NAD^+ site and high affinity formate site) under the conditions of low formate concentration.

When the formate concentration becomes high (> 0.3 mM) the regulatory subunit now binds formate and acts as catalytic subunit with a higher K_m for formate (low affinity formate site). The other subunit possibly also changes to the same high formate K_m form. The NAD^+ K_m of the former regulatory site now changes to the low affinity value (the same value as the catalytic subunit). Since the maximal velocity of the dimer is now higher, once the former concentration approaches the high K_m value (0.3 mM) the linear phase of low formate plots breaks off and enters the linear phase of high formate plots (Fig. 16).

The model is a modification of the negative cooperativity model of Koshland (Conway and Koshland, 1968), since it is assumed that the first binding of a substrate has a higher affinity than the second binding. Other possible models such as a Random Bi Bi model could not explain the kinetic results obtained, especially the product inhibition studies.

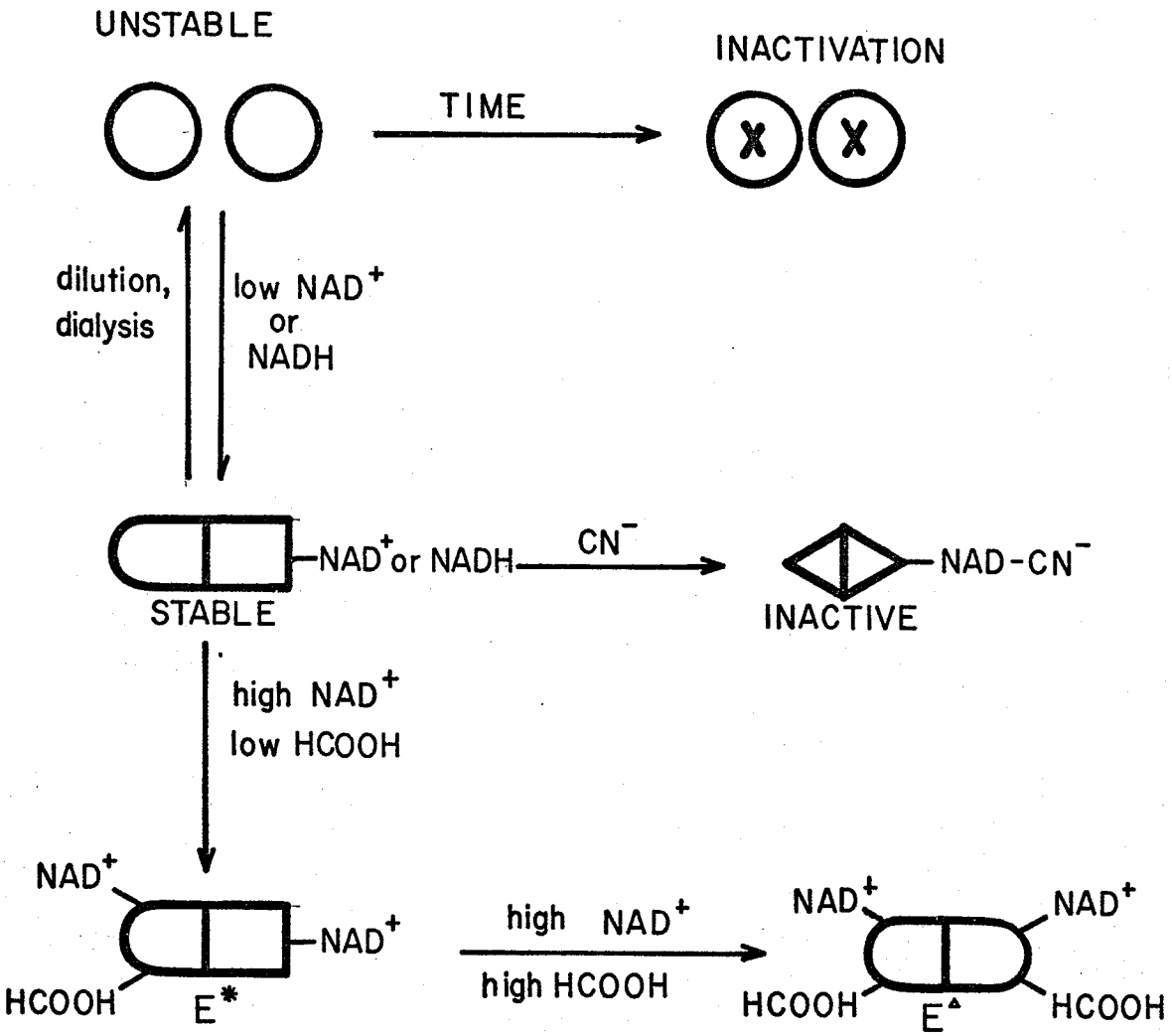
The model presented is a highly speculative one and requires substantiation at a molecular level, but a difficulty in obtaining a large quantity of purified enzyme prevented a total elucidation of the FDH mechanism at the molecular level. There is, however, some evidence in support of the molecular model presented in addition to the data published on the pea enzyme by Ohyama and Yamazaki (1974 and 1975). They found that a conformational change of FDH might be induced by NAD^+ binding since the rate of inhibition by the sulfhydryl inhibitor PCMB was retarded, whereas the rate by another sulfhydryl inhibitor DTNB was greatly accelerated, by the presence of NAD^+. Their data on NAD^+ binding indicated saturation of the first NAD^+ site at about 11 μM NAD^+ whereas the second site was not saturated until about 47 μM NAD^+. Their enzyme was a dimer binding 2 moles of NAD^+ per mole of enzyme and the double reciprocal formate-velocity plots were biphasic.

