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Purification and Properties of a Diacetyl Reductase from *Escherichia coli*

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A reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase with the ability to reduce diacetyl has been isolated from *Escherichia coli* and has been purified 800-fold to near homogeneity. The product of the reduction of diacetyl was shown to be acetoin. The enzyme proved to catalyze the oxidation of NADPH in the presence of both uncharged α- and β-dicarbonyl compounds. Even monocarbonyl compounds showed slight activity with the enzyme. On the basis of its substrate specificity, it is suggested that the enzyme functions 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 this 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 μmol per min per mg of protein and the K_m values for diacetyl and NADPH were found to be 4.44 mM and 0.02 mM, respectively. The molecular weight was estimated by gel filtration on Sephadex G-100 to be approximately 10,000.

The enzyme diacetyl reductase (acetoin: NAD^+ oxidoreductase EC 1.1.1.5) catalyzes the conversion of diacetyl (2,3-butanedione) to acetoin (3-hydroxy-2-butanone) according to equation 1:

$$CH_3-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-CH_3 + NADH + H^+ \rightarrow$$

$$CH_3-\overset{\overset{O}{\|}}{C}-\underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}}-CH_3 + NAD^+ \quad (1)$$

It was first observed by Strecker and Harary in the microorganisms *Aerobacter aerogenes* and *Staphylococcus aureus* (29), although later it was found in other microorganisms (3, 24) but not in *Escherichia coli*. Thus far only the diacetyl reductase of *A. aerogenes* has been purified to homogeneity and extensively studied (1, 11-13, 18, 19). Recently a diacetyl reductase activity has also been observed in mammalian liver (2, 9, 21, 22).

In this report we demonstrate the presence of an NADPH-dependent diacetyl reductase in *Escherichia coli* B. This 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 Schwarz-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 (7). The product was recrystallized from ether/hexane (melting point 58 to 61 C, literature melting point 60 C [17]; nuclear magnetic resonance, δ =

$$1.4\ (S, 6H)\ (CH_3)_2C\begin{smallmatrix} / C \\ \\ \backslash C \end{smallmatrix}, \quad \delta = 4.5\ \ (S, 2H)-CH_2-O-).$$

The following substrates were synthesized by established procedures, and their respective melting and boiling points agreed with the values in the literature: ethyl levulinate (8), ethyl thiolacetoacetate (23), acetoacetyl *N*-acetylcysteamine (25), and methyl β-ketohexanoate (28).

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 treated with a Branson sonifier 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 Determinations." 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 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 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) containing 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 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 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 1.

Protein and enzyme determinations. Protein concentrations were determined according to Lowry et al. (20) or by the biuret method (10). 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 (4). 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

Purification of diacetyl reductase. During the course of the purification of β-ketoacyl acyl carrier protein reductase (23), an ethyl acetoacetate-dependent NADPH dehydrogenase was detected and separated on a Sephadex G-100 column (Fig. 1). Because this enzyme was thought to be a β-ketoacyl ester reductase and thus possibly involved in fatty acid degradation or synthesis, it was further purified and studied. The enzyme was isolated from *E. coli* B cells

TABLE 1. *Summary of the purification of diacetyl reductase*

Purification step	Total activity[a] (μmol/min)	Total protein (mg)	Specific activity (μmol/min mg of protein)	Purification (fold)	Yield (%)
1. Crude homogenate	220	72,000	0.003	1	100
2. $(NH_4)_2SO_4$ (45–75% saturated)	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)
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.

