PURIFICATION AND PROPERTIES OF A DIACETYL REDUCTASE FROM *ESCHERICHIA COLI*

and

A PRELIMINARY STUDY OF A FATTY ACID PERMEASE IN *ESCHERICHIA COLI*

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfilment of the requirements for the degree of Doctor of Philosophy, The City University of New York

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

3-26-76
date

Chairman of Examining Committee

3/26/76
date

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ABSTRACT

An ethyl acetoacetate-dependent NADPH-oxidase was found in Escherichia coli as a contaminant of β-ketoacyl acyl carrier protein reductase. This enzyme was purified 800-fold to near homogeneity and characterized. An extensive substrate study revealed that the enzyme catalyzed the oxidation of NADPH in the presence of both uncharged α- and β-dicarbonyl compounds. However, the best substrate proved to be diacetyl which is reduced to acetoin. On the basis of its substrate specificity, it is suggested that the enzyme functions in vivo as a diacetyl reductase. In contrast to other diacetyl reductases, the one reported here is specific for NADPH and does not possess acetoin reductase activity. The pH optimum of the enzyme was found to be between 6 and 7. The maximal velocity for the NADPH-dependent reduction of diacetyl was determined to be 9.5 μmoles per min per mg of protein and the Km values for diacetyl and NADPH were found to be 4.44 mM and 0.02 mM, respectively. The molecular weight was estimated by gel filteration on Sephadex G-100 to be approximately 10,000.

In a preliminary study of fatty acid uptake and activation in E. coli the following observations were made. Induced cells grown on oleate as sole carbon source contained levels of the enzyme acyl-CoA synthetase which were 10-fold higher than in uninduced cells grown on acetate as sole carbon source. Triton X-100 extracts of the cytoplasmic membrane

of induced cells did not show the presence of the acyl-CoA synthetase. In a double label experiment, no convincing evidence for the existence of a fatty acid permease was found.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Horst Schulz whose constant guidance and encouragement made this all possible.

I also would like to thank my fellow graduate students and teachers not only for making my stay at City College a pleasant one, but also for their helpful advice.

Last, but by no means least, I would like to thank my dear wife Shirley for her constant patience and understanding while this work was in progress and for the many hours she spent typing this manuscript.

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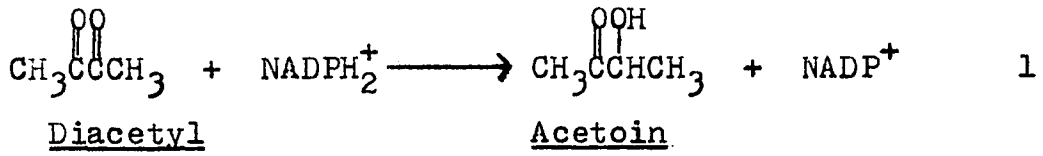
INTRODUCTION

The initial aim of this thesis was to study some aspects of fatty acid metabolism in the bacterium Escherichia coli. Specifically, the aim was to purify and characterize an enzyme activity which was detected during the purification of the enzyme β-ketoacyl acyl carrier protein reductase (1) and which catalyzed the oxidation of NADPH in the presence of β-ketoesters and β-ketothioesters. The reason for studying this newly detected β-ketoester NADPH-oxidase was its possible involvement in fatty acid metabolism. However, while determining the enzyme's substrate specificity with a number of different compounds it became apparent that the enzyme was most active with diacetyl (2,3-butanedione). The enzyme catalyzed the reduction of one keto group of diacetyl to a hydroxyl group with the simultaneous oxidation of NADPH. Thus, the products of the reaction are acetoin (3-hydroxy-2-butanone) and $NADP^+$ as shown in equation 1.



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Diacetyl and acetoin are biologically important compounds because of their role as flavor ingredients in milk, butter and such fermented foods as cheese and beer. The microorganisms responsible for the production of the compounds are primarily the bacterial strains Streptococci, Lactobacilli,

and Leuconostoc as well as yeast. Diacetyl and acetoin are produced through the fermentation of pyruvate.

Pyruvate can be metabolized in several different ways. (See Fig. 1.) Under aerobic conditions pyruvate is usually completely oxidized via the Krebs cycle to CO_2 and H_2O. However, under anaerobic conditions pyruvate can be metabolized through a number of pathways called collectively fermentations. The pathway used and the products formed depend on the organism and the growth conditions. In one of these pathways pyruvate is reduced to lactate with the simultaneous oxidation of NADH to NAD^+. Other metabolic pathways lead to the formation of a number of aldehydes, ketones, organic acids and alcohols. Diacetyl and acetoin are formed by certain of these fermentative pathways.

A central compound in the fermentative pathways leading to diacetyl and acetoin formation is 2-hydroxyethyl thiamine pyrophosphate (HETPP). HETPP is formed as a result of the reaction of pyruvate and thiamine pyrophosphate which is catalyzed by the enzyme pyruvate decarboxylase (See Fig. 1). This can be further converted directly to acetoin by either of two pathways. In bacteria the HETPP can react with pyruvate to give α-acetolactate which is decarboxylated to give acetoin (2). In mammals and yeast, however, the HETPP reacts with acetaldehyde to form acetoin (3). Another pathway of acetoin formation in bacteria requires the prior formation of diacetyl through the condensation of HETPP with acetyl CoA.

The diacetyl is then reduced to acetoin in the presence of NADH by the enzyme diacetyl reductase (4,5). (For the different reactions see Fig. 1.)

Chuang and Collins in a survey of diacetyl and acetoin metabolism in a number of microorganisms reported that E. coli did not produce diacetyl but only acetoin (6). Furthermore, E. coli was found not to possess a diacetyl reductase (6). Instead, cell-free extracts of E. coli produced acetoin from pyruvate only when free acetaldehyde was added (6), a fact also reported by Juni and Heyms (7). Thus, the formation of acetoin in E. coli is different from that of other bacteria which are able to produce acetoin from pyruvate via ∝-acetolactate and are also able to form diacetyl which can be reduced by diacetyl reductase to acetoin.

Diacetyl reductases have been reported in different bacterial systems (8-16) as well as in mammalian and avian liver (17-21). The diacetyl reductase of Aerobactor aerogenes has been extensively studied by Stormer and his associates (10-15). The enzyme was found to be highly specific with respect to the diacetyl substrate (13) and also the enzyme had an absolute requirement for NADH as cofactor (13). A beef liver and pigeon liver enzyme found and studied by Burgos and Martin also was very specific for the substrate diacetyl, but could use NADH and NADPH interchangeably (19,20). The molecular weights of the enzymes were 100,000 for the A. aerogenes enzyme (12) and 76,000 and 110,000 for the beef liver (19) and pigeon liver (20) enzyme respectively. The

subunit composition of the enzymes was also investigated. The A. aerogenes diacetyl reductase was found to be composed of four equal subunits of 25,000 molecular weight (11) and also consisted of several isoelectric species (12). The beef liver diacetyl reductase on the other hand comprised of three subunits of 26,000 molecular weight (19). The kinetic parameters of the different diacetyl reductases have been found to vary considerably. Whereas, the Km for diacetyl for the diacetyl reductases from A. aerogenes and pigeon liver are 1.2 mM and 3.5 mM respectively (13,20), the beef liver enzyme had a Km of 40 uM indicating a high affinity for diacetyl (18). In a report on a diacetyl reductase from rat liver an unusually high Km value for diacetyl of 48 mM was found (21). The diacetyl reductase from A. aerogenes is unusual in being able to catalyze the reversible NADH-dependent reduction of acetoin to 2,3-butanediol as well as the irreversible reduction of diacetyl to acetoin (10). (See Table 1 for a comparison of the different diacetyl reductases.)

In the present report the existence of an NADPH-dependent diacetyl reductase in E. coli B is shown. The enzyme was purified to near homogeneity and characterized.

MATERIALS AND METHODS

Chemicals. NADH and NADPH were purchased from PL-Biochemicals. Ethyl acetoacetate was bought from Fisher Scientific Co. Ethyl pyruvate, oxalacetic acid, lithium acetoacetate,

2-oxoglutaric acid and methyl acetoacetate were obtained from Sigma Chemical Co. Dehydroascorbate was bought from Schwartz-Mann. 2,4-Pentanedione, pyruvic acid, diacetyl, and acetoin were purchased from Aldrich Chemical Co. Diacetyl, acetoin, and ethyl acetoacetate were distilled before use whereas, all other chemicals were used without further purification.

Synthesis of substrates. α -Ketopantolactone was prepared by chromic acid oxidation of pantolactone analogously to a procedure by Fieser (22). The product was recrystallized from ether/hexane (melting point 58° to 61° C, literature melting point 60°C (23); nuclear magnetic resonance, δ = 1.4(S,6H) $(CH_3)_2C\begin{matrix}C\\C\end{matrix}$, δ = 4.5 (S,2H)-CH_2-O-).

The following substrates were synthesized by established procedures, and their respective melting and boiling points agreed with the values in the literature; ethyl levulinate (24), ethyl thiolacetoacetate (1), acetoacetyl N-acetylcysteamine (25), and methyl β -ketohexanoate (26).

Organism. E. coli B ATCC 11303 cells were purchased as a frozen cell paste from Grain Processing Corp., Muscatine, Iowa. These cells were grown on a medium containing casein hydrolysate, yeast extract, and dextrose as carbon source and were harvested at three-quarter log.

Purification of diacetyl reductase. Frozen E. coli cells (1 kg) were suspended in 1 liter of 0.05 M potassium phosphate (pH 7.0). The suspension was irradiated with a Branson

sonifier, at its maximum setting, for 10 min at 4°C. All further operations were carried out at 4°C. Reductase activity assays throughout this purification were performed with ethyl acetoacetate as substrate, as described under "Protein and Enzyme Determination." The resulting crude homogenate was centrifuged at 16,000 g for 30 min, and the precipitate was discarded. The supernatant was fractionated with solid ammonium sulfate, and the protein fraction that precipitated between 45% to 75% saturation was collected. The precipitate was suspended in a minimal volume of 0.01 M potassium phosphate (pH 7.0) buffer and dialyzed extensively against the same buffer. The dialyzed solution was applied onto a diethylaminoethyl (DEAE)-cellulose column (6.5 by 40 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). The column was washed with the same buffer until no more ultraviolet absorbing material was eluted. The column was then washed with 0.01 M potassium phosphate (pH 7.0), 0.1 M NaCl, until all reductase activity had been eluted. The enzyme was precipitated by bringing the eluate to 90% saturation with solid ammonium sulfate. The precipitated protein was isolated by centrifugation, suspended in a minimal volume of 0.01 M potassium phosphate (pH 7.0) and dialyzed overnight against several changes of the same buffer. The dialyzed protein solution was then chromatographed on another DEAE-cellulose column (4 by 45 cm) with a linear gradient made from 1 liter of 0.01 M potassium phosphate (pH 7.0) and 1 liter of 0.01 M potassium phosphate (pH 7.0) containig 0.3 M NaCl. Fractions of 20 ml each were collected and assayed

for reductase activity. The fractions containing active enzyme were pooled and brought to 90% saturation with solid ammonium sulfate. The precipitated protein was isolated by centrifugation, suspended as before in 0.01 M potassium phosphate (pH 7.0) and chromatographed on a Sephadex G-100 column (5 by 45 cm) previously equilibrated with 0.01 M potassium phosphate (pH 7.0). Fractions of 15 ml were collected and assayed for reductase activity. Fractions with high activity were pooled, precipitated by saturation to 90% with ammonium sulfate, and isolated by centrifugation. The precipitated protein was suspended as before in 0.01 M potassium phosphate (pH 7.0) and dialyzed overnight against 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0). The dialyzed solution was then chromatographed on a DEAE-Sephadex A-50 column (2.5 by 30 cm) which was previously equilibrated with 0.01 M Tris-hydrochloride (pH 7.0) and eluted with a linear gradient made of 1 liter of 0.01 M Tris-hydrochloride (pH 7.0) and 1 liter of 0.01 M Tris-hydrochloride (pH 7.0) containing 0.3 M NaCl. Fractions of 20 ml were collected and assayed. Again the fractions with the most activity were pooled and the protein was precipitated by saturation to 90% with ammonium sulfate. The protein was isolated by centrifugation and stored in this form at -20°C. Data for this purification procedure are presented in Table 2.

Protein and enzyme determinations. Protein concentrations were determined according to Lowry et al. (27) or by the biuret method (28). The enzyme assays were based on the

substrate-dependent oxidation of NADPH and were followed by measuring the decrease in light absorbance at 340 nm at 25°C on a Gilford recording spectrophotometer, model 240. A typical assay contained 100 μmol of potassium phosphate (pH 7.0), 0.11 μmol of NADPH, and 20 μmol of substrate in a total volume of either 0.6 ml or 0.8 ml. The reaction was started by the addition of enzyme except where otherwise indicated.

Disc gel electrophoresis. Electrophoresis of diacetyl reductase was performed with a standard 7.5% acrylamide gel and Tris-glycine buffer (pH 8.5) at 20°C by the procedure of Davis (29). Between 50 μg to 100 μg of protein were applied to each gel. Gels were run in duplicate. One gel was stained with amido black and then destained in 7% acetic acid. The other gel was sliced and each slice was eluted with 1 ml of 0.01 M potassium phosphate buffer (pH 7.0) for 1 h and then assayed for reductase activity as described above.

RESULTS

The diacetyl reductase, which was isolated from E. coli B cells was purified by ammonium sulfate precipitation, followed by chromatography on DEAE-cellulose, Sephadex G-100 and DEAE Sephadex A-50 as summarized in Table 2. The chromatography on DEAE-cellulose (Fig. 2) did not give an optimal purification but allowed for some separation of the reductase

from the bulk protein. A better separation of the enzyme from the bulk protein was achieved by chromatography on Sephadex G-100 (Fig. 3). Additionally, the fact that the enzyme appeared late in the elution profile indicated that the reductase activity was associated with a protein of relatively low molecular weight.