In the present work, there was the effect of low concentrations of NAD^+ (5-10 μM) on the stability and activation of the enzyme. Also, the presence of NAD^+ mitigated the effect of dissociation of the enzyme by urea and guanidine hydrochloride as illustrated in Fig. 43 and 44. The "activation" of the enzyme by low concentrations of NAD^+ and that by NADH differ slightly in their effects, based on the maximum rate of reaction after activation. As can be seen in Figs. 10 and 11, NADH eliminated the

lag period completely, whereas a very short lag of about 5-10 seconds remained with low NAD^+. However, the maximum rate was higher with NAD^+ than the NADH, which was the same as the rate of the diluted control (after lag). This point seems to be supported by the plot obtained in Fig. 20 where even when the concentration of NADH was low, the inhibition by it with variable NAD^+ remained even when NAD^+ was saturating. NAD^+ on the other hand seems to be able to reverse what appears to be lowering of the maximal rate due to oxidation during dilution of the enzyme to create the lag (Fig. 10). Also in the same figure it can be seen that a substrate level (0.5 mM) concentration of NAD^+ only shortened the lag period to 1-2 minutes, whereas 0.005 mM to 0.01 mM concentrations of NAD^+ all but eliminated the lag. At the high concentration of NAD^+, the regulatory (high affinity) site would already be saturated and the catalytic (low affinity) site itself would also be occupied. The molecule might then tend to resist steric change and this might possibly be responsible for making the activating process more difficult, resulting in the 1-2 minute lag.

Another point is the requirement of the enzyme for the presence of a very high (0.01 M) NAD^+ concentration for its relative stability and possibly its integrity during sucrose density gradient centrifugation. Even with 0.5 mM NAD^+, there was complete loss of activity after centrifugation. The precise reason why this is so is not clear; very

Figure 54. Diagrammatic representation of the hypothetical model proposed for the *P. oxalaticus* FDH.


UNSTABLE
INACTIVATION
TIME
dilution,
dialysis
low NAD+
or
NADH
NAD+ or NADH
CN−
NAD-CN−
STABLE
INACTIVE
high NAD+
low HCOOH
NAD+
NAD+
HCOOH
E*
high NAD+
high HCOOH
NAD+
NAD+
HCOOH
HCOOH
E▲
ACTIVE ENZYME I
ACTIVE ENZYME II
K_{NAD^+} = 0·025 mM
K_{HCOOH} = 0·12 mM
K_{NAD^+} = 0·025 mM
K_{HCOOH} = 0·33 mM

high NAD^+ concentrations seem to assist in stabilizing the enzyme molecule under the conditions of sucrose density gradient centrifugation.

*Note

Although in the speculative hypothetical model presented a dimer was chosen to represent FDH, it must be stressed that this was mainly for convenience and in no way implies that a tetramer or an octomer is not probable. The fact that the molecular weight for FDH obtained in this work is double the value for Ohyama and Yamazaki's enzyme (1974) but only half that of the main species of Hoepner and Trautwein's (1972) preparation, might even suggest the tetramer. Obviously, however, a further study of the P. oxalaticus enzyme at the molecular level is required before a more firmly based model can be proposed.

Adaptation of Glutamate-grown cells to Formate

Glutamate-grown whole cells showed a rapid rise in the specific activity of formate oxidizing capability (when challenged with an external source of formate) between 12-16 hours after transfer into formate medium. This is illustrated in Fig. 46. The rise in the specific activity of toluenized cells exactly paralleled that of untreated cells, as shown in Fig. 47. It therefore appears that this rise

was not due to the removal of a permeability barrier to formate, but rather to an increase in the cellular formate oxidizing activity.

However, it appears that additional factors become important when glutamate cells are challenged to grow on formate agar instead of the liquid formate medium. As illustrated in Figs. 52 and 53 , the cells required exposure to liquid formate for a period of time before they were fully capable of growth on the formate agar medium. This requirement was unnecessary if small concentrations (1-3 mM) of pyruvate were incorporated into the formate agar medium. The precise reason for this is not obvious but it appears that the changeover from glutamate to the autotrophic formate mode involves some major cellular reorganization which requires time. During this time there is a sufficient diffusion of formate into the cells in the liquid medium to sustain the necessary cellular changes. In the agar medium, however, the diffusion rate is retarded and might therefore not be rapid enough to enable the cells to complete the necessary changes. If such was the case, pyruvate might accelerate the process by supplying energy and reducing power.

Oxalate-grown cells and formate oxidation

As illustrated in Fig. 48 , cells growing in liquid oxalate medium showed a sharp rise in the specific activity

of formate oxidizing capability at the time they entered the logarithmic phase of growth. The specific activity of the oxalate-oxidizing capability of these cells did not rise dramatically during this period. The experiment illustrated by Fig. 49, comparing toluenized and untreated cells showed that the toluenized cells were able to oxidize external formate at a high rate long before the untreated cells were able to do so. It therefore appears that there is initially a permeability barrier against the influx of external formate. The sharp rise in formate oxidizing activity of untreated oxalate cells comes shortly after the efflux of a small but discernible amount of formate into the liquid oxalate medium as illustrated in Fig. 50.

Use of the uncouplers CCCP and DNP (Fig. 51) indicated that formate oxidizing activity of the whole cells was not energy dependent whereas oxidation of oxalate was energy dependent. The experiment also showed that the cells were relatively impermeable to DNP, since there was a significant difference in the inhibition of the oxalate oxidizing activity of toluenized and untreated cells by DNP. Requirement of energy for oxalate metabolism is probably related to the activation of oxalate to oxalyl CoA before further metabolism (Quayle et al, 1960).

From these experiments it therefore appears that the permeability barrier, preventing what is probably the facilitated diffusion of formate into the cells, is removed after the efflux of the small amount of formate into the oxalate medium, thereby unmasking the formate oxidizing activity of the cells.

Physiological roles of formate dehydrogenase (FDH) and formate oxidase in Pseudomonas oxalaticus.