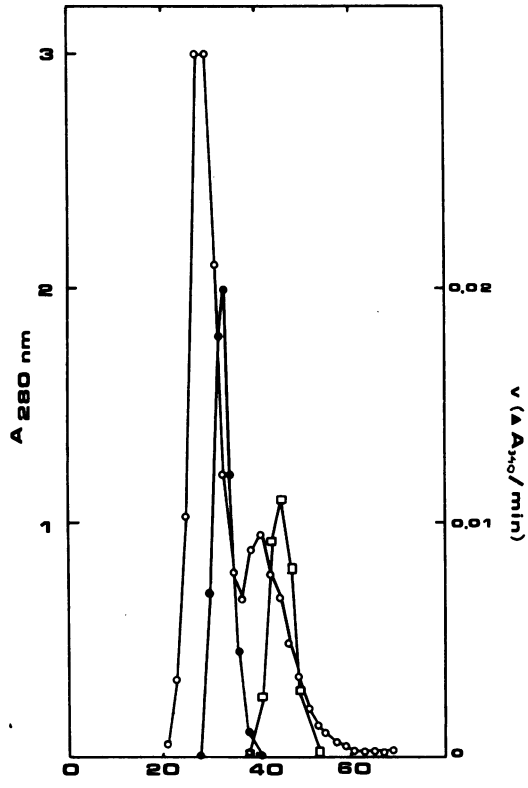

FIG. 1. *Separation of diacetyl reductase from β-ketoacyl acyl carrier protein reductase by chromatography on a Sephadex G-100 column (2 by 45 cm). Potassium phosphate (0.5 M) (pH 7.0) was used as buffer, and fractions of 2.5 ml each were collected. For both reductase assays, portions of the indicated fractions were diluted 1:100 with 10 mM potassium phosphate buffer (pH 7.0). Portions of 5 μliters of these diluted fractions were used for assaying β-ketoacyl acyl carrier protein reductase activity as described by Schulz and Wakil (23), and portions of 0.1 ml were used for assaying diacetyl reductase. The values of ΔA_{340}/min thus obtained are plotted above. Symbols: ●, β-ketoacyl carrier protein reductase; □, diacetyl reductase; ○, absorbance at 280 nm.*

and purified by ammonium sulfate precipitation and chromatography on DEAE-cellulose, Sephadex G-100, and DEAE-Sephadex A-50 as summarized in Table 1. Throughout the procedure the enzyme was assayed with ethyl acetoacetate as substrate. The final purification step on DEAE-Sephadex A-50 gave a good separation of the reductase from the bulk protein (Fig. 2). 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 3 shows that the activity peak coincides with the protein peak, and other evidence (not presented) proved that the ethyl acetoacetate reductase and diacetyl reductase activities were superimposable. However, as seen in Fig. 3 the protein peak is broad and has shoulders not seen in the activity peak. This observation indicates

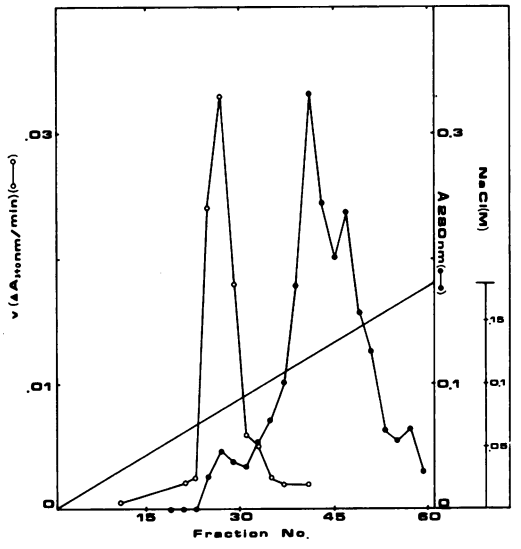

FIG. 2. *The final purification of diacetyl reductase on DEAE-Sephadex A-50. Diacetyl reductase was chromatographed and fractions of 20 ml each were collected. Reductase activity assays were performed with 0.1 ml of each of the indicated fractions. The values of ΔA_{340}/min thus obtained are plotted above.*

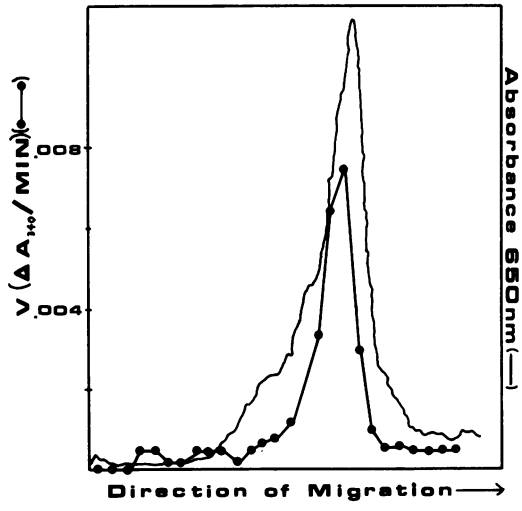

FIG. 3. *Disc gel electrophoresis of diacetyl reductase. Photometric scan of gel after staining with amido black (——); diacetyl reductase activity (●).*

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.

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. It was found to be 10,000, an unusually small molecular weight for 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 2). Since the enzyme was highly active also with ethyl pyruvate, but showed little activity with pyruvate, oxalacetate, or α-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* (16) 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* (1), 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 $NADP^+$.