The final purification step on DEAE-Sephadex A-50 gave a good separation of the reductase from the bulk protein (Fig. 4). The resulting enzyme preparation was purified over the original homogenate 800-fold but was obtained in low yield. The purity of this reductase preparation was evaluated by disc gel electrophoresis on polyacrylamide gels. Figure 5 shows that the activity peak coincides with the protein peak. However, as seen in Fig. 5 the protein peak is broad and has shoulders not seen in the activity peak. This observation indicates that there were still noticeable impurities in the preparation and, in subsequent gel electrophoresis runs, evidence was obtained that the broad protein band was due to the presence of two proteins. However, as demonstrated in Fig. 6, both ethyl acetoacetate reductase activity and diacetyl reductase activity are associated with the same band on disc gel electrophoresis.

The molecular weight of the enzyme was estimated by chromatography on Sephadex G-100 using bovine serum albumin, ovalbumin, pepsin, chymotrypsinogen A, myoglobin, ribonuclease, and cytochrome c as standards (Fig. 7). It was found to be

10,000, an unusually small molecular weight for a pyridine nucleotide-dependent dehydrogenase. Since this reductase has such a low molecular weight, it is unlikely that the enzyme is composed of subunits, although this point has not yet been clarified.

Substrate studies. The specificity of the reductase was investigated by testing a series of different compounds, containing at least one keto group, for their ability to serve as substrates of this enzyme. The highest activity was found with diacetyl as substrate (Table 3). Since the enzyme was highly active also with ethyl pyruvate, but showed little activity with pyruvate, oxalacetate, or 2-oxoglutarate, it can be concluded that the enzyme recognizes an uncharged α-dicarbonyl structure. A recent report demonstrating the presence of an α-ketopantolactone reductase in E. coli (30) led us to test α-ketopantolactone as a possible substrate. However, this compound proved to be a very poor substrate for this enzyme, and thus it is unlikely that the enzyme purified by us is identical with the described α-ketopantolactone reductase. The reductase also showed significant activity with β-keto esters, such as ethyl acetoacetate and ethyl thiolacetoacetate, but again showed negligible activity with a free β-keto acid such as acetoacetate. An increase in the chain length of the substrate leads to a decrease in the activity as shown by the negligible activity observed with methyl β-ketohexanoate as compared to the activity found with methyl acetoacetate. The β-diketone 2,4-pentanedione

was a poor substrate when compared to β-keto esters. The fact that this enzyme showed a very small but definite amount of activity with monocarbonyl compounds such as acetone and acetaldehyde is indicative of an enzyme with a broad substrate specificity. Since the best substrate found during the course of this investigation was diacetyl and since the other potential substrates are unlikely metabolites in E. coli, it is suggested that the enzyme is a diacetyl reductase, which in contrast to the one reported in A. aerogenes (11) does not possess any acetoin reductase activity. This reductase is highly specific for NADPH as evidenced by the fact that the enzyme was 10 times more active with NADPH than it was with NADH. All attempts to measure the reverse reaction both at pH 7 and 9 met with negative results even in the presence of increased amounts of enzyme. The same results were obtained when NAD^+ was substituted for $NADP^+$.

Effect of pH and buffers. To determine the optimal pH for diacetyl reductase, the rate of NADPH oxidation in the presence of diacetyl was determined between pH 5 and 8. The optimal pH was found to be between 6 and 7 as shown in Fig. 8.

When, during the course of the pH-activity study, Tris-hydrochloride buffer was substituted for potassium phosphate at pH 7.5, the activity was reduced by a factor of 7, while with ethyl acetoacetate as substrate, the activity remained unchanged. Since this finding might suggest the presence of

separate diacetyl and ethyl acetoacetate reductase activities in the enzyme preparation, it was decided to investigate the effect of Tris-hydrochloride on the reduction of diacetyl. When the reaction was started by the addition of diacetyl to the assay mixture, a rapid initial velocity (Fig. 9, curve 2) comparable to that determined in potassium phosphate buffer (Fig. 9, curve 1) was observed. The velocity rapidly decreased and became identical to the velocity observed when the reaction was started by the addition of enzyme (Fig. 9, curve 4). These observations suggest that diacetyl reacted with Tris-hydrochloride to give a compound which either no longer functioned as a substrate of diacetyl reductase or was a very poor substrate analogue. This compound probably results from the condensation of the amino group of the Tris-hydrochloride and one of the carbonyls of diacetyl to give a Schiff base. Support for this suggestion comes from the finding that the addition of Tris-hydrochloride to diacetyl resulted in the disappearance of the diacetyl absorbance near 400 nm. This change in absorbance, which can also be followed at 340 nm, was completed in 2 to 3 min as shown in Fig. 9, curve 5. The rapid reaction between diacetyl and Tris-hydrochloride was reflected by the fast decrease in the rate of NADPH oxidation, due to the disappearance of free diacetyl (Fig. 9, curve 2). The constant rate of oxidation which was reached after 2 to 3 min was nearly identical to the rate observed when diacetyl and Tris-hydrochloride were preincubated (Fig. 9, curve 4). This rate then reflected the concentration of the remaining

unreacted diacetyl or the rate of reduction of the Tris-diacetyl derivative. The difference between curves 2 and 5 is represented by curve 3, which reflects the decrease in the rate of diacetyl-dependent NADPH oxidation as a function of the decreasing diacetyl concentration.

Identification of product. To determine the product of the diacetyl-dependent oxidation of NADPH, the reaction was allowed to proceed for 2 days, NADPH was added successively to minimize its inhibition at higher concentrations. Even under these conditions only a small portion of diacetyl was reduced. The incubation mixture as well as the control which did not contain enzyme were then chromatographed on a Dowex 1-X8 column as described by Speckman and Collins (36). Two peaks (I, II) were observed when the reaction mixture was chromatographed (Fig. 10A). Peak II coincides with the peak in Fig. 10C which represents the unreacted diacetyl of the control. Peak I was eluted at the same position as an acetoin standard which was separately chromatographed on the same column (See Fig. 10B). Although the method used for determining the concentrations of diacetyl and acetoin is not very reliable, a reasonably good agreement was observed between the amount of NADPH (0.293 µmol) oxidized and the amount of acetoin (0.247 µmol) formed. This result indicated a 1:1 stoichiometric relationship between the reduction of diacetyland the oxidation of NADPH.

An attempt was also made to identify the product of the

ethyl acetoacetate-dependent oxidation of NADPH. For this purpose the reaction mixture, as well as a control which did not contain enzyme, were incubated under the conditions described for the reduction of diacetyl. After 2 days, both the reaction mixture and the control were reacted with an alkaline hydroxylamine solution (pH 11.5) for 2.5 h at 25°C. The pH of the solutions were then brought to 7 and the water was removed under reduced pressure. The resulting solid residues were extracted with ether, and the ether extracts were concentrated and applied to a silica gel thin-layer plate together with a 3-hydroxybutyryl hydroxamic acid standard. The thin-layer plate was developed with a solvent system containing $CHCl_3$, CH_3OH, and acetic acid in the ratic of 79:20:1 and was sprayed with an aqueous $FeCl_3$ solution to visualize the hydroxamic acids. However, no 3-hydroxybutyryl hydroxamic acid was detected in the reaction mixture, indicating that the ethyl acetoacetate had not been reduced to ethyl 3-hydroxybutyrate as expected. Since this method has been successfully used to show that 3-hydroxyacyl-CoA dehydrogenase catalyzes the reduction of ethyl acetoacetate to ethyl 3-hydroxybutyrate, I am confident that no ethyl 3-hydroxybutyrate was formed in the diacetyl reductase catalyzed reaction.

Kinetic study of diacetyl reductase. The optimal substrate concentrations for the diacetyl-dependent oxidation of NADPH were determined. Substrate inhibition was observed at diacetyl concentrations above 40 to 60 mM (Fig. 11A).

More pronounced substrate inhibition was observed when the concentration of NADPH was increased beyond the optimal concentration of 0.15 mM (Fig. 11B). A similar inhibition was found with NADH as the coenzyme at concentrations above 0.3 mM in the presence of 20 mM diacetyl (data not shown).

An initial velocity pattern was obtained for the oxidation of NADPH as a function of the diacetyl concentration at various fixed levels of NADPH (Fig. 12). Measurements were made at an enzyme concentration at which the velocity of the reaction was proportional to the enzyme concentration. A replot of the slopes and intercepts versus the concentration of NADPH (Fig. 12, insert) yielded the kinetic parameters (Km, Vmax) for the diacetyl-dependent oxidation of NADPH (Table 4). The Km value for diacetyl was found to be 4.44 mM, a value which is of the same order of magnitude as the Km obtained with the diacetyl (acetoin) reductase of A. aerogenes (13). Additionally, the kinetic constants obtained with ethyl acetoacetate and acetoacetyl N-acetylcysteamine as substrates are listed in Table 4. These data clearly show that diacetyl is the preferred substrate and that NADPH and not NADH serves as the coenzyme in this reaction.

DISCUSSION

The diacetyl reductase described in this paper was not first recognized as such. As mentioned earlier, the enzyme was first detected by its ability to catalyze the oxidation

of NADPH in the presence of ethyl acetoacetate. It was only after testing several possible substrates, that the enzyme's ability to reduce diacetyl in the presence of NADPH was recognized. Thus, since the enzyme showed maximum activity with diacetyl as substrate and since diacetyl is a known metabolite in several organisms, it is proposed that the enzyme functions as a diacetyl reductase.

The E. coli diacetyl reductase reported here seems to be quite different from other known diacetyl reductase. In contrast to the diacetyl reductase from beef liver (19) and A. aerogenes (14), this enzyme has a broad substrate specificity as evidenced by its small but definite activities with monocarbonyl compounds such as acetone and acetaldehyde.

Another distinctive feature of this diacetyl reductase is its preference for NADPH as the coenzyme, a finding which contrasts with the absolute specificity of the A. aerogenes reductase (15) for NADH and with the ability of the beef liver and pigeon liver reductases to utilize equally well NADH and NADPH (19, 20).

The molecular weight of the enzyme was estimated by gel filtration to be 10,000. This value is surprisingly low for a pyridine nucleotide-dependent dehydrogenase, especially when it is compared to such values as 100,000, 110,000 and 76,000 which have been obtained for the molecular weights of the diacetyl reductase of A. aerogenes, pigeon and beef liver, respectively (12,19, 20).

An interesting aspect is the finding that ethyl acetoacetate, although supporting the oxidation of NADPH, did not yield the expected product, ethyl 3-hydroxybutyrate. Thus, it is concluded that either the carbonyl group of the ester function is reduced or that the reduced product formed a covalent bond with $NADP^+$. The latter possibility is supported by the known condensation of pyruvate with NAD^+ in the presence of lactate dehydrogenase to give a compound in which pyruvate is covalently bound to NAD^+ (33).

E. coli's unusual coenzyme requirement for NADPH may be understood in the context of its carbohydrate metabolism. It has been reported by Eagon that the extent to which E. coli utilizes the pentose phosphate pathway for glucose oxidation is dependent upon the availability of $NADP^+$ (34). Thus it is possible that the reduction of diacetyl is important for the formation of $NADP^+$ to be utilized in the pentose phosphate pathway. Additionally, the possibility exists that by combined action of diacetyl reductase and pyridine nucleotide transhydrogenase, which is known to exist in E. coli (42), NADH is oxidized in the presence of diacetyl.

In order to understand the function of the enzyme diacetyl reductase it is also necessary to look at how the organism metabolizes pyruvate. Ordinarily most of the cell's pyruvate is produced from glucose during glycolysis. Under anaerobic conditions though, pyruvate has a specific metabolic role, namely to regenerate the NAD^+ that is needed for glycolysis. Most bacteria accomplish this by reducing

pyruvate to lactate with the simultaneous oxidation of NADH to NAD^+. Thus, glycolytic pyruvate has an important function and is not available for acetoin formation. However, if the organism forms pyruvate by other pathways the pyruvate can be converted to acetoin.

This is indeed the case when many bacteria including E. coli are grown on citrate. This is due to the presence of an enzyme citratase which cleaves citrate to oxalacetate and acetate (9, 35-39). The oxalacetate is then decarboxylated to pyruvate (9, 35).

As a result of this discussion the importance of acetoin formation can be seen as a mechanism to reduce excessive levels of intracellular pyruvate (40). In the case where diacetyl is formed first, its subsequent reduction to acetoin may serve the two additional functions of regenerating the cell's supply of NAD^+ (4) and, also of removing diacetyl whose polymers are known to be toxic substances that react with arginine residues of proteins.

It would be useful to reinvestigate the mechanism of acetoin and diacetyl biosynthesis in this bacterium in order to answer the many questions that were raised.

INTRODUCTION

A cell membrane is a barrier which functions to protect the cell and to confine the cell's soluble contents while at the same time allowing nutrients to enter. Some nutrients like water can enter by diffusion. Other nutrients, however, require transport systems which are more complicated. These transport systems show such properties as substrate specificity, saturability with substrate, competitive and non-competitive inhibition and genetic determination. In recent years many studies have been done on bacterial transport systems for sugars, amino acids, and inorganic ions (41, 42). However, few studies have been done on the transport system involved in fatty acid uptake in bacteria. Recently though, the problem has been studied by a number of investigators and some of their findings are summarized below.

The specificity of fatty acid uptake has been studied by Overath (43). Working with an E. coli mutant constitutive for the enzymes of β-oxidation, he found a long-chain specific, irreversible fatty acid uptake system (43). Salanitro and Wegener reported that on the basis of specific growth rates and uptake inhibition studies that three physiologically distinct entry and/or activation systems for fatty acids seem to exist in E. coli: one for short-chain (C_4-C_5); one for medium chain (C_6-C_9); and one for long-chain (C_{10}-C_{18}) fatty acids (44, 45).

In an interesting study of fatty acid uptake Overath

demonstrated that mutants of E. coli lacking any of the β-oxidation enzymes or the acyl-CoA synthetase exhibited greatly reduced rates of fatty acid uptake (42). This along with the findings of the coinducibility of both fatty acid uptake and of the β-oxidation enzymes and the similar substrate specificities of both the acyl-CoA synthetase and fatty acid uptake suggest that fatty acid uptake is intimately tied to fatty acid oxidation (43). As a result of these observations Overath proposed that the synthetase was an integral part of the fatty acid uptake system and in order for fatty acids to be taken up they have to be converted to their CoA esters by the synthetase (43).