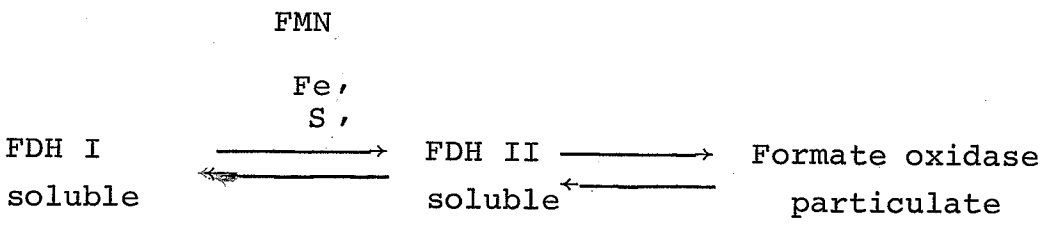
In the work (by various groups) thus far on P. oxalaticus and its FDH, there are some differing and apparently contradictory results that have been reported. Blackmore and Quayle (1968 b) concluded that oxalate-grown cells had a specific activity for FDH four times higher than that in formate-grown cells. Hoepner and Knappe (1970) on the other hand reported that their formate-grown cells had a specific activity three-and-half times that of the oxalate-grown cells. Their values given for the specific activity of the formate-grown cell extracts were indeed much higher than that obtained by Quayle (1966) or by this writer, from oxalate-grown cells. Certain properties of FDH as reported by Hoepner and Trautwein (1972) also differs from the FDH as obtained in this work. A possible explanation for these discrepancies regarding the FDH levels in P. oxalaticus might lie in the possibility

that the two types of formate oxidizing systems, formate dehydrogenase (FDH) reducing NAD^+ and formate oxidase reducing oxygen, involve the same basic enzyme protein.

That two systems exist in P. oxalaticus, which can oxidize formate, is apparent from the work of both Blackmore and Quayle (1968 a, b) and Hoepner and Trautwein (1972). Blackmore and Quayle suggested the existence of a "formate oxidase" system, separate and distinct from the "soluble" NAD^+-linked FDH system. This was because their evidence showed that when P. oxalaticus was grownoon an equimolar mixture of "slow"-growth (see "Historical") substrate and formate, NAD^+-dependent FDH was found in the cell extracts but when the organism was grown on an equimolar mixture of "fast"-growth substrate and formate, then "soluble" NAD^+-dependent FDH found in the extracts was negligible. However, these cells were able to oxidize formate without a lag period and it was shown that a considerable amount of formate had been consumed during growth. Formate was used at 60% of the rate of formate consumption in an equimolar formate-oxalate mixture (where formate was consumed at approximately ten times the rate of oxalate). Hoepner and Trautwein (1972) like Quayle and Keech (1959 d) before, found "particles" which could oxidize formate. However, they could cause no release of "soluble" NAD^+-dependent FDH from these particles. No details of extraction procedures

attempted were given. These particles were very oxygen-labile and were protected by the presence of formate, whereas their soluble FDH preparation showed slow inactivation by formate.

If one makes the assumption that, in vivo, the basic FDH protein can participate in both systems, then much of the data gathered so far can be reasonably accomodated. A graphical representation is given below:



(FDH I and FDH II are not necesarily equivalent to that of Hoepner and Trautwein (1972) with the same notation)

FDH I is the soluble basic enzyme protein that oxidizes formate with NAD^+ producing NADH, the main role being the generation of reducing power. Formate oxidase is a particular (membrane) complex of FDH and electron transport components catalyzing the oxidation of formate with oxygen, the main role being the generation of energy through oxidative phosphorylation. FDH II is derived from formate oxidase and consists of the basic enzyme protein and FMN, non-heme iron and labile sulfur, the last three being the components of electron transport. It oxidizes formate either with

NAD^+ or with oxygen.

The enzyme purified in this thesis is possibly mainly FDH I and is similar to the pea enzyme purified by Ohyama and Yamazaki (1974). The P. oxalaticus FDH purified by Hoepner and Trautwein (1972) is assumed to be mainly FDH II.

The soluble FDH I would be the main form encountered in oxalate-grown cells where FDH would be produced largely for the purpose of generating reducing power required for the catabolism as well as the heterotrophic assimilation of oxalate carbon. From the diagram shown in "Historical" we can see that there is a heavy demand for reducing power at two points early in the pathway of oxalate metabolism. NADPH (from NADH via transhydrogenase enzyme) is required for the formation of glyoxylate from two oxalyl CoA's and NADH is required to reduce tartronyl semialdehyde to glycerate. Some formate oxidase or a combination of FDH I and NADH oxidase is probably required to generate energy necessary for the oxalate metabolism and biosynthesis of cell materials unless glycerate oxidation possibly through pyruvate and the tricarboxylic acid cycle can generate enough ATP for these purposes. The second possibility appears likely since the "crude extracts" of oxalate-grown cells have higher levels of NADH oxidase activity than formate-grown cells (not reported in "Results").

Growth on formate as sole carbon and energy source requires both systems; that is, energy via formate oxidase, and reducing power via soluble NAD^+-linked FDH for CO_2 assimilation. Since in this case formate is the sole energy source, the particulate formate oxidase system would be more efficient than the combination of the soluble FDH and NADH oxidase in the generation of energy. The level of soluble FDH activity reported in cell-free extracts possibly depends not only on the level of FDH I in the cells but also on the degree of solubilization of FDH II from the formate oxidase during breakage of cells. This could account for the fact that NAD^+-linked "soluble" FDH levels in formate-grown cells depends very crucially on time of harvesting. It is possible that the efficiency of solubilization of FDH II from the formate oxidase complex is influenced by the age of cells. Hoepner and Knappe collected the cells very early; in fact, just when the turbidity of the liquid medium became visible, whereas it appears that Quayle (1966) did not collect his cells quite that early (0.8 g wet cells per litre). Under the conditions stated in "Methods", the cells collected in this thesis (0.7 to 1.0 g per litre) were about the same age as Quayle's.