TABLE 2. *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 α-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] 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 μmol/min/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.

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. 4.

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. 5, curve 2)

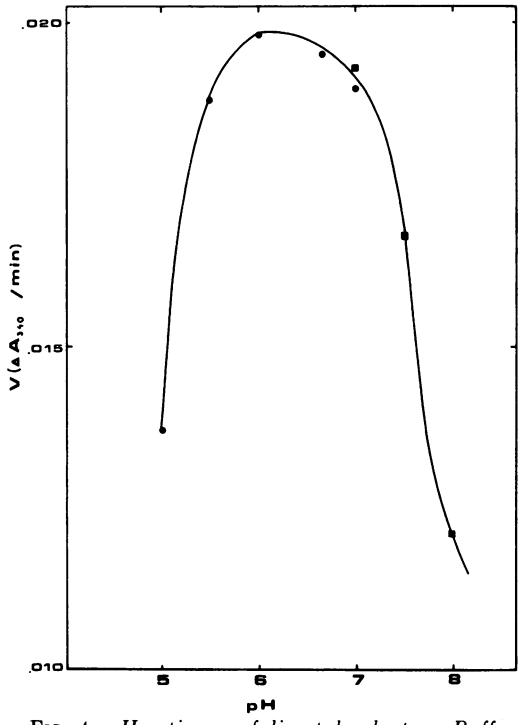

FIG. 4. *pH optimum of diacetyl reductase. Buffers used were citrate phosphate (●) and potassium phosphate (■). The assay mixture contained 100 μmol of the indicated buffer, 10 μmol of diacetyl, 56 nmol of NADPH, and 0.45 μg of diacetyl reductase in a total volume of 0.6 ml.*

comparable to that determined in potassium phosphate buffer (Fig. 5, 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. 5, 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. 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. 5, 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. 5, 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. 5, 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 Speck-

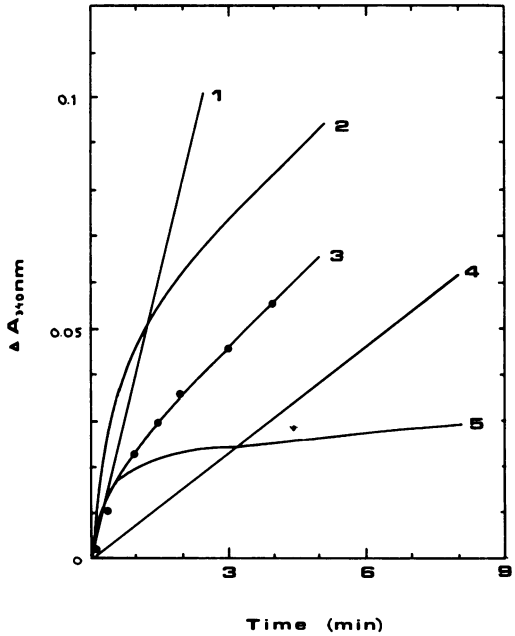

FIG. 5. *Time curves of NADPH oxidation catalyzed by diacetyl reductase in the presence of diacetyl. (Curve 1) With potassium phosphate (pH 7.8) as buffer. (Curve 2) With Tris-hydrochloride (pH 7.8) as buffer when the reaction was started by the addition of diacetyl. (Curve 3) Difference between curves 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.*

man and Collins (27). Two peaks (I, II) were observed when the reaction mixture was chromatographed (Fig. 6A). Peak II coincides with the peak in Fig. 6C 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. 6B). 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 diacetyl and 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 ratio 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 we have successfully used this method to show that 3-hydroxyacyl-CoA dehydrogenase catalyzes the reduction of ethyl acetoacetate to ethyl 3-hydroxybutyrate, we are confident that no ethyl 3-hydroxybutyrate was formed in the diacetyl reductase catalyzed reaction.