This proposal is certainly interesting and is supported by the fact that the acyl-CoA synthetase is partially membrane-bound (43). Although localization in the cytoplasmic membrane is required for a transport protein, Frerman and Bennett found evidence that the acyl-CoA synthetase is not localized on the outside of the membrane (46). Obviously, the exact nature of fatty acid uptake and the involvement of the acyl-CoA synthetase in it need to be clarified and in fact this has led to comparative studies on fatty acid uptake and fatty acid activation.

In one such comparative study, Overath found that the chain length specificity of both fatty acid uptake and fatty acid activation were the same (43). The kinetic constants of both uptake and activation were also compared. Working

with partially purified preparations of the acyl-CoA synthetase different Km values for oleate were obtained by several investigators: 0.46 mM (46), 0.5 mM (47), and 20 μM (48). Of these values only the last one is similar to the value of 15 μM (43) found for oleate transport. However, the two higher values should be interpreted with caution. As Frerman and Bennett pointed out, these values were obtained by measuring the in situo formation of oleylhydroxamate from hydroxylamine which has been shown to have adverse affects on acyl-CoA synthetases (46). The value of 20 μM was obtained by Ailhaud using a radioactive assay (48). Another complicating factor is that the chain-length specificities of these crude acyl-CoA synthetase preparations obtained by Overath and Ailhaud were not the same. Whereas, Overath was working with an enzyme preparation specific for long-chain fatty acids (47), Ailhaud's enzyme preparation was active with both medium, and long-chain fatty acids (48).

These studies leave many unanswered questions both about fatty acid uptake and activation. To clear up these questions it would be necessary to further characterize the fatty acid uptake system as well as the acyl-CoA synthetase. Of particular importance would be an investigation of the possible involvement in fatty acid uptake of a specific protein or permease distinct from the acyl-CoA synthetase.

In the following report, preliminary findings of a study are presented which were aimed at localizing and

studying the acyl-CoA synthetase and a proposed fatty acid permease.

MATERIALS AND METHODS

Chemicals. CoA in its reduced form was purchased from PL-Biochemicals. Lysozyme, DNAase, ATP, leucine and Triton X-100 were bought from Sigma Chemical Co. Oleic acid was obtained from Fisher Scientific Co. (4,5^{3}H)-leucine and ^{14}C(U)-leucine were purchased from New England Nuclear.

Organisms. E. coli B (ATCC11775) was grown in M-9 mineral salts medium with either oleate or acetate as the sole carbon source, as previously described (47). Cells were grown in a New Brunswick gyrotory shaker incubator. All cells were grown with shaking to late log phase which corresponds to an absorbance of 1.8 at 420 nm when measured on a Zeiss M4 QIII spectrophotometer.

Incorporation of ^{14}C and ^{3}H-leucine into the cytoplasmic membrane. Cells were grown on radioactive leucine as follows. M-9 mineral salts media with either oleate or acetate as sole carbon source were prepared as described above. Then 10 mg of DL-leucine containing 100 μCi of (4,5^{3}H)-leucine was added to 500 ml of oleate containing media and then to 500 ml of the acetate containing media 10 mg of DL-leucine containing 50 μCi of ^{14}C(U)-leucine were added. To each of the above culture media was added 30 ml of a late log culture of either

oleate or acetate grown cells, as appropriate. The initial absorbance of the cells was 0.2-0.3 at 420 nm. Cells were grown to late log phase and harvested. Equal weights (1.3 g each) of ^{14}C and ^{3}H grown cells were then mixed. Cytoplasmic membranes of the mixed cells were prepared according to Kaback's procedure (49).

Solublization of cytoplasmic membrane with Triton X-100. Cytoplasmic membranes were first prepared as described above. The membranes were then suspended in 2% Triton X-100 in Tris-HCl (7.5) to a final concentration of 10 mg protein/ml. Two equal portions were taken and treated as follows. One portion was sonicated for 50 sec. (5x10 sec.) at 0-5°C using a Branson Sonifier with microtip attachment at a setting of 4 and incubated for 2 hrs. (includes sonication time) in the Triton X-100. The other portion was not sonicated but only incubated in the Triton X-100 for 2 hrs. The two portions were then centrifuged at 166,500 x g in a Spinco Model L2-65B preparative ultracentrifuge for one hour. The supernatents were used for enzyme assays.

Preparation of Acyl-CoA synthetase. 0.3 g of either oleate or acetate grown cells were suspended in 1.5 ml containing 0.1M Tris-hydrochloride (7.7), 0.01M 2-mercaptoethanol, 1% Triton X-100 and 0.002M EDTA and sonicated for 50 sec. (5x10 sec.) at 0-5°C using a Branson Sonifier with a microtip attachment at a setting of 4 (47). This preparation was centrifuged at 31,300 x g for 30 minutes in a Beckman

J-21 centrifuge. The supernatants were used to assay for the acyl-CoA synthetase.

SDS-acrylamide gel electrophoresis and scintillation counting. The SDS-disc gels were prepared and run according to the procedure of Weber and Osborne (50). 100 µg or 200 µg of membrane protein was applied to each gel. After electrophoresis the gels were removed from the glass tubes and either stained in Coomasie brilliant blue for 2 hrs or cut into 2 mm slices and prepared for liquid scintillation counting as follows. Each slice was placed into a glass counting vial containing 0.7 ml of NCS solublizing cocktail (NCS:H_2O 9:1) (51). The slices were then incubated in the tightly closed vials at 50°C in an oven overnight. After the incubation the vials were allowed to cool down and then 10 ml of toluene based scintillation solution were added. The vials were then counted in a Beckman LS-150 liquid scintillation counter using narrow ^{3}H and ^{14}C isosets.

Protein and enzyme determination. Protein was determined by a modified biuret procedure (28). Incubation with the biuret reagent was as usual, but was done in the presence of 2% Triton X-100. Acyl-CoA synthetase activity was assayed according to Overath (47). The assay mixture contained 4 µmoles oleate, 40 µmoles NaF, 0.5 mg Triton X-100 and H_2O to a final volume of 2 ml. To this was added a 1 ml solution containing 250 µmoles Tris-hydrochloride (8.5), 1 mmole hydroxylamine (neutralized to pH 8.5), 10 µmoles ATP, 0.6 mg CoA, 20 µmoles $MgCl_2$ and 40 µmoles 2-mercaptoethanol.

RESULTS AND DISCUSSION

Induction and localization of the acyl-CoA synthetase. In this investigation the acyl-CoA synthetase was initially studied because of its importance in fatty acid uptake (47). It was thought necessary to identify the synthetase, study some of its characteristics and to compare these data with those of earlier reports.

Two previous reports have demonstrated that the growth of E. coli on a long-chain fatty acid as sole carbon source causes the induction of the β-oxidation enzymes as well as the acyl-CoA synthetase (47, 52). However, two different strains were used in these studies and the extent of induction was different in each strain. Hence, it seemed necessary to determine the extent of induction in the strain used in this study. Table 5 shows that the specific activity of the acyl-CoA synthetase in the induced cells are 10-fold higher than in non-induced cells. These values are in general agreement with those reported by Weeks et al (52). Furthermore, the acyl-CoA synthetase activity was found to be linear with respect to both time and protein concentration (Figs. 13 and 14).

In view of earlier reports that the acyl-CoA synthetase was partially membrane bound (47, 53) it was decided to determine whether this enzyme is localized on the cytoplasmic membrane. For this purpose a cytoplasmic membrane prepara-

tion was obtained from oleate grown cells according to an established procedure (49). The membranes were solublized by a modified procedure initially used by Schnaitman (54). According to this procedure membranes were incubated in 2% Triton X-100 either with or without sonication as described in "Materials and Methods." These Triton extracts, however, exhibited no acyl-CoA synthetase activity as shown in Table 6. This indicated that there was no acyl-CoA synthetase activity associated with the cytoplasmic membrane. The above result is not surprising in view of the fact that the acyl-CoA synthetase is only partially membrane bound and it could have been removed during the preparation of the cytoplasmic membranes.

A preliminary search for a fatty acid permease. An attempt was made to detect the presence of a fatty acid permease on or in the cytoplasmic membrane. A double-label experiment was performed as follows. Two cultures of E. coli cells were grown, one on oleate in the presence of ^{3}H-leucine and one on acetate in the presence of ^{14}C-leucine. Thus, both cultures should incorporate equally their respective labels into protein except the culture grown on oleate should also incorporate ^{3}H-leucine into the β-oxidation enzymes. The two cultures were then mixed. As a result, all proteins should have the same $^{3}H:^{14}C$ ratio except those proteins involved in β-oxidation which should have higher $^{3}H:^{14}C$ ratios. This experiment was carried out as described in "Materials and Methods." The preparation of cytoplasmic

membranes was expected to result in the removal of all the soluble enzymes of β -oxidation. The cytoplasmic membranes were treated with SDS and subjected to SDS gel electrophoresis. The gels were then analyzed for the presence of protein and radioactivity. As can be seen in Fig. 15 the extent of ^{3}H and ^{14}C incorporation does not seem to vary much and consequently, the $^{3}H:^{14}C$ ratio is fairly constant over the length of the gel. There are two areas (peaks I and II) with increased $^{3}H:^{14}C$ ratios. However, the increase in $^{3}H:^{14}C$ ratio are not comparable to the ten-fold increase expected for proteins associated with fatty acid uptake and degradation. However, if the bands on the gel were composed of several proteins of similar molecular weight, a change in concentrations of a single protein would not significantly change the overall protein composition of the band and might not be detected in a double-label experiment as described here. Thus, unless the permease contributed much to the protein concentration of one band no dramatic increase in the $^{3}H:^{14}C$ ratio would be expected.

A disturbing aspect of Fig. 15 is the fact that not all the peaks of radioactivity coincide with the protein bands. The reason for this is not known and remains unexplainable. Another anamolous aspect of Fig. 15 is the low level of radioactive incorporation associated with the large stained area towards the end of the gel. However, this band is thought to be an artifact because the stain associated with this band fades faster than the stain associated with the

other bands. The nature of the artifact is not known. Thus, the question of whether there exists a fatty acid permease in E. coli cannot be answered on the basis of the results of this study but the evidence obtained here does not appear encouraging.

A final point worth considering is the likelihood of the involvement of a fatty acid permease in bacterial fatty acid uptake. The possibility exists that fatty acids enter the cell by simple diffusion of their protonated lipophilic forms across the membrane. The free fatty acids would then be converted to their CoA esters by acyl-CoA synthetase and in this fashion a favorable concentration gradient would be maintained. If this were the mechanism of fatty acid uptake and if the diffusion would be rate-limiting one would expect to observe maximal rates of fatty acid uptake at low pH's where a greater percentage of fatty acids exist in their protonated forms. However, fatty acid uptake exhibited a pH optimum of 7 (46), a finding which does not disprove a mechanism of simple diffusion because of the involvement of acyl-CoA synthetase, which has a pH optimum of 8.5 in the overall process.

Support for the involvement of a specific transferring protein in fatty acid uptake comes from mammalian systems. The best known example of a fatty acid uptake and transferring system is the carnitine dependent translocations of fatty acids across the inner mitochondrial membrane (55). Furthermore, recent evidence indicates that isolated rat liver cells may have a protein which is involved in the

transfer of fatty acids across the cytoplasmic membrane (56, 57). In view of the requirement in mammalian systems for a specific fatty acid transferring protein, it is not unreasonable to assume that such a protein exists in a bacterial system as well.

Table 1. Properties of diacetyl reductases

Source of Diacetyl Reductase	Molecular Weight and Subunit Composition	Coenzyme Requirement	Km (Diacetyl) mM
A. Aerogenes	100,000 4 equal subunits	NADH	1.2
Beef Liver	76,000 3 subunits	NADH, NADPH	0.04
Pigeon Liver	110,000	NADH, NADPH	3.5

Table 2. Summary of the purification of diacetyl reductase from E. coli.

Purification Step	Total[a] Activity	Total Protein	Specific Activity	Purification	Yield
	μmoles/min	mg	μmoles/min and mg of protein	-fold	%
1) Crude Homogenate	220	72,000	0.003	1	100
2) $(NH_4)_2SO_4$ 45-75% sat.	170	24,000	0.007	2.3	77
3) DEAE-Cellulose I	71	3,000	0.024	8	32
4) DEAE-Cellulose II	(27.5)[b]	350	0.078	26	(12.5)[b]
5) Sephadex G-100	46.1	80	0.58	193	20.9
6) DEAE-Sephadex	3.3	1.36	2.45	800	1.5

[a] Enzyme activity was determined using ethyl acetoacetate as substrate.

[b] The low activity after the second DEAE-cellulose chromatography step was surprising and remains unexplained.

Table 3. Substrate specificity of diacetyl reductase.

Substrate	Activity[a] %
Diacetyl (2,3 butanedione)	100
Ethyl pyruvate	70.9
Pyruvate	4.3
Oxalacetate	7.0
2-Oxogluterate	3.2
α-Ketopantolactone	6.6
Dehydroascorbate	0
Ethyl acetoacetate	31.6
Methyl acetoacetate	10.4
Ethyl thiolacetoacetate	55.5[b]
Acetoacetyl N-acetylcysteamine	19.1[b]
Acetoacetate	1.6
Methyl β -ketohexanoate	2.5
2,4-Pentanedione	4.9
Ethyl levulinate	3.9
Acetaldehyde	2.5
Acetone	2.3
Acetoin (3-hydroxy-2-butanone)	2.2

[a] Assays were performed as described in "Materials and Methods". The assay mixtures contained 167 mM potassium phosphate (pH 7.0), 0.18 mM NADPH, 33 mM substrate and between 1.8 µg and 18 µg of diacetyl reductase (specific activity 0.5 µmoles per min and mg of protein) in a total volume of 0.6 ml. The amount of reductase per assay was varied to give an observed ΔA_{340}/min between 5×10^{-3} and 35×10^{-3}.