In the experiments by Blackmore and Quayle (1968 b), when the organism was presented with an equimolar mixture of formate and a "fast"-growth carbon source like citrate,

lactate or succinate, it was only reasonable to expect the organism to utilize the "fast"-growth carbon source preferentially because it would be economical (energy-wise) to do so. However, it would also be sensible for the organism to utilize the formate purely on the basis of obtaining energy if it were efficient to do so. This was apparently the case as formate was used (in the mixture) as ancillary energy source. The "formate oxidase" system where the FDH involved could handle the transfer of electrons via the electron transport chain, without being interrupted by release of NADH, would be a very-efficient energy-generating system.

The apparently surprising and unexpected results obtained by Blackmore and Quayle (1968 b) with mixtures of the "equi"-growth substrate and formate can also be accomodated. Even though glycollate and glyoxylate produces the same growth rates of the organism, there is one important step in the metabolic paths involved. Glyoxylate as we have seen, necessitates a source of NADH, required for the reduction of tartronyl semialdehyde to glycerate. Hence the presence of the "soluble", NAD^+-linked FDH in the extracts of glyoxylate-formate mixture cells. However, if glycollate is the carbon source, we have one additional step involved; that of the oxidation of glycollate to glyoxylate by glyoxylate reductase (glycollate : NAD^+ oxidoreductase EC 1.1.1.26), with the production of NADH.

This would serve naturally as the source of NADH for the tartronyl semialdehyde reductase and of course the necessity for the "soluble" NAD^+-linked FDH would then be eliminated. Hence there would be negligible "soluble" NAD^+-dependent FDH in the extracts of the glycollate-formate mixture cells, although these cells would still oxidize formate purely for use as an energy source via the "formate oxidase" system. This was precisely the results reported by Blackmore and Quayle (1968 b).

Evidence gathered thus far on certain properties of the various FDH preparations alsoaagree with the concept developed above, as will be discussed in detail in the following paragraphs.

Hoepner and Trautwein's (1972) preparation of the enzyme from P. oxalaticus was done anaerobically at pH 5.6 and resulted in a FDH with at least one mole FMN, in addition to Fe and S, per mole enzyme protein, which was only slowly inactivated by its substrate formate. The FDH obtained here; aerobically at pH 7.5 and in the presence of NADH, had less than 0.1 mole flavin per mole enzyme protein and was inactivated very quickly by formate. Again using the model of the two "systems" involving the same enzyme protein, a plausible explanation for the differences can be given. It must be remembered that Hœpner and Trautwein's organism was grown in the presence of pyruvate (1 mM to 5 mM) when formate (100 mM) was the carbon source.

Although the actual amount of pyruvate was small, it is likely that its presence in the cell would be sufficient to create a "push" to the FDH protein molecule in the direction of the formate oxidase, since pyruvate is an easy source for generation of reducing power to drive biosynthesis. Hence the FDH, when solubilized, would be mostly the FDH II form. On the other hand, the FDH in this work was obtained from cells grown in oxalate or formate alone. This would tend to favour the presence of the form which is not a part of the formate oxidase system. The presence of NADH and the neutral pH used (pH 7.5) during purification might have also contributed to the purification of a FDH preparation containing most of the enzyme in the "free" form (FDH I) catalyzing the oxidation of formate with the reduction of NAD^+ but not oxygen.

From the data gathered in this work it would appear that FMN does not influence the FDH activity, measured in terms of NAD^+ reduction. This is in contrast to the NAD^+-dependent FDH from R. palustris which was greatly stimulated by the addition of FMN or FAD in the reaction cuvette (Yoch and Linstrom, 1969), but is supported by the homogenous FDH obtained from peas by Ohyama and Yamazaki (1974). Their pea enzyme had a specific activity comparable to the enzyme preparation of Hoepner and Trautwein (1972) and this writer's, but was colourless and had no flavin. The basic enzyme

protein molecule itself thus appears to be active in the oxidation of formate coupled to NAD^+ reduction. There is a significant difference between the FDH obtained here and Hoepner and Trautwein's enzyme in regards to the apparent "inactivation" by formate. Hoepner and Trautwein's enzyme was only slowly "inactivated" whereas the enzyme obtained here was rapidly "inactivated" by formate, in the absence of NAD^+. Since it is suicidal and futile for a substrate to inactivate its own enzyme, it is tempting to speculate that the presence of formate merely causes a steric change in the enzyme protein that results in the site involving the binding of NAD^+ and its subsequent release as NADH being changed. This would serve to facilitate the efficient functioning of FDH as part of the formate oxidase system in transferring electrons via the electron transport chain and would naturally result in loss of "activity", viz a viz activity measured by NAD^+ reduction. Indeed, Hoepner and Trautwein (1972) noted that the "formate oxidase" particles they obtained were oxygen-labile and required the addition of formate for protection against loss of formate oxidizing activity. It is probable that the association of FMN, Fe and labile sulfur with the basic enzyme protein offerred some protection to their preparation of enzyme against the effects of formate.

As mentioned earlier, the pH of the environment during purification of the enzyme may also influence the form of enzyme that is recovered. Yoch and Lindstrom (1969) in purifying a NAD^+-dependent soluble FDH from the facultative phototroph R. palustris (the first NAD^+-dependent FDH in which flavin nucleotide involvement was implicated) found that slightly acid conditions (pH 6.8) favoured cytochrome C and DCPIP coupling by the enzyme, whereas an alkaline conditions (pH 8.0) favoured coupling to NAD^+. Although the FDH from R. palustris is similar to the P. oxalaticus enzyme, the specific activity is rather low by comparison.