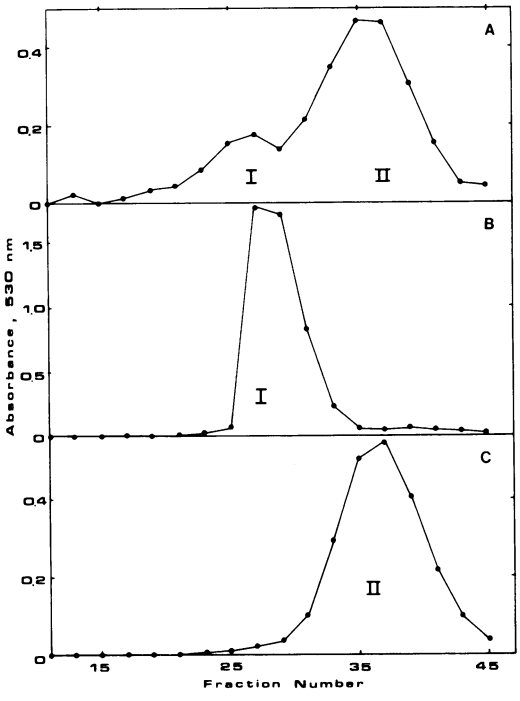

FIG. 6. *Identification of the product of the diacetyl reductase reaction. The assay mixture contained 2.5 μmol of diacetyl, 40 μmol of potassium phosphate, 0.15 mg of diacetyl reductase (added in two portions), and 0.5 mg of NADPH (added in three portions) in a total volume of 1 ml. The control contained 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 (27). 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 (30). (A) Reaction mixture; (B) 2.5 μmol of acetoin; (C) control.*

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. 7A). More pronounced substrate inhibition was observed when the concentration of NADPH was increased beyond the optimal concentration of 0.15 mM (Fig. 7B). 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. 8). 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. 8, insert) yielded the kinetic parameters (K_m, V_{max}) for the diacetyl-dependent oxidation of NADPH (Table 3). The K_m value for diacetyl was found to be 4.44 mM, a value which is of the same order of magnitude as the K_m 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 3. These data clearly show

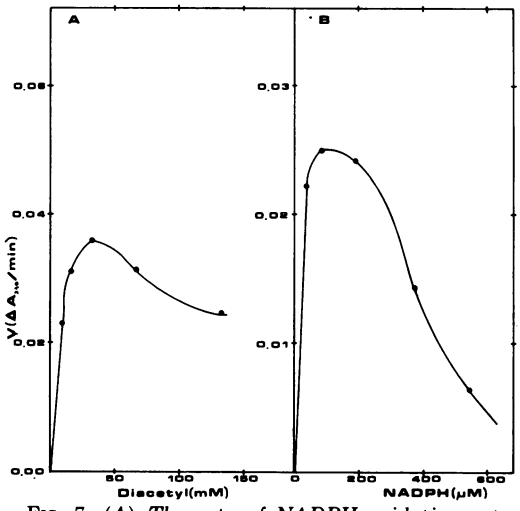

FIG. 7. *(A) The rate of NADPH oxidation catalyzed by diacetyl reductase as a function of the diacetyl concentration. The assay mixture contained, in a total volume of 0.6 ml, 100 μmol of potassium phosphate (pH 7.0), 45 nmol of NADPH, 0.9 μg of diacetyl reductase, and the indicated amounts of diacetyl. (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.*

that diacetyl is the preferred substrate and that NADPH and not NADH serves as the coenzyme in this reaction.

DISCUSSION

The diacetyl reductase was initially recognized by its ability to catalyze the oxidation of NADPH in the presence of ethyl acetoacetate. Since this property indicated a possible role of the enzyme in fatty acid metabolism, it was decided to purify and characterize the enzyme. However, the testing of various possible substrates showed that diacetyl was the most active. Since diacetyl was the only substrate with which the enzyme showed appreciable activity and which is known to exist in vivo in microorganisms, it is suggested that the enzyme functions as a diacetyl reductase and is therefore not involved in fatty acid metabolism. In contrast to the diacetyl reductase from beef liver (2) and *A. aerogenes* (18), this enzyme has a broad substrate specificity as evidenced by its small but definite activity with monocarbonyl compounds such as acetone and acetaldehyde.