[b] Assays were as described in footnote a except that the substrate concentrations were 3.3 mM. Percent activity was calculated based on the activity observed with an assay mixture containing 3.3 mM diacetyl.

Table 4. Kinetic Constants

Variable Substrate	Fixed Substrate	K_m (mM)	V_m (μmoles/min and mg of protein)
Diacetyl[a]	NADPH	4.44	9.5
NADPH[a]	Diacetyl	0.02	
NADH	Diacetyl	0.46	2.34
Ethyl acetoacetate	NADPH	15.4	3.63
NADPH	Ethyl Acetoacetate	0.008	
Acetoacetyl N-acetylcysteamine	NADPH	7.15	1.63
NADPH	Acetoacetyl N-acetylcysteamine	0.008	

[a]Constants for these substrates were obtained from Fig. 12.

Table 5. Acyl-CoA synthetase assay[a]

Source	Specific Activity $\frac{\mu moles\ hydroxamic\ acid}{(mg\ protein)\ (min)}$
Oleate grown cells	2.6×10^{-3}
Acetate grown cells	2.5×10^{-4}

[a]The assay mixtures were incubated for 2 hrs. as described in "Materials and Methods." For assaying oleate grown cells 0.28-0.84 mg of protein were used for each assay and for assaying acetate grown cells 1.29-2.58 mg of protein were used for each assay.

Table 6. Acyl-CoA synthetase assay[a] of solublized cytoplasmic membrane.

Sonicated membrane preparation[b] mg protein	μmoles hydroxamic acid/min
0.132	0
0.264	0
0.528	0

Unsonicated membrane preparation[b] mg protein	μmoles hydroxamic acid/min
0.108	0
0.216	0
0.432	0

[a]The acyl-CoA synthetase assay was performed as described in "Materials and Methods."

[b]Membranes were prepared as described in "Materials and Methods."

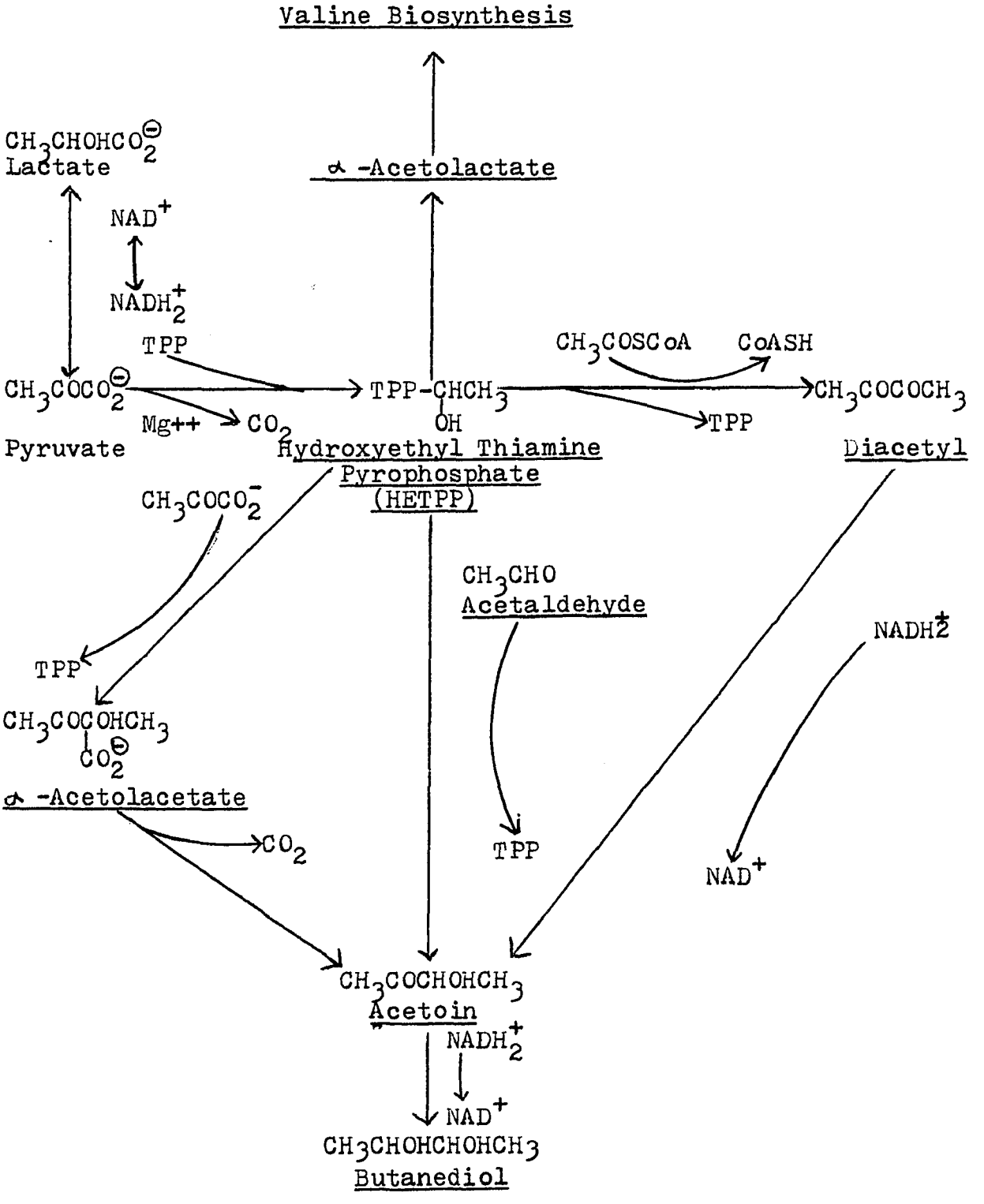
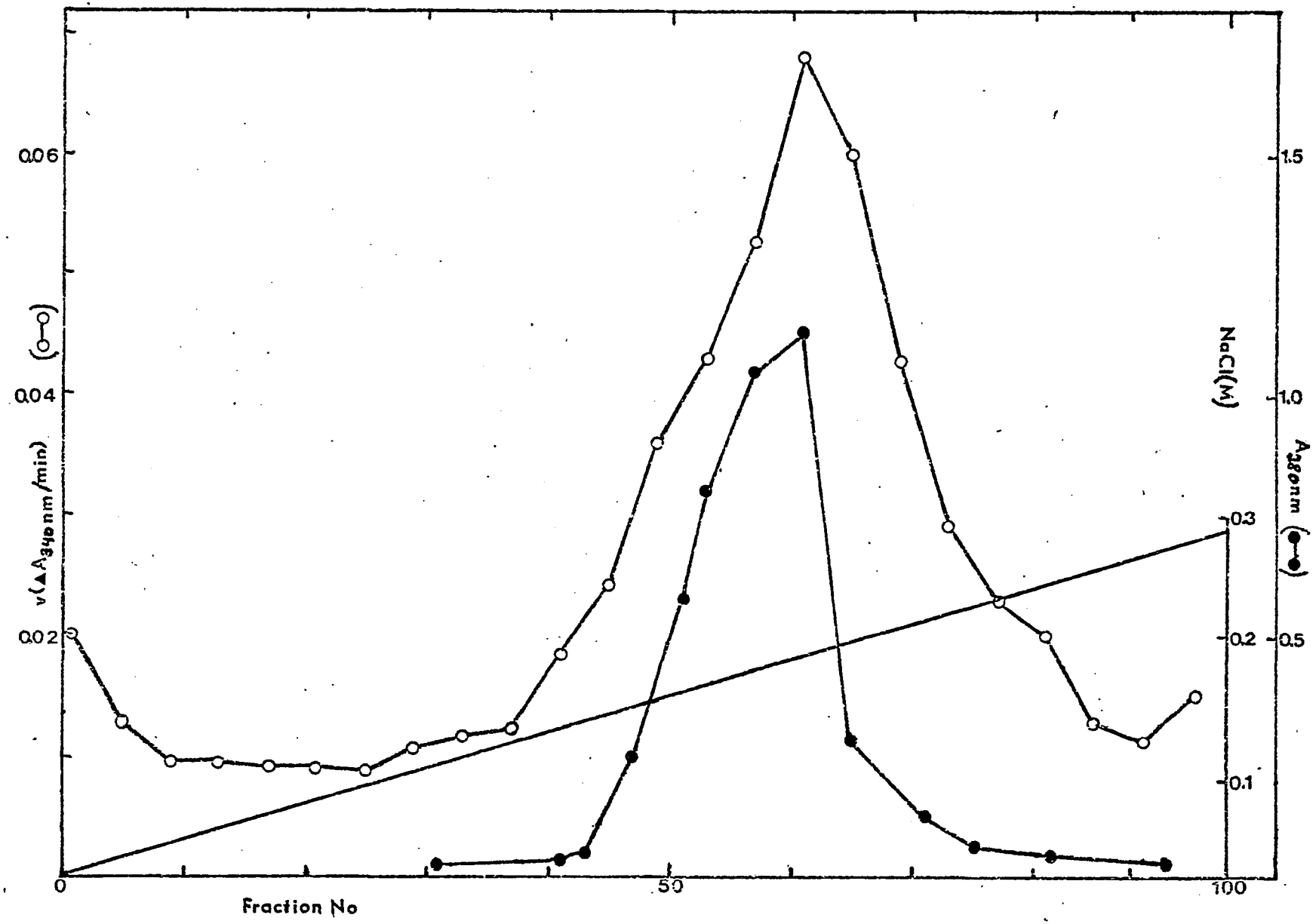


Fig. 1. Alternative pathways of pyruvate metabolism

Fig. 2. Chromatography of diacetyl reductase on DEAE-cellulose. Fractions of 20 ml each were collected. Reductase activity assays were performed with 0.1 ml of each of the indicated fractions. The values of $\blacktriangle A_{340}$/min thus obtained are plotted above.


0.06
0.04
0.02
0
50
100
Fraction No
v(ΔA340nm/min) (o—o)
NaCl(M)
A280nm (•—•)
1.5
1.0
0.5
0.3
0.2
0.1

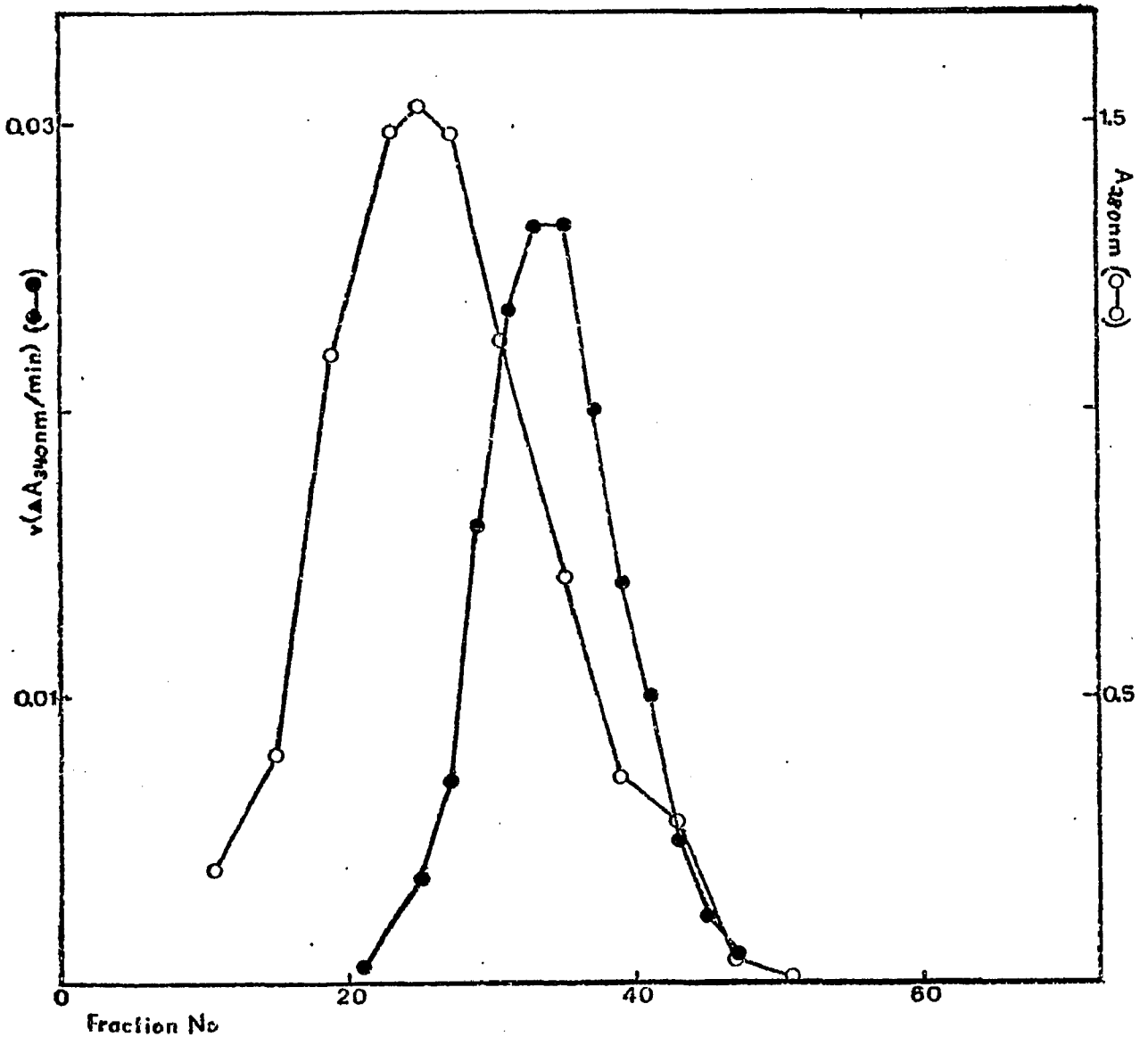


Fig. 3. Chromatography of diacetyl reductase on a Sephadex G-100 column (5 x 45 cm). Fractions of 15 ml each were collected. Reductase activity assays were performed with 0.1 ml of each of the indicated fractions. The values of A_{340}/min thus obtained are plotted above.