The molecular weights obtained by Hoepner and Trautwein (1972) for their enzyme preparation was 200,000 and 300,000 daltons, by sucrose density gradient centrifugation. The larger species constituted the major portion of their enzyme preparation. This value is close to double that value which was obtained by this writer with his preparation. Peacock and Boulter's (1970) active mung-bean enzyme was 92,000 daltons and Ohyama and Yamazaki's homogenous pea enzyme was even smaller at 70,000 daltons. This pea FDH could be dissociated into two subunits of 42,000 daltons each by SDS polyacrylamide gel electrophoresis but they were not biologically active. The fact that the mung-bean FDH is quite small and that of Ohyama and Yamazaki (the purest FDH preparation obtained thus far) even smaller, suggests that the basic enzyme protein

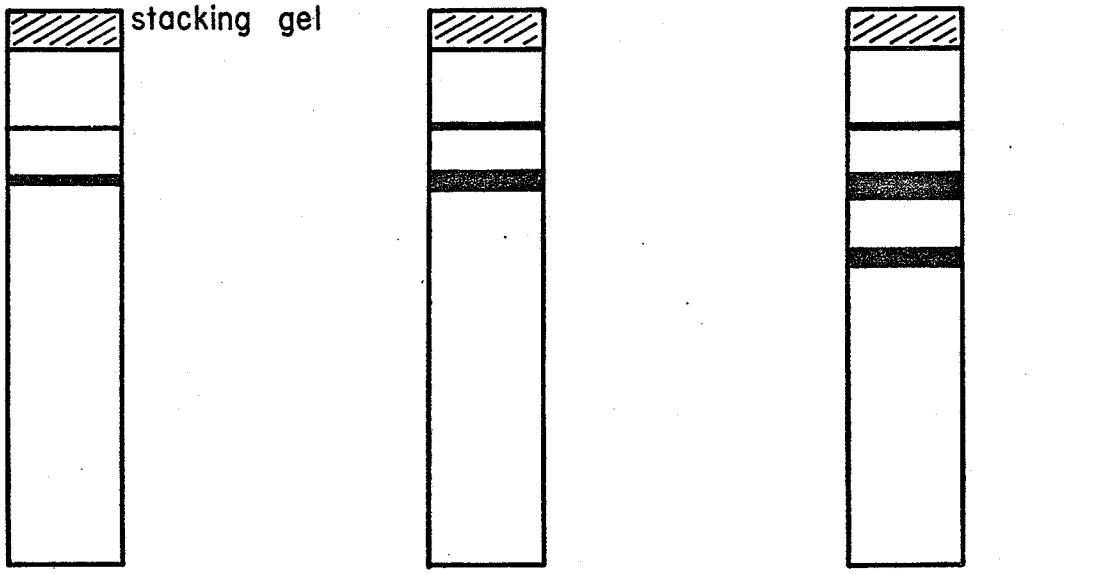
molecule which is capable of catalyzing NAD^+-dependent oxidation of formate is quite small. Despite the different sources of FDH, the great similarity in catalytic activity, kinetic behaviour and response to inhibitors indicates the closeness in enzyme structure as far as the basic protein portion (catalytically active) of the FDH is concerned. It then appears plausible that the P. oxalaticus enzyme has been modified to serve specific purposes in the different systems it may be a part of. This could account for the value of 200,000 daltons for Hoepner and Trautwein's minor species against the value of just over 150,000 daltons obtained here. The difference could then lie in the associated electron transport components such as FMN, non-heme iron and labile sulfur. It is even possible that these components might be present in a form of a flavoprotein moiety which is associated with the basic FDH protein, but there is no direct evidence to that effect available at the present time. Since their major species has a molecular weight double the value which was obtained here, it could be due to an aggregation of the enzyme since the enzyme in this study showed polydispersive tendencies in the absence of NAD^+.

Another interesting point which was noticed by Ohyama and Yamazaki (1974) and which was also encountered by this writer in the early part of the work, was the fact that certain "crude extracts" after being electrophoresized on

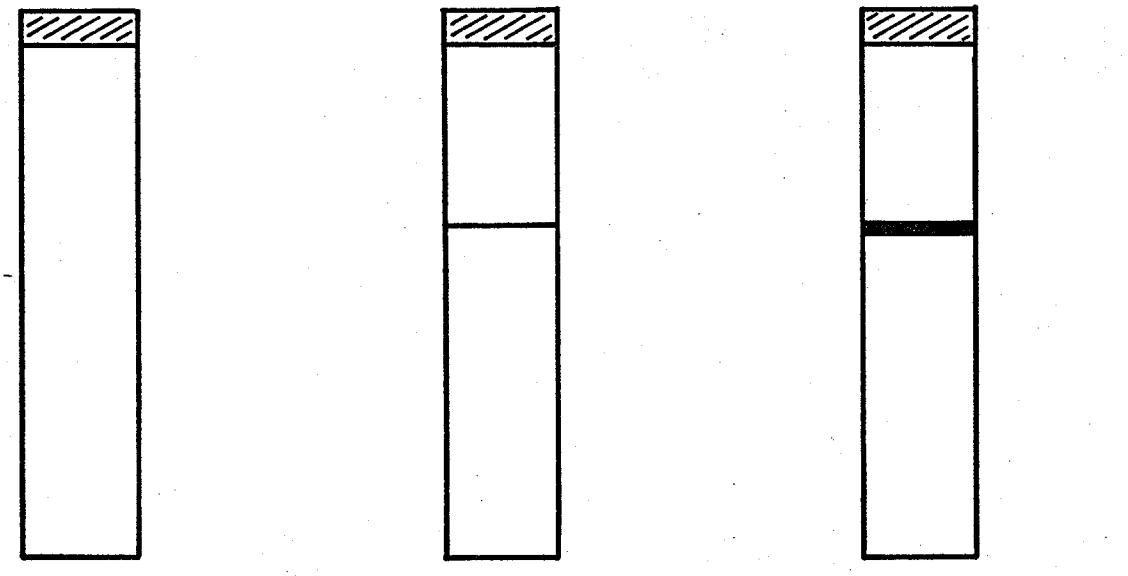
Figure 55. Diagrams of polyacrylamide gels specifically stained for FDH activity. (see "Methods")