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

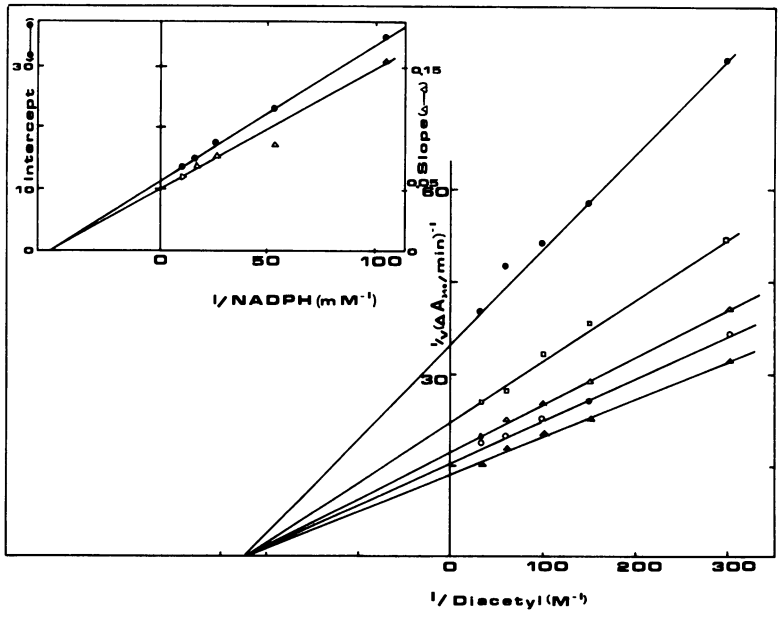

FIG. 8. *The rate of NADPH oxidation catalyzed by diacetyl reductase as a function of diacetyl concentration at several fixed levels of NADPH. Data are plotted on reciprocal coordinates. (Insert) Replot of slopes and intercepts versus the concentrations of NADPH. Each assay mixture contained, in a total 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 (●).*

TABLE 3. *Kinetic constants*

Variable substrate	Fixed substrate	K_m (mM)	V_{max} (μmol/min/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. 8.

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^+ (5).

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 (19) for NADH and with the ability of the beef liver reductase to utilize equally well NADH and NADPH (2).

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 and 76,000 which have been obtained for the molecular weights of the diacetyl reductases of *A. aerogenes* and beef liver, respectively (2, 12).

The biosynthesis of acetoin has been shown to take place via three pathways. Juni demonstrated that it is formed in bacteria by the condensation of 2-hydroxyethyl thiamine pyrophosphate and pyruvate, followed by the decarboxylation of the resulting α-acetolactate (14). In yeast and mammals, acetoin was shown to be formed by the reaction of 2-hydroxyethyl thiamine pyrophosphate with acetaldehyde (15). The third pathway which occurs in bacteria was demonstrated by Speckman and Collins to involve the formation of diacetyl from 2-hydroxyethyl thiamine pyrophosphate and acetyl CoA and the subsequent reduction of diacetyl to acetoin, catalyzed by diacetyl reductase (26). It has been suggested that acetoin formation, by the third pathway, functions to reduce high intracellular concentrations of pyruvate with the concurrent formation of NAD^+ which would be reutilized in glycolysis (26).

The formation of acetoin in *E. coli* was reported to occur by the pathway typical for mammals and yeast which does not involve the reduction of diacetyl. However, since we have demonstrated that diacetyl reductase is present in *E. coli* and since the formation of acetoin in this organism does not occur by the typical bacterial pathway, a reinvestigation of the mechanism of acetoin biosynthesis in this bacterium is warranted.

The unusual coenzyme requirement for NADPH may be understood in the context of the carbohydrate metabolism in *E. coli*. 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 $NADP^+$ (6). 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 the combined action of diacetyl reductase and pyridine nucleotide transhydrogenase, which is known to exist in *E. coli* (6), NADH is oxidized in the presence of diacetyl.

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LITERATURE CITED

1. Bryn, K., O. Hetland, and F. C. Stormer. 1971. The reduction of diacetyl and acetoin in *Aerobacter aerogenes*. Evidence for one enzyme catalyzing both reactions. Eur. J. Biochem. **18**:116-119.
2. Burgos, J. and R. Martin. 1972. Purification and some properties of diacetyl reductase from beef liver. Biochim. Biophys. Acta **268**:261-270.
3. Chuang, L. F., and E. B. Collins. 1968. Biosynthesis of diacetyl in bacteria and yeast. J. Bacteriol. **95**:2083-2089.
4. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404-427.
5. Di Sabato, G. 1971. On the nature of the lactic dehydrogenase-oxidized coenzyme-pyruvate complex. Biochemistry **10**:395-401.
6. Eagon, R. G. 1963. Rate limiting effects of pyridine nucleotides on carbohydrate catabolic pathways of microorganisms. Biochem. Biophys. Res. Commun. **12**:274-279.
7. Fieser, L. F. 1953. Cholesterol and companions. II.