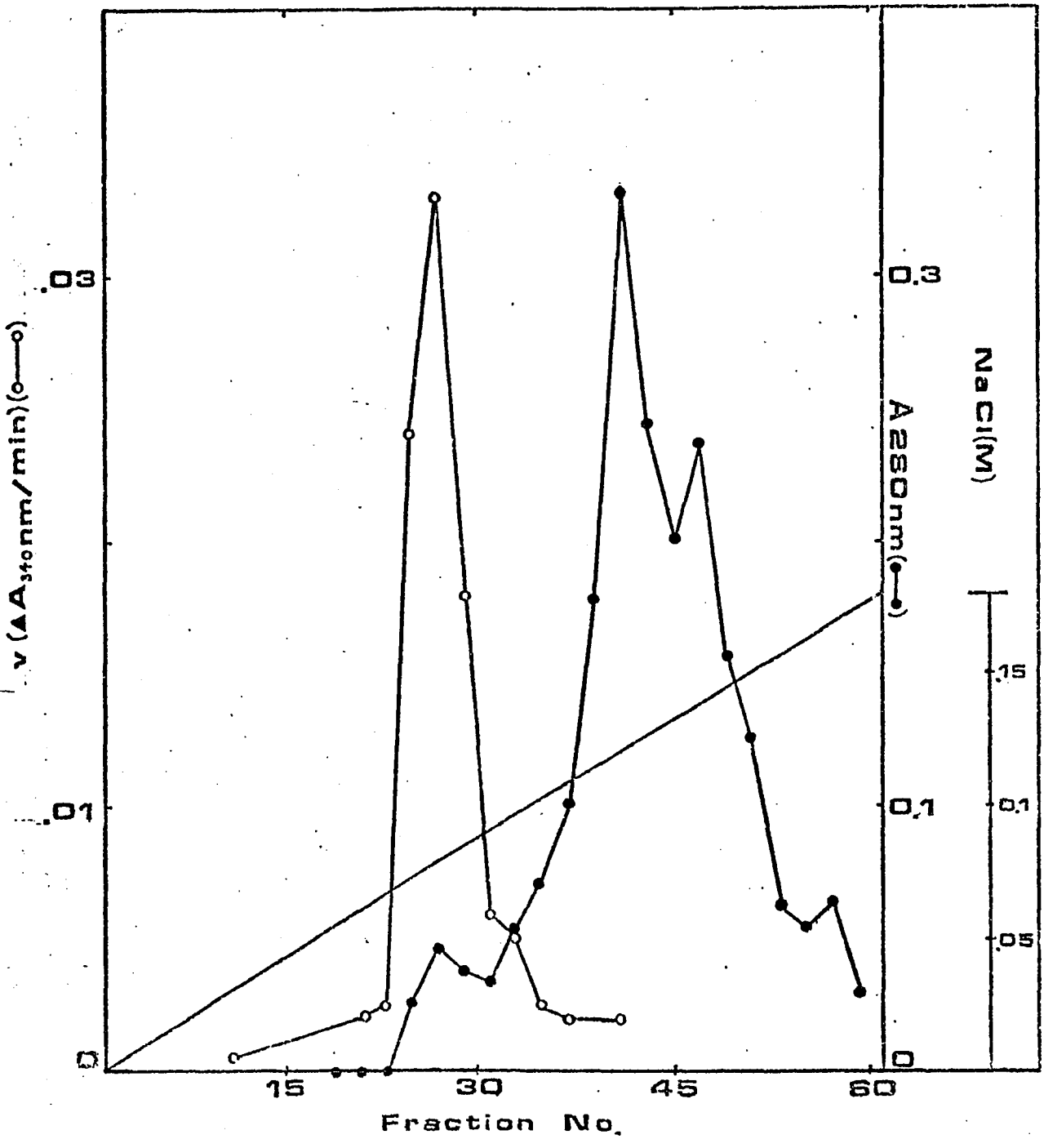


Fig. 4. The final purification of diacetyl reductase on DEAE-Sephadex A-50. Diacetyl reductase was chromatographed and fractions of 20 ml each were collected. Reductase activity assays were performed with 0.1 ml of each of the indicated fractions. The values of $\blacktriangle A_{340}$/min thus obtained are plotted above.

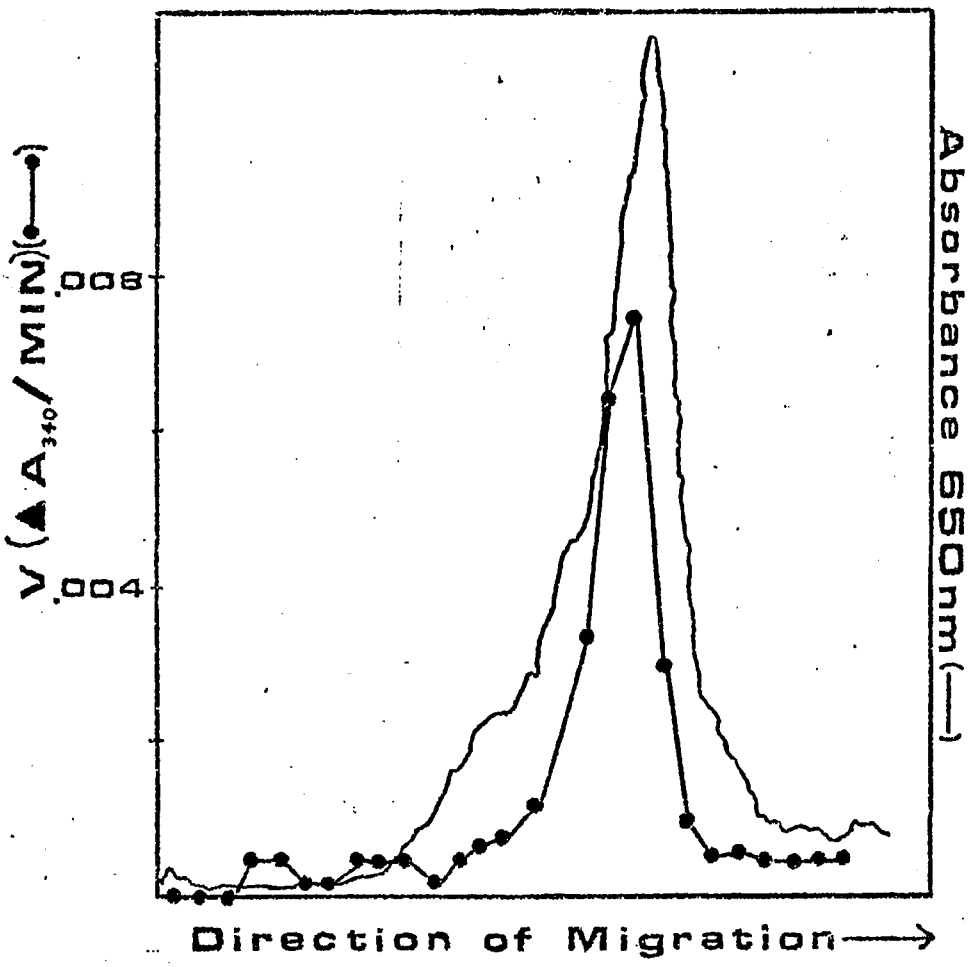


Fig 5. Disc gel electrophoresis of diacetyl reductase. Photometric scan of gel after staining with amido black.

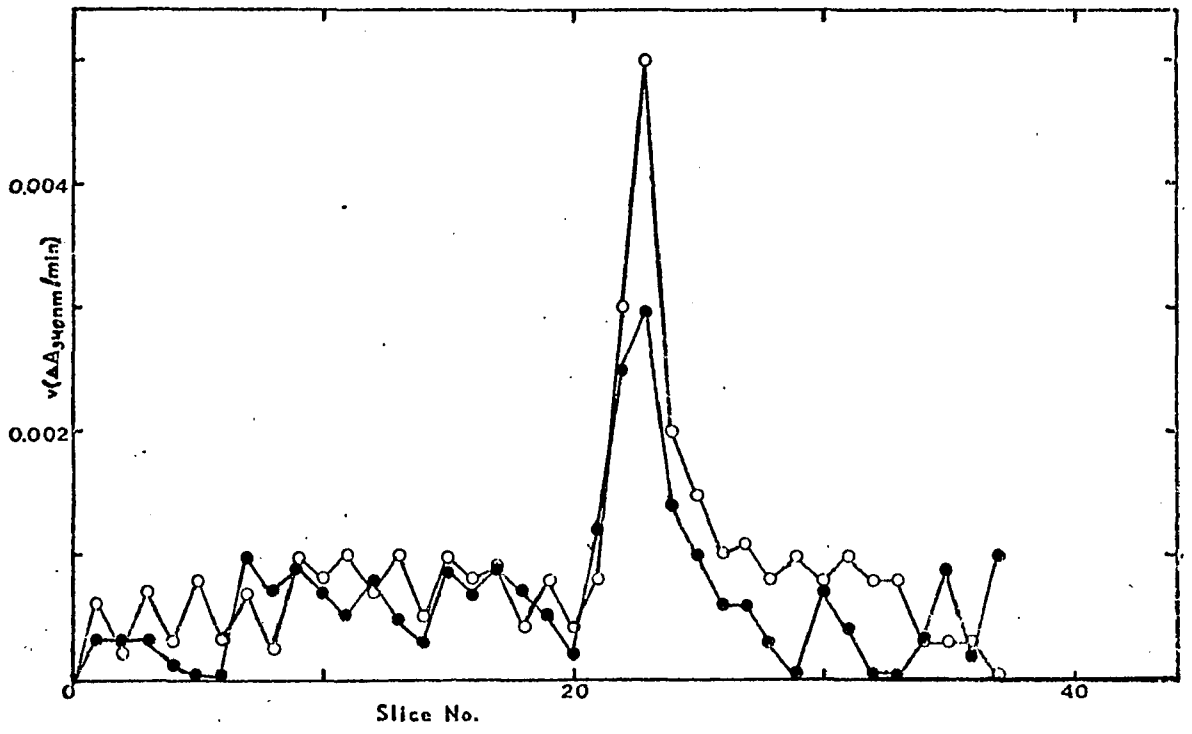


Fig. 6. Disc gel electrophoresis of diacetyl reductase, and ethyl acetoacetate reductase. Gel was cut into 2 mm slices and each slice was eluted with 1 ml of 0.01 M, KPi (pH 7.0). In the diacetyl reductase assays 0.3 ml of the eluate of each slice was used, in the ethyl acetoacetate reductase assay 0.5 ml of each eluate was used. The values of $\blacktriangle A_{340}$/min thus obtained are plotted above. (●)Ethyl acetoacetate reductase, (o) diacetyl reductase.

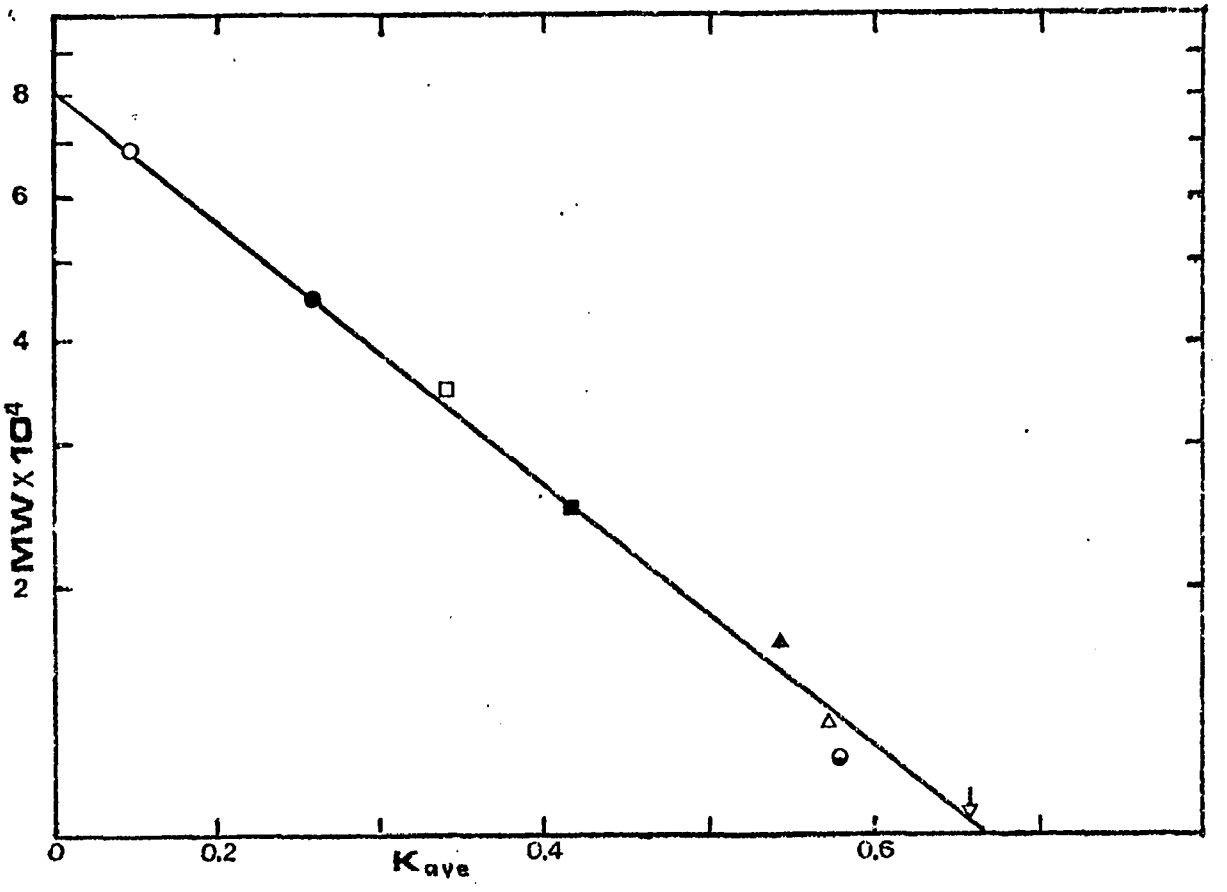


Fig. 7. Molecular weight determination of diacetyl reductase on Sephadex G-100. The following standards were used: (○) bovine serum albumin, (●) ovalbumin, (□) pepsin, (■) chymotrypsinogen A, (▲) myolobin, (△) ribonuclease, and (◒) cytochrome c. Arrow indicates position of diacetyl reductase.