POLYACRYLAMIDE DISC GELS WITH SPECIFIC FDH ACTIVITY STAIN

FORMATE - GROWN EXTRACTS



OXALATE - GROWN EXTRACTS



polyacrylamide gels and specifically stained for FDH activity (see "Methods") showed more than one activity band. This would lend support to the theory of the existence, *in vivo*, of more than one system involving FDH. With formate-grown cell extracts there were three activity bands, one of which would only stain in the presence of NAD^+. The oxalate-grown cell-extracts, however, always exhibited only one activity band. Purified preparations from both extracts, however, only showed one activity band. A diagrammatic representation of these gels is shown in Fig. 55 . Most of the time, the formate cell extracts showed apparent non-specific staining (weak) in the absence of added formate but this was probably due to the formate in the cell-extracts (Johnson *et al*, 1964 b). Ohyama and Yamazaki's crude preparations also showed three activity bands but their purified preparations exhibited only one activity band. Their enzyme also had a tenacious faint yellow colour until their final purification steps when it disappeared to yield a colourless preparation. There was no loss of enzyme activity by the removal of colour as far as the reduction of NAD^+ was concerned.

The three activity bands of the formate-grown cells would agree with the proposal that the basic enzyme protein is utilized in more than one system by cells growing on formate. The evidence is obviously preliminary and circumstantial and other interpretations might be possible. It would be interesting to study in greater detail the

molecular relationship of the different enzyme forms.

B I B L I O G R A P H Y

BIBLIOGRAPHY

Adler, E. and Sreenivasaya, M. (1937). The Components of Dehydrase Systems XVI. The formic acid and alcohol dehydrases of seeds (transl.). Z. physiol. chem. 249: 24-29.

Andersson, B. (1934). Cozymase as coenzyme in enzymic dehydrogenations (transl.). Fermentforschung 11: 469-489.

Andreesen, J. P. and Ljungdahl, L. (1971). Conversion of CO_2 (HCO_3^-) to formyltetrahydrofolate by formate dehydrogenase and formyl tetrahydrofolate synthetase from Clostridium thermoaceticum. Bact. Proc. (1971): 166.

Andrews, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91: 222-233.

Bassalik, K. (1913). "Uber die Verarbeitung der Oxalsaure durch Bacillus extorquens, n. sp.". Jahrb. Wiss. Botan. 53: 255-302.

Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T. and Calvin, M. (1954). The Path of Carbon in Photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. Amer. Chem. Soc. J. 76:

1760-1770.

Bhat, J. V. and Barker, H. A. (1948). Studies on a new oxalate-decomposing bacterium, Vibrio oxalaticus. J. Bact. 55: 359-368.

Blackmore, M. A., Quayle, J. R. and Walker, I. O. (1968 a). Choice between Autotrophy and Heterotrophy in Pseudomonas oxalaticus. Utilization of oxalate by cells after adaptation from growth on formate to growth on oxalate. Biochem. J. 107: 699-704.

Blackmore, M. A., and Quayle, J. R. (1968 b). Choice between Autotrophy and Heterotrophy in Pseudomonas oxalaticus. Growth in mixed substrates. Biochem. J. 107: 705-713.

Bradshaw, W. H. and Reeder, D. J. (1964). Ferredoxin coupling of formate oxidation to urate reduction in extracts of Clostridium cylindrosporium. Bact. Proc. (1964): 110.

Brill, W. J., Wolin, E. A. and Wolfe, R. S. (1964). Anaerobic formate oxidation: A ferredoxin-dependent reaction. Science 144: 297-298.

Chambrach, A., Reisfield, R. A., Wycoff, M. and Zacavi, J. (1967). A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal. Biochem. 20: 150-154.

Chung, S. T., Tan, R. T. Y. and Suzuki, I. (1971). Glyoxylate Carboligase of Pseudomonas oxalaticus. A possible structural role for Flavin-Adenine Dinucleotide.

Biochemistry 10: 1205-1209.

Cleland, W. W. (1963 a). The kinetics of enzyme-catalysed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim. Biophys. Acta., 67: 104-137.

_____ (1963 b). Ibid. II. Inhibition: Nomenclature and theory. Biochim. Biophys. Acta 67: 173-187.

_____ (1963 c). Ibid. III. Predictions of initial velocity and inhibition patterns by inspection. Biochim. Biophys. Acta, 67: 188-196.

Colowick, S. P., Kaplan, N. O. and Ciotti, M. M. (1951). The reaction of pyridine nucleotide with cyanide and its analytical use. J. Biol. Chem. 191: 447-459.

Conway, A. and Koshland, D. E. (1968). Negative Cooperativity in Enzyme Action. The binding of diphosphopyridine nucleotide to glyceraldehyde-3-phosphate dehydrogenase. Biochemistry 7: 4011-4022.

Davis, B. J. (1964). Disc Electrophoresis II. Method and application to human serum proteins. Annals. N. Y. Acad. Sci. 121 404-427.

Davison, D. C. (1951). Studies on plant formate dehydrogenase. Biochem. J. 49: 520-526.

Fawcett, C. P., Ciotti, M. M. and Kaplan, N. O. (1961). Inhibition of dehydrogenase reactions by a substance formed from reduced diphosphopyridine nucleotide. Biochim. Biophys. Acta 54: 210-212.

Fodor, A. and Frankenthal, L. (1930). The Mode of Action of Dehydrogenases. I. The dehydrogenase of the pea and its so-called coenzyme (transl.). Fermentforschung 11: 469-489.

Frieden, C. (1970). Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept. J. Biol. Chem. 245: 5788-5799.