Exhaustive dichromate oxidation. J. Amer. Chem. Soc. **75**:4386-4394.
8. Frank, R. L., P. G. Arvan, J. W. Richter, and C. R. Vanneman. 1944. The preparations of cyclopentenones from lactones. J. Amer. Chem. Soc. **66**:4-6.
9. Gabriel, M. A., H. Jabara, and U. A. S. al-Khalidi. 1971. Metabolism of acetoin in mammalian liver slices and extracts. Interconversions with butane-2,3-diol and biacetyl. Biochem. J. **124**:793-800.
10. Gornall, A. G., G. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751-766.
11. Hetland, O., K. Bryn, and F. C. Stormer. 1971. Diacetyl (acetoin) reductase from *Aerobacter aerogenes*. Evidence for multiple forms of the enzyme. Eur. J. Biochem. **20**:206-208.
12. Hetland, O., B. R. Olsen, T. B. Christensen, and F. C. Stormer. 1971. Diacetyl (acetoin) reductase from *Aerobacter aerogenes*. Structural properties. Eur. J. Biochem. **20**:200-205.
13. Johansen, L., S. H. Larsen, and F. C. Stormer. 1973. Diacetyl (acetoin) reductase from *Aerobacter aerogenes*. Kinetic studies of the reduction of diacetyl to acetoin. Eur. J. Biochem. **34**:97-99.
14. Juni, E. 1952. Mechanisms of formation of acetoin by bacteria. J. Biol. Chem. **195**: 715-726.
15. Juni, E. 1952. Mechanisms of the formation of acetoin by yeast and mammalian tissue. J. Biol. Chem. **195**:727-734.
16. King, H. L., Jr., and D. R. Wilken. 1972. Separation and preliminary studies on 2-ketopantoyl lactone and 2-ketopantoic acid reductases of yeast. J. Biol. Chem. **247**:4096-4098.
17. Kuhn, R., and T. Wieland. 1942. Zur Biogenes der Pantothensaure. Ber. Deut. Chem. Gesamte **75B**:121-123.
18. Larsen, S. H., L. Johansen, F. C. Stormer, and H. J. Storesund. 1973. Formation of 2,3-pentanediol from 2,3-pentanedione and acetylethylcarbinol by diacetyl (acetoin) reductase from *Aerobacter aerogenes*. A possible new pathway. FEBS Lett. **31**:39-41.
19. Larsen, S. H., and F. C. Stormer. 1973. Diacetyl (acetoin) reductase from *Aerobacter aerogenes*. Kinetic mechanisms and regulation by acetate of the reversible reduction of acetoin to 2,3-butanediol. Eur. J. Biochem. **34**:100-106.
20. Lowry, O. H., N. J. Rosebrough, N. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
21. Martin, R., and J. Borgos. 1970. Diacetyl reductase in animal tissues and its intracellular distribution. Biochim. Biophys. Acta **212**:356-358.
22. Martin, R., and J. Borgos. 1972. Kinetic studies of beef liver diacetyl reductase. Biochim. Biophys. Acta **289**:13-18.
23. Schulz, H., and S. J. Wakil. 1971. Studies on the mechanism of fatty acid synthesis. XXV. On the mechanism of β-ketoacyl acyl carrier protein reductase from *Escherichia coli*. J. Biol. Chem. **246**:1895-1901.
24. Seitz, E. W., W. E. Sandine, P. R. Elliker, and E. A. Day. 1963. Studies on the diacetyl biosynthesis by *Streptococcus diacetilactis*. Can. J. Microbiol. **9**:431-441.
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. Speckman, R. A., and E. B. Collins. 1968. Diacetyl biosynthesis in *Streptococcus diacetilactis* and *Leuconostic citrovorum*. J. Bacteriol. **95**:174-180.
27. Speckman, R. A., and E. B. Collins. 1968. Separation of diacetyl, acetoin and 2,3-butylene glycol by salting-out chromatography. Anal. Biochem. **22**:154-160.
28. Stallberg-Stenhagen, S. 1945. Synthesis of higher β-keto esters. Methyl esters of normal chain β-keto acids with 9 to 24 carbon atoms. Arkiv. Kemi Mineral. Geol. **20A**, no. 19.
29. Strecker, H. J., and I. Harary. 1954. Bacterial butylene glycol dehydrogenase and diacetyl reductase. J. Biol. Chem. **211**:263-270.
30. Westerfeld, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161**:495-502.