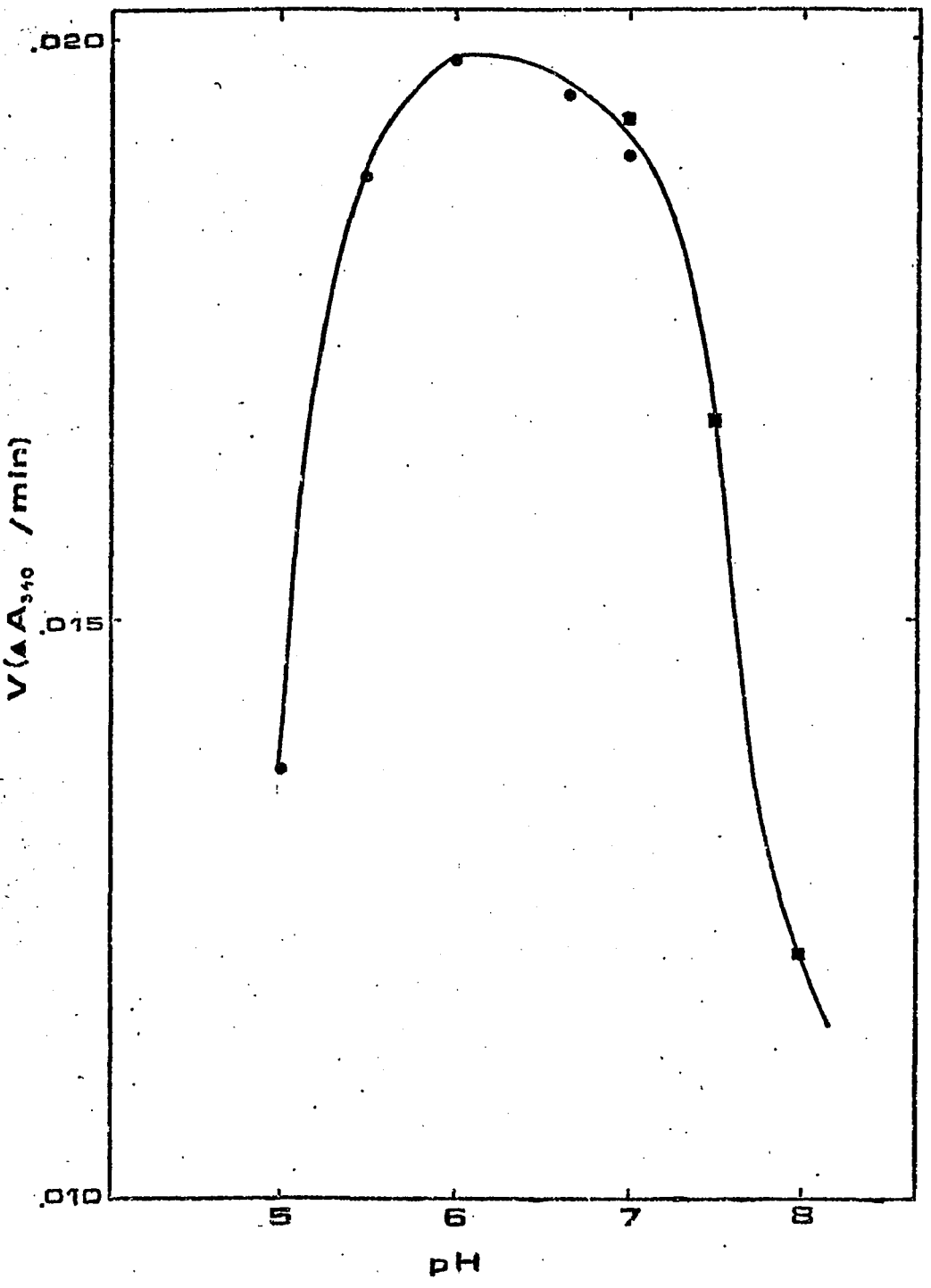


Fig. 8. pH optimum of diacetyl reductase. Buffers used were citrate phosphate (●) and potassium phosphate (■). The assay mixture contained 100 µmol of the indicated buffer, 10 µmol of diacetyl, 56 nmol of NADPH, and 0.45 µg of diacetyl reductase in a total volume of 0.6 ml.

Fig. 9. Time curves of NADPH oxidation catalyzed by diacetyl reductase in the presence of diacetyl. (Curve 1) With potassium phosphate (pH 7.8) as buffer. (Curve 2) With Tris-hydrochloride (pH 7.8) as buffer when the reaction was started by the addition of diacetyl. (Curve 3) Difference between curves 2 and 5. (Curve 4) With Tris-hydrochloride (pH 7.8) as buffer, when the reaction was started by the addition of enzyme. (Curve 5) Change in absorbance of control. Assays contained as follows: curve 1, 100 μmol of potassium phosphate, 0.11 μmol of NADPH, 20 μmol of diacetyl, and 9 μg of diacetyl reductase in a total volume of 0.6 ml; curves 2 and 4, 100 μmol of Tris-hydrochloride, 180 μmol of potassium chloride, 0.113 μmol of NADPH, 20 μmol of diacetyl, and 9 μg of diacetyl reductase in a total volume of 0.6 ml; curve 5, control contained the same as assays 2 and 4 except that no enzyme was present.

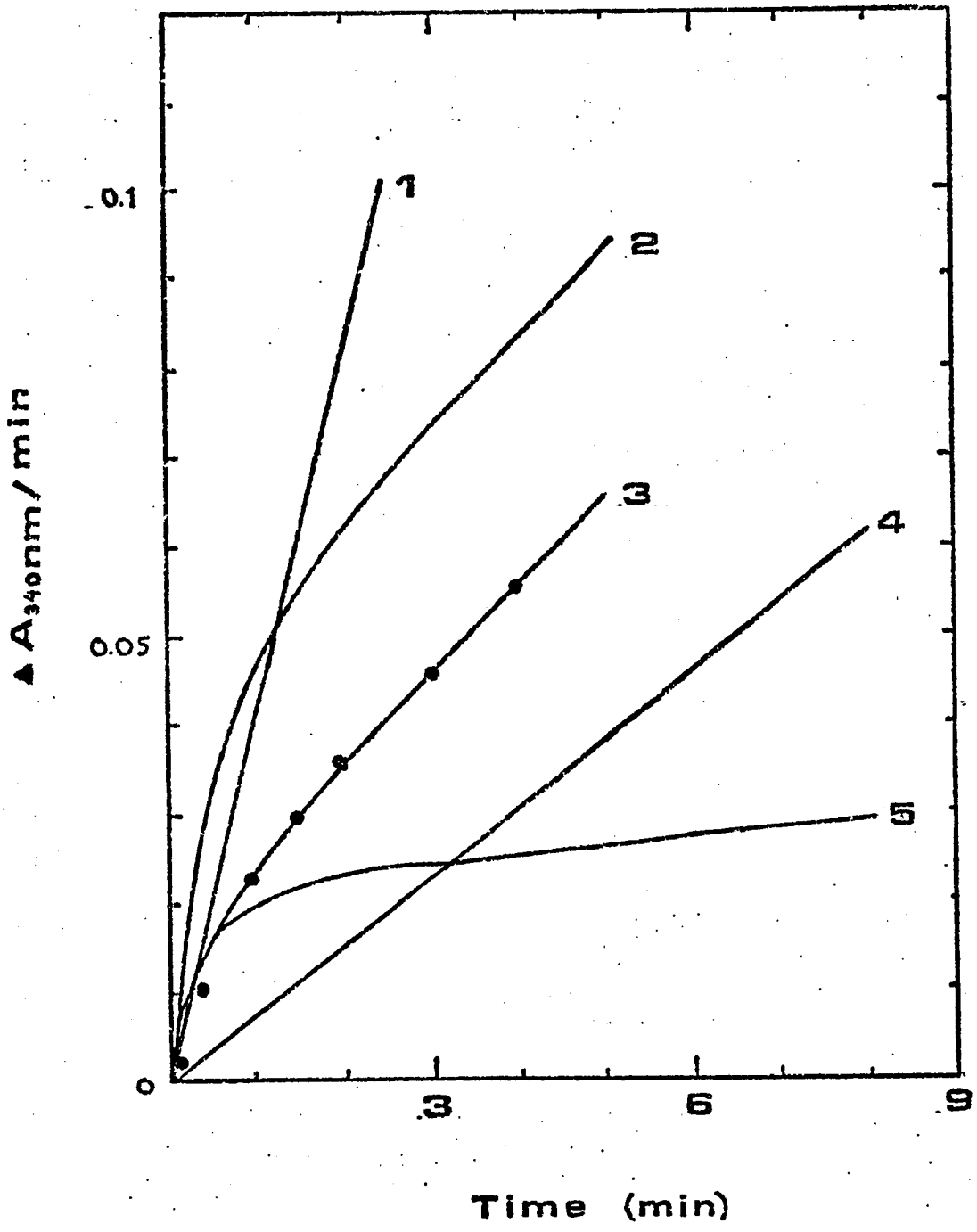
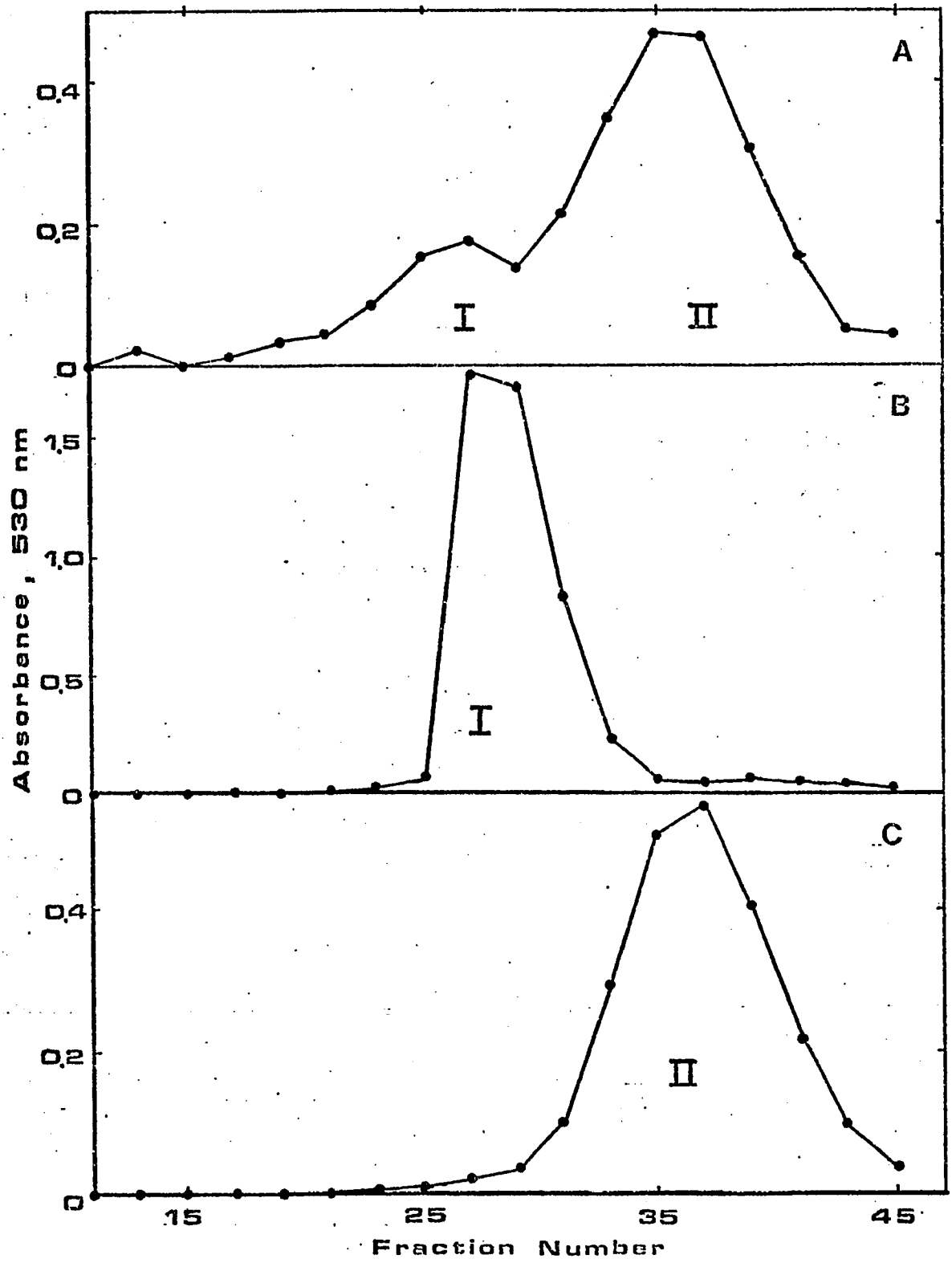

0.1
0.05
0
3
6
9
1
2
3
4
5
▲ A340nm / min
Time (min)

Fig. 10. Identification of the product of the diacetyl reductase reaction. The assay mixture contained 2.5 μmol of diacetyl, 40 μmol of potassium phosphate, 0.15 mg of diacetyl reductase (added in two portions), and 0.5 mg of NADPH (added in three portions) in a total volume of 1 ml. The control contained the same components except that no enzyme was added. After a total reaction time of 40 h the reaction mixture was chromatographed on a Dowex 1-X8 (sulfate form) column (1.9 by 27 cm) as described by Speckman and Collins (31). Subsequently, the control followed by the acetoin standard was chromatographed on the same column. Fractions of 3 ml each were collected and assayed for diacetyl and acetoin by the procedure of Westerfeld (32). (A) Reaction mixture; (B) 2.5 μmol of acetoin; (C) control.