Gale, E. F. (1939). CXXV. Formic Dehydrogenase of Bacterium coli: Its inactivation by oxygen and its protection in the bacterial cell. Biochem. J. 33: 1012-1027.

Gest, H. (1951). Phosphorus metabolism. Vol. II. ed. McElroy W.D. and Glass, B. : 522.

Gest, H. and Peck, H. D. (1955). A study of the hydrogenlyase reaction with systems derived from normal and anaerogenic coli-aerogenes bacteria. J. Bacteriol. 70: 326-334.

Gray, C. T. and Gest, H. (1965). Biological formation of molecular hydrogen. Science 148: 186-191.

Greaves, R. I. N. (1960). Preservation of living cells by freeze-drying. Annals N.Y. Acad. Sci. 85: 723-728.

Hauge, J. G. (1957). Formic acid oxidation in Aspergillus niger. Biochim. Biophys. Acta 25: 148-155.

Hess, W. M. (1966). Fixation and staining of Fungus hyphae and host plant root tissue for electron microscopy. Stain. Technol. 41: 27-35.

Hoepner, T. and Knappe, J. (1970). Formate. Determination with Formate dehydrogenase. Methods of Enzymatic Analysis (transl.). ed. H. U. Bergmeyer, 2nd ed. : 1509-

1513.

Hoepner, T. and Trautwein, A. (1971). Pseudomonas oxalaticus: Requirement of a cosubstrate for growth on formate. Arch. Mikrobiol. 77: 26-35.

_____. (1972). Some properties of Formate Dehydrogenase. Z. Naturforsch. 27b: 1075-1076.

Hug, D. H. and Sagers, R. D. (1957). Properties of a soluble formic dehydrogenase from Clostridium acidurici. Bact. Proc. (1957): 111.

Iida, K. and Taniguchi, S. (1959). Nitrate reductase system of Escherichia coli. I. Particulate electron transport system to nitrate and its solubilization. J. Biochem. 46: 1041-1055.

Ishimoto, M., Koyama, J., Omura, T. and Nagai, Y. (1954). Sulfate-reducing bacteria III. Sulfate reduction by cell suspension. J. Biochem. 41: 537-546.

Itagaki, E., Fujita, T. and Sato, R. (1961). Solubilization and some properties of formic dehydrogenase from Escherichia coli. Biochem. Biopnys. Res. Comm. 51: 30-34.

Itagaki, E. (1964). The role of lipophilic quinones in the electron transport system of Escherichia coli. J. Biochem. 55: 432-445.

Janota, L. (1950). Med. Doswindezalna i Mikrobiologie 2: 131-132. transl. in (1951) Chem. Abstr. 45: 7637.

Jayasuriya, G. C. N. (1955). The isolation and characteristics of an oxalate-decomposing organism. J. Gen. Microbiol. 12: 419-428.

Johnson, P. A. and Quayle, J. R. (1964 a). Microbial growth on c_1 compounds. 6. Oxidation of methanol, formaldehyde and formate by methanol-grown Pseudomonas AM 1. Biochem. J. 93: 281-289.

Johnson, P. A., Jones-Mortimer, M. C. and Quayle, J. R. (1964 b). Use of a purified bacterial formate dehydrogenase for the micro-estimation of formate. Biochim. Biophys. Acta 89: 351-353.

Kaneda, T. and Roxburgh, J. M. (1959). Methanol-utilizing bacterium. II. Studies on the pathway of methanol assimilation. Can. J. Microbiol. 5: 187-195.

Kaplan, N. O., Ciotti, M. M. and Stolzenbach, F. E. (1954). The action of hydroxylamine and cyanide on alcohol dehydrogenase of horse liver. J. Biol. Chem. 211: 419-429.

Kaplan, N. O. and Ciotti, M. M. (1954). Direct evidence for a diphosphopyridine nucleotide-hydroxylamine complex with horse-liver alcohol dehydrogenase. J. Biol. Chem. 211: 447-463.

Kellenberger, E., Sechaud, J. and Ryter, A. (1959). Electron Microscopical Studies on Phage Multiplication. IV. The establishment of the DNA pool of vegetative phage and the maturation of phage particles. Virology 8: 478-498.

King, E. L. and Altman, C. (1956). A schematic method of deriving the rate laws for enzyme catalysed reactions. J. Phy. Chem. 60: 1375-1378.

Khambata, S. R. and Bhat, J. V. (1953 a). Studies on a new oxalate-decomposing bacterium Pseudomonas oxalaticus. J. Bacteriol. 66: 505-507.

Khambata, S. R. and Bhat, J. V. (1953 b). Bacterium oxalaticum, a new oxalate-decomposing bacterium isolated from the intestine of earthworms. Proc. Indian Acad. Sci. (B) 38: 157-160.

Kornberg, H. L. and Reeves, R. E. (1972). Correlation between hexose transport and phosphotransferase activity in Escherichia coli. Biochem. J. 126: 1241-1243.

Lederberg, J. and Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399-406.

Lentz, K. and Wood, H. G. (1955). Synthesis of acetate from formate and carbon dioxide by Clostridium thermoaceticum. J. Biol. Chem. 215: 645-654.

Li, L. F., Ljundahl, L. and Wood, H. G. (1966). Properties of NADP-dependent formate dehydrogenase from Clostridium thermoaceticum. J. Bacteriol. 92: 405-412.

Lichenstenstein, N. (1936). The effect of enzymic hydrolysis of phosphoric acid from Cozymase and its effect on pea dehydrogenase systems (transl.). Fermentforschung 15: 44-48.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56: 658-666.

Lowry, O. H., Rosebrough, N. J. Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Martin, R. G. and Ames, B. N. (1961). A method for determining the sedimentation behaviour of enzymes: Application to protein mixtures. J. Biol. Chem. 236: 1372-1379.

Mathews, M. B. and Vennesland, B. (1950). Enzymic oxidation of formic acid. J. Biol. Chem. 186: 667-682.

Monod, J. (1949). The growth of bacterial cultures. Ann. Rev. Micro. 3: 371-394.

Nakada, H. I. and Weinhouse, S. (1953). Studies of glycine oxidation in rat tissues. Arch. Biochem. Biophys. 42: 257-270.

Ohyama, T. and Yamazaki, I. (1974). Purification and some properties of formate dehydrogenase. J. Biochem. 75: 1257-1263.

Ohyama, T. and Yamazaki, I. (1975). Formate dehydrogenase. Subunit and Mechanism of Inhibition by cyanide and azide. J. Biochem. 77: 845-852.

Ornstein, L. (1964). Disc Electrophoresis. I. Background and theory. Annals N.Y. Acad. Sci. 121: 321-349.

Peacock, D. and Boulter, D. (1970). Kinetic studies of Formate Dehydrogenase. Biochem. J. 120: 763-769.

Peck, H. D. and Gest, H. (1957). Formic dehydrogenase and the hydrogenlyase enzyme complex in coli-aerogenes bacteria. J. Bacteriol. 73: 706-721.

Poiger, H., Falkner, G. and Michl, H. (1969). Enzymatic formate determination with formate dehydrogenase from peas (transl.). Mikrochimica Acta (Wien) : 1165-1169.

Quayle, J. R. and Keech, D. B. (1958). Carbon dioxide and formate utilization by formate-grown Pseudomonas oxalaticus. Biochim. Biophys. Acta, 29: 223-225.

_____. (1959, a). Carboxydismutase activity in formate- and oxalate-grown Pseudomonas oxalaticus (strain OX 1) Biochim. Biophys. Acta, 31: 587-588.

_____. (1959, b). Formation of glycerate from oxalate by Pseudomonas oxalaticus (OX 1) grown in oxalate. Nature 183: 1794-1795.

_____. (1959, c). Carbon Assimilation by Pseudomonas oxalaticus (OX 1). 1. Formate and carbon dioxide utilization during growth on formate. Biochem. J. 72: 623-630.

_____. (1959, d). Ibid. 2. Formate and carbon dioxide utilization by cell-free extracts of the organism grown on formate. Biochem. J. 72: 631-637.

_____. (1960). Ibid. 3. Oxalate utilization during growth on oxalate. Biochem. J. 75: 515-523.

Quayle, J. R., Keech, D. B. and Taylor, G. A. (1960). Metabolism of oxalate in oxalate-grown Pseudomonas oxalaticus (OX 1). Biochem. J. 76: 3p.

_____. (1961, a). Carbon Assimilation by Pseudomonas oxalaticus (OX 1). 4. Metabolism of oxalate in cell-free extracts of the organism grown on oxalate. Biochem. J. 78: 225-236.

Quayle, J. R. and Taylor, G. A. (1961, b). Ibid. Purification and properties of glyoxylic dehydrogenase. Biochem. J. 78: 611-615.

Quayle, J. R. (1962). Chemical synthesis of oxalyl-coenzyme A and its enzymic reduction to glyoxylate. Biochim. Biophys. Acta 57: 398-400.

_____. (1963, a). Enzymatic decarboxylation of oxalyl-coenzyme A to formyl-coenzyme A. Biochem. J. 87: 40p.

_____. (1963, b). Carbon Assimilation by Pseudomonas oxalaticus (OX 1). 6. Reactions of oxalyl-coenzyme A. Biochem. J. 87: 368-373.

_____. (1963, c). Ibid. 7. Decarboxylation of oxalyl-coenzyme A to formyl-coenzyme A. Biochem. J. 89: 492-503.

_____. (1966). Formate dehydrogenase assay method. Methods of Enzymology. IX. Edit. Colowick, S.P. and Kaplan, N. O. : 360-364.

Rapporport, D. A., Green, J. A. and Gest, J. A. (1956). Formate oxidation by erythrocytes. Arch. Biochem. Biophys. 63: 343-351.

Schlenk, F., van Euler, H., Heiwinkel, H., Glein, W. and Nystrom, H. (1937). The action of alkali on Cozymase. Z. Physiol. Chem. 247: 23-33.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Research 26: 31-43.

Thunberg, T. (1921). The presence of certain oxidizing enzymes in the seeds of Phaseolus vulgaris (transl.). Arch. Int. Physiol. 18: 601-606.

Van Eys, J., Stolzenbach, F. E., Sherwood, L. and Kaplan, N. O. (1958). Enzyme-coenzyme-substrate complexes of pyridine nucleotide-dependent dehydrogenases. Biochim. Biophys. Acta 27: 63-83.

Warburg, O., Christian, W. and Griese, A. (1935). Hydrogen-transferring coenzyme, its composition and mode of action. Biochem. Z. 282: 157-205.

Wrigley, C. W. and Linnane, A. W. (1961). Formic acid dehydrogenase-cytochrome b_1 complex from Escherichia coli. Biochem. Biophys. Res. Comm. 4: 66-70.

Yagi, T. (1969). Formate : Cytochrome oxidoreductase of Desulfovibrio vulgaris. J. Biochem. 66: 473-478.

Yamamoto, Y. (1954). On formic dehydrogenase of bean seed embryo in the germination stage. J. Biochem. 41: 551-565.

Yoch, D. C. and Lindstrom, E. S. (1969). Nicotinamide adenine dinucleotide-dependent formate dehydrogenase from Rhodopseudomonas palustris. Arch. Mikrobiol. 67: 182-188.

Yue, R. H., Noltmann, E. A. and Kuby, S. A. (1967). Glucose-6-phosphate dehydrogenase (Zwischenferment). II. Homogeneity measurements and physical properties of the crystalline apoenzyme from yeast. Biochemistry 6: 1174-1183.