A
B
C
I
II
I
II
0.4
0.2
0
1.5
1.0
0.5
0
0.4
0.2
0
15
25
35
45
Absorbance, 530 nm
Fraction Number

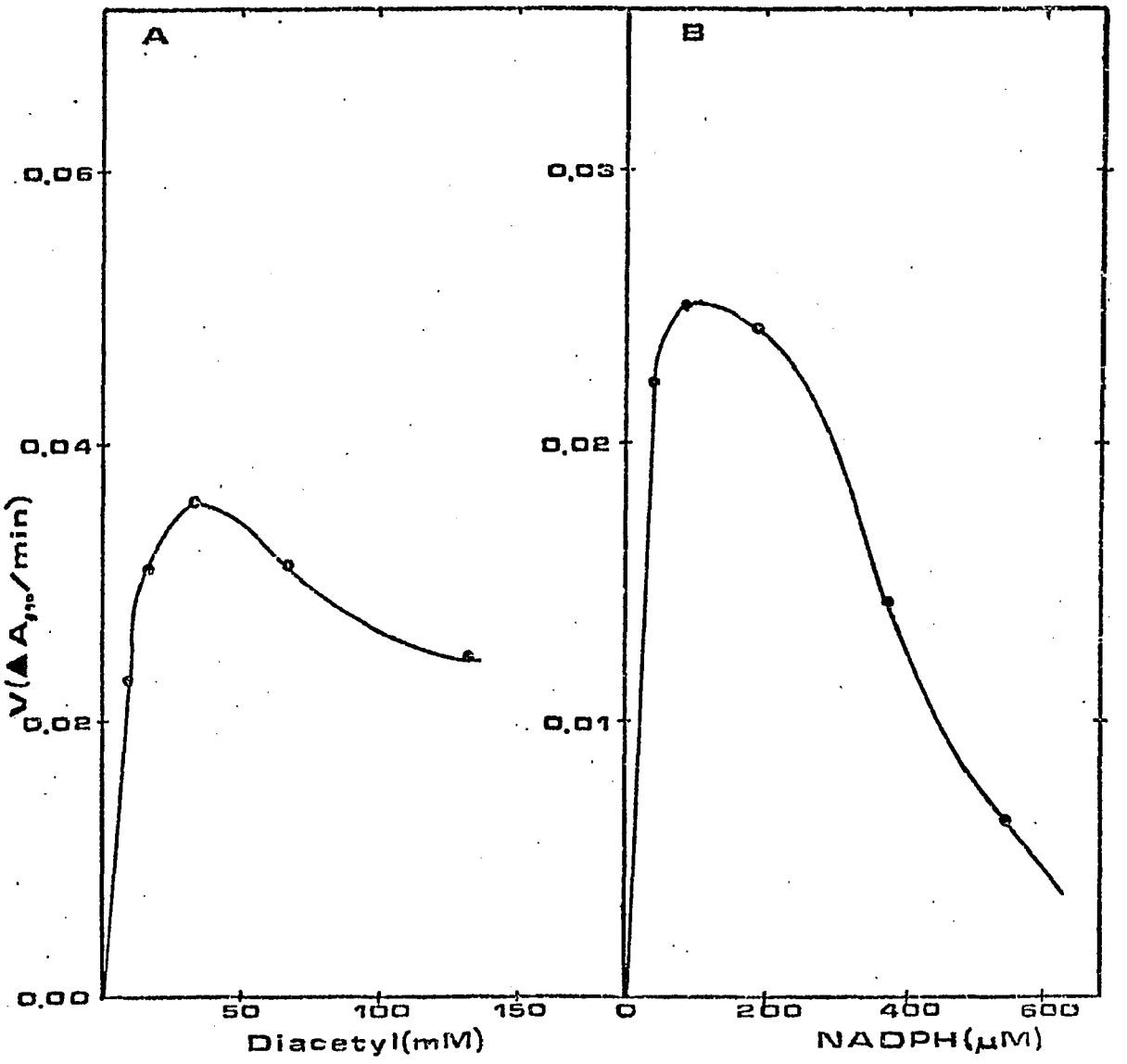


Fig. 11. (A) The rate of NADPH oxidation catalyzed by diacetyl reductase as a function of the diacetyl concentration. The assay mixture contained, in a total volume of 0.6 ml, 100 μmol of potassium phosphate (pH 7.0), 45 nmol of NADPH, 0.9 μg of diacetyl reductase, and the indicated amounts of diacetyl. (B) The rate of NADPH oxidation catalyzed by diacetyl reductase as a function of the NADPH concentration. The assay mixture contained, in a total volume of 0.6 ml, 100 μmol of potassium phosphate (pH 7.0), 40 μmol of diacetyl, 0.9 μg of diacetyl reductase, and the indicated amounts of NADPH.

Fig. 12. The rate of NADPH oxidation catalyzed by diacetyl reductase as a function of diacetyl concentration at several fixed levels of NADPH. Data are plotted on reciprocal coordinates. (Insert) Replot of slopes and intercepts versus the concentrations of NADPH. Each assay mixture contained, in a total volume of 0.6 ml, 100 µmol of potassium phosphate (pH 7.0), 0.9 µg of diacetyl reductase, diacetyl as indicated, and the following amounts of NADPH: (▲) 56.3 nmol, (○) 33.8 nmol, (△) 22.6 nmol, (□) 11.3 nmol, (●) 5.6 nmol.

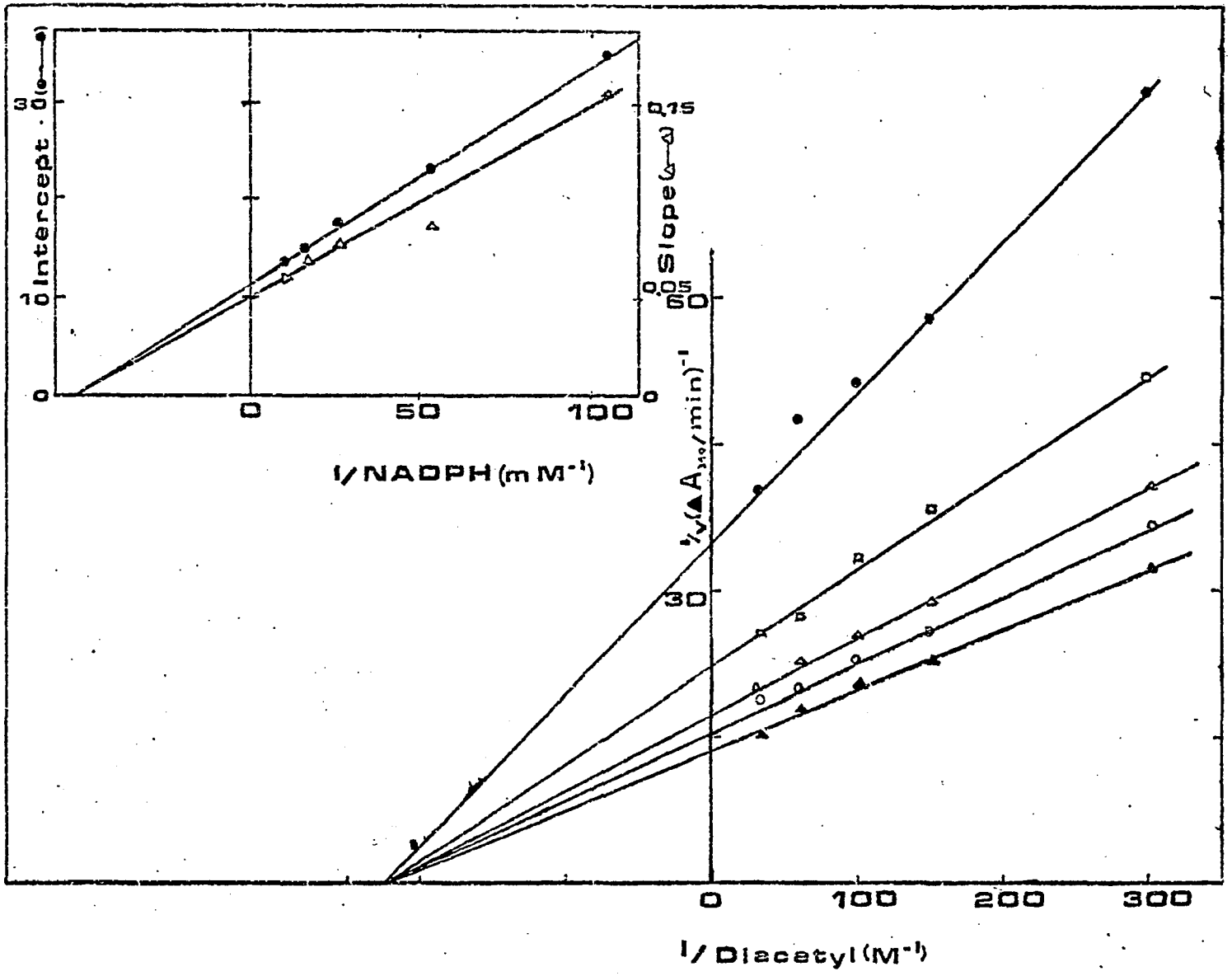

Intercept
Slope
0
50
100
0
0,05
0,15
1/ NADPH (m M⁻¹)
1/v(ΔA340/min)⁻¹
30
60
0
100
200
300
1/ Diacetyl (M⁻¹)

Fig. 13. The rate of formation of oleylhydroxamic acid as a function of time. Each assay was done as described in "Materials and Methods" and contained the following amounts of protein. (○) 0.28 mg, (●) 0.56 mg, (□) 0.84 mg.

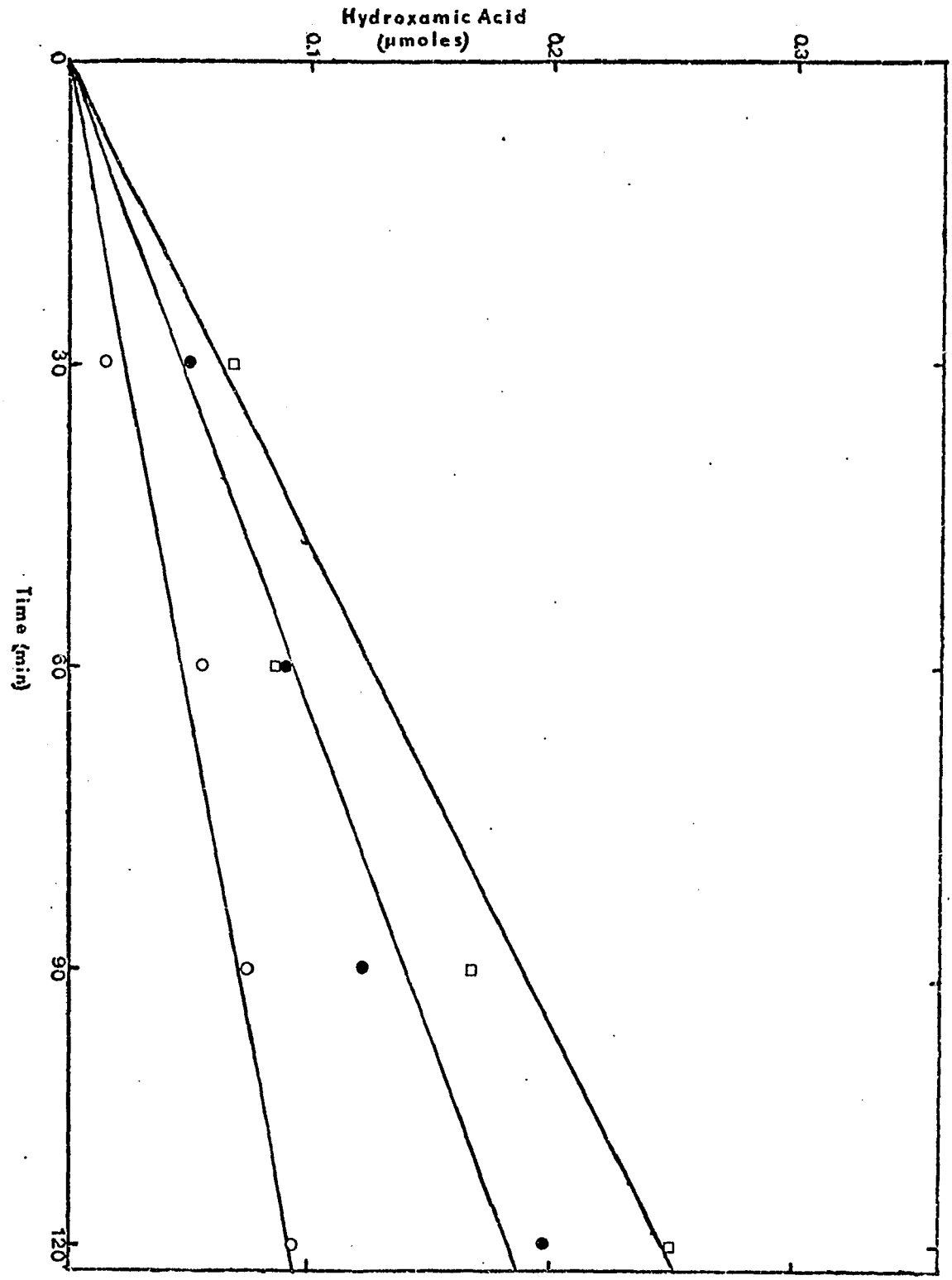

Hydroxamic Acid
(μmoles)
0.1
0.2
0.3
0
30
60
90
120
Time (min)

Fig. 14. The rate of formation of oleylhydroxamic acid as a function of protein concentrations. Each assay was done as described in "Materials and Methods" and was run for the following times. (○) 30 min, (●) 60 min, (□) 90 min, (■) 120 min.

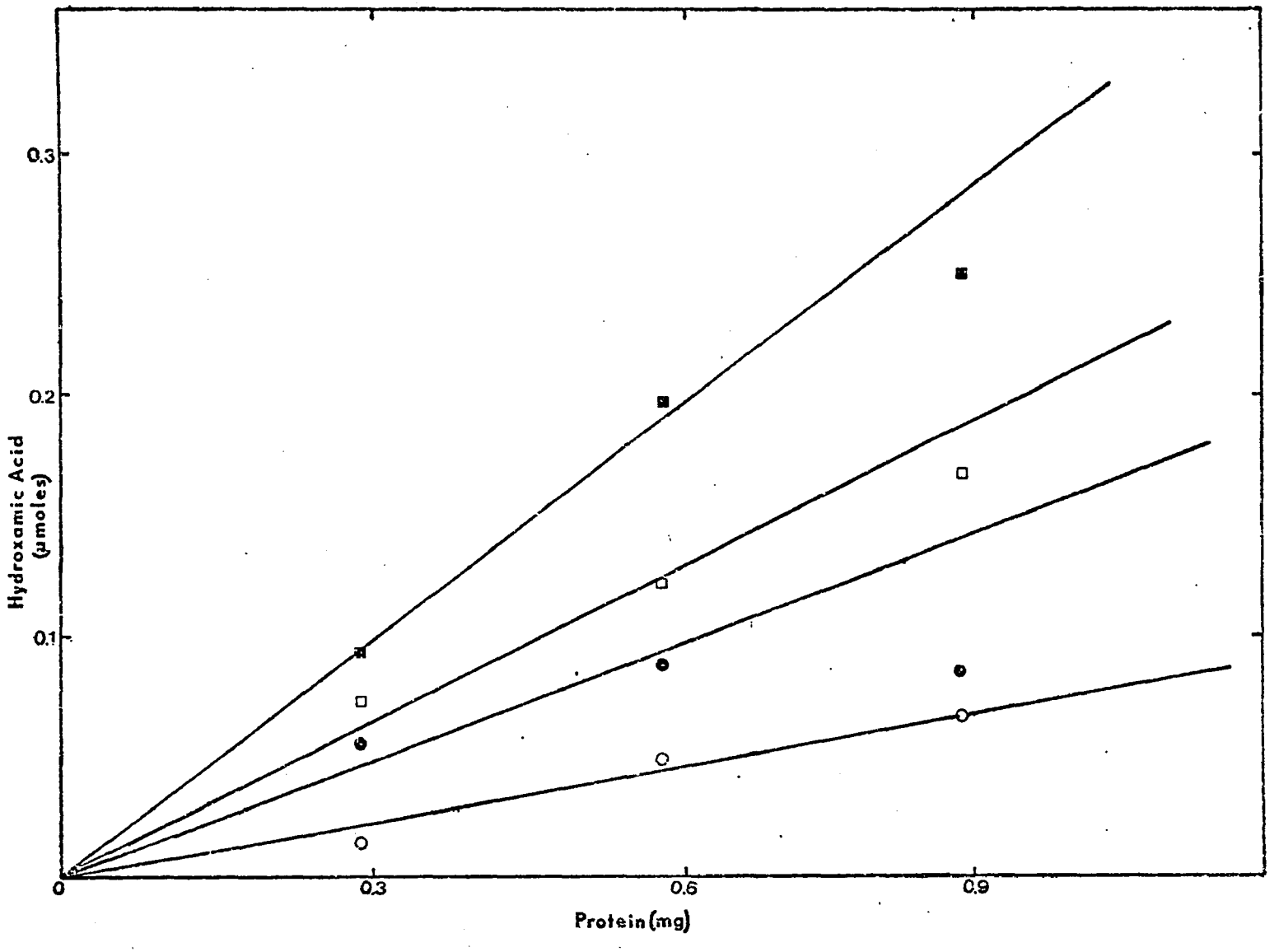
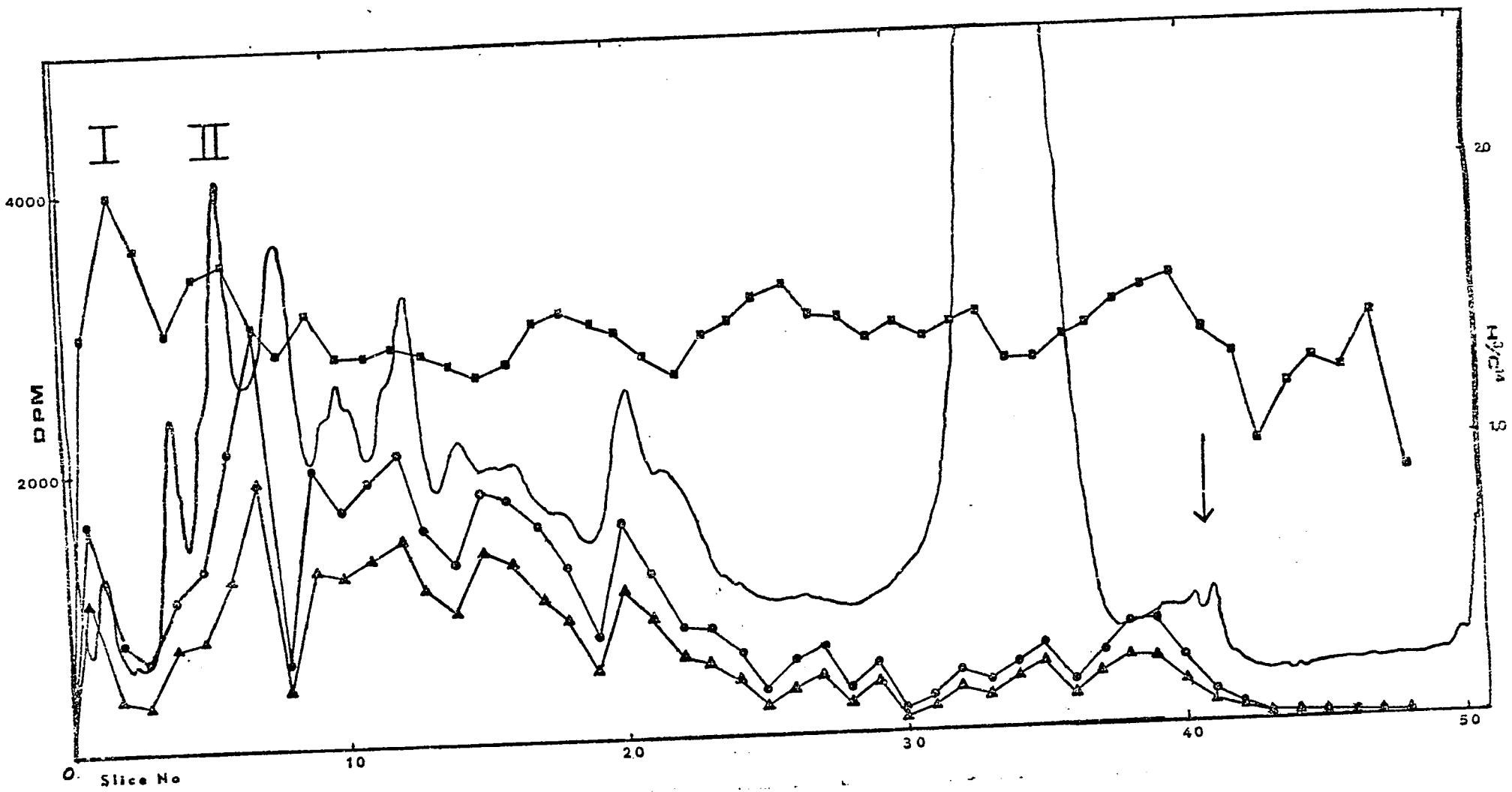

0.3
0.2
0.1
0
0.3
0.6
0.9
Hydroxamic Acid (μmoles)
Protein (mg)

Fig. 15. SDS gel electrophoresis of double labelled E. coli cytoplasmic membranes. Cytoplasmic membrane were prepared from a mixture of induced cells grown on ^{3}H-leucine and uninduced cells grown on ^{14}C-leucine. The gels were either stained in Coomasie Brilliant Blue or cut into 2 mm slices and counted as described in "Materials and Methods." (——) Absorbance at 650 nm, (▲) ^{3}H, (●) ^{14}C, (■) $^{3}H/^{14}C$.


I
II
4000
2000
cpm
20
10
H^3/C^{14}
0
10
20
30
40
50
Slice No

REFERENCES

(1) Schulz, H. and S. J. Wakil. 1971. J. Biol. Chem. 246 1895.

(2) Juni, E. 1952. J. Biol. Chem. 195 715.

(3) Juni, E. 1952. J. Biol. Chem. 195 727.

(4) Speckman, R. A. and E. B. Collins. 1968. J. Bacteriol. 95 175.

(5) Speckman, R. A. and E. B. Collins. 1973. Appl. Microbiol. 26 744.

(6) Chuang, L. F. and E. B. Collins. 1968. J. Bacteriol. 95 2083.

(7) Juni, E. and E. A. Heyms. 1956. J. Biol. Chem. 218 365

(8) Strecker, H. J. and I. Harry. 1954. J. Biol Chem. 211 263.

(9) Seitz, E. W., W. E. Sandine, P. R. Elliker, and E. A. Day. 1963 Can. J. Microbiol. 9 431.

(10) Bryn, K., O. Hetland and F. C. Stormer. 1971. Eur. J. Biochem. 18 116.

(11) Hetland, O., K. Bryn, and F. C. Stormer. 1971. Eur. J. Biochem. 20 206.

(12) Hetland, O., B. R. Olson, T. B. Christensen and F. C. Stormer. 1971. Eur. J. Biochem. 20 200.

(13) Johansen, L., S. H. Larsen and F. C. Stormer. 1973. Eur. J. Biochem. 34 97.

(14) Larsen, S. H., L. Johansen, F. C. Stormer and H. J. Storesund. 1973. FEBS Lett. 31.39.

(15) Larsen, S. H. and F. C. Stormer. 1973. Eur. J. Biochem. 34 100.

(16) Branen, A. L. and T. W. Keenan. 1970. Can. J. Microbiol. 16 947.

(17) Martin, R. and J. Burgos. 1970. Biochim. Biophys. Acta. 212 356.

(18) Martin, R. and J. Burgos. 1972. Biochim. Biophys. Acta. 89 13.

(19) Burgos, J. and R. Martin. 1972. Biochim. Biophys. Acta. 268 261.

(20) Diez, V., J. Burgos, and R. Martin. 1974. Biochim. Biophys. Acta. 350 253.

(21) Gabriel, M. A., H. Jabara, and U.A.S. al-Khalidi. 1971 Biochem. J. 124 793.

(22) Feiser, L. F. 1953. J. Amer. Chem. Soc. 75 4386.

(23) Kuhn, R. and T. Wieland. 1942. Ber. Deut. Chem. Gesamte. 73B 121.

(24) Frank, R. L., P. G. Arvan, J. W. Richterand, and C. R. Vanneman. 1944. J. Amer. Chem. Soc. 66 4.

(25) Seubert, W. 1960. S-Palmityl coenzyme A. P. 80-83. In H. A. Lardy (ed). "Biochemical Preparations" vol. 7. John Wiley and Sons, Inc. New York.

(26) Stallberg-Stenhagen, S. 1945. Arkiv Kemi Minerl. Geol. 20A no. 19.

(27) Lowry, O. H., N. J. Rosebrough, N. J. Farr and R. J. Randall. 1951. J. Biol. Chem. 193 265.

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

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

(30) King, H. L., Jr., and D. R. Wilken. 1972. J. Biol. Chem. 247 4096.

(31) Speckman, R. A. and E. B. Collins. 1968. Anal. Biochem. 22 151.

(32) Westerfeld, W. W. 1945. J. Biol. Chem. 161 495.

(33) Di Sabato, G. 1971. Biochemistry. 10 395.

(34) Eagon, R. G. 1963. Biochem. Biophys. Res. Commun. 12 274.

(35) Mizuno, W. G. and J. J. Jezeski. 1961. J. Dairy Sci. 44 579.

(36) Harvey, R. J. and E. B. Collins. 1961. J. Bacteriol. 82 954.

(37) Wheat, R. W. and S. J. Ajl. 1955. J. Biol. Chem. 217 897.

(38) Wheat, R. W. and S. J. Ajl. 1955. J. Biol. Chem. 217 909.

(39) Dageley, S. and E. B. Dawes. 1955. Biochim. Biophys. Acta. 17 177.

(40) Harvey, R. J. and E. B. Collins. 1963. J. Bacteriol. 86 1301.

(41) Simoni, R. D., In "Membrane Molecular Biology" C. F. Fox and A. D. Kieth (eds) p. 289. Sinauer Associates Inc., Stamford, Conn. 1972.

(42) Lin, E. C. C., In "Structure and Functions of Biological Membranes". L. I. Rothfield (ed) p. 286. Academic Press, N. Y. 1971.

(43) Klein, K., R. Steinberg, B. Fiethen and P. Overath. 1971. Eur. J. Biochem. 19 442.

(44) Salanitro, J. P. and W. S. Wegener. 1971. J. Bacteriol. 108 885.

(45) Salanitro, J. P. and W. S. Wegener. 1971. J. Bacteriol. 108 893.

(46) Frerman, F. E. and W. Bennett. 1973. Arch. Biochem. Biophys. 159 434.

(47) Overath, P., G. Pauli and H. U. Schairer. 1969. Eur. J. Biochem. 7 559.

(48) Samuel, D. and G. Ailhaud. 1969. FEBS Lett. 2 213.

(49) Kaback, H. R. 1968. J. Biol. Chem. 243 3711.

(50) Weber, K. and M. Osborn. 1969. J. Biol. Chem. 244 4406.

(51) Zoutlin, M. and V. Hariharasabramanian. 1970. Anal. Biochem. 35 296.

(52) Weeks. G., M. Shapiro, R. O. Burns, and S. J. Wakil. 1969. J. Bacteriol. 97 827.

(53) Samuel, D., J. Estroumza, and G. Ailhaud. 1970. Eur. J. Biochem. 12 576.

(54) Schnaitman, C. A. 1971. J. Bacteriol. 108 545.

(55) Brosman, J. T., B. Kopec and I. B. Fritz. 1973. J. Biol. Chem. 248 4075.

(56) Mahadevan, S. and F. Sauer. 1971. J. Biol. Chem. 246 5862.

(57) Mahadevan, S. and F. Sauer. 1974. Arch. Biochem. Biophys. 164 185.