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PURIFICATION AND SOME PROPERTIES OF DIACETYL REDUCTASE FROM BEEF LIVER

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SUMMARY

1. Diacetyl reductase (acetoin:NAD^+ oxidoreductase, EC 1.1.1.5) from beef liver has been partially purified by a series of steps involving acetone precipitation of water extracts of acetone cakes, adsorption on calcium phosphate gel and chromatography on DEAE-cellulose. By following this procedure we have reached a specific activity 300-fold that of beef liver water extracts.

2. Enzyme purified in this manner is highly specific for one of the substrates, diacetyl (butane-2,3-dione), which can not be replaced by pentane-3-one, pentane-2,4-dione, hexane-2,5-dione, pyruvate, oxaloacetate, α-ketoglutarate or acetoin (acetyl methyl carbinol), but not for the other; NADPH can be substituted for NADH. Since beef liver crude extracts catalyze acetoin reduction by NADH to butyleneglycol, it is deduced that butyleneglycol dehydrogenase (2,3-butyleneglycol:NAD^+ oxidoreductase, EC 1.1.1.4) and diacetyl reductase are two different enzymes in beef liver.

3. Diacetyl reductase shows maximal activity at pH 6.1 and does not require metallic ions. At pH 6.1 the reaction in the diacetyl–acetoin direction follows Arrhenius's law, at least from 3.5 °C to 28–30 °C; activation energy has been estimated to be 14 400 cal/mole. It has not been possible to detect any activity in the backward direction (acetoin–diacetyl).

4. Sephadex-gel filtration resolved the enzyme preparations into two peaks of activity; the molecular weights of the enzymatic species were estimated as 26 000 and 76 000. The ratio of the peaks sizes varied with the eluant (phosphate buffer pH 6.3) molarity. This is taken as indicative that diacetyl reductase can be in two different states of association (as a monomer and a trimer), the equilibrium being somehow regulated by the ionic strength of the buffer.

INTRODUCTION

The ability of the extracts of a large number of bacteria to reduce diacetyl to acetoin and butyleneglycol has been known for a long time. Whether a single or two different enzymes catalize this stepwise reduction is still a controversial subject. The existence of two different enzyme systems in *Staphilococcus aureus* and *Aerobacter*

aerogenes, one (diacetyl reductase; acetoin:NAD^+ oxidoreductase, EC 1.1.1.5) catalyzing the reduction of diacetyl to acetoin by NADH and other (butyleneglycol dehydrogenase; 2,3-butyleneglycol:NAD^+ oxidoreductase, EC 1.1.1.4) which catalyzes the reversible oxidation of butyleneglycol to acetoin by NAD^+, was first reported in 1954 by Strecker and Harary[1], who purified butyleneglycol dehydrogenase 80-fold and diacetyl reductase 14-fold. Nevertheless, Juni and Heym[2] reported that bacterial acetoin reductase and diacetyl reductase activities reside in a single enzyme and Bryn *et al.*[3] purified 124-fold an *A. aerogenes* extract and could not observe any change in diacetyl reductase/butyleneglycol dehydrogenase activities, which was taken as proof that diacetyl reductase and butyleneglycol dehydrogenase are one and the same enzyme in *A. aerogenes*.

Although it is well known that significant amounts of acetoin are produced in animal tissues[4,5,6], little attention has been paid to its metabolism in animal species. Martin and Burgos[7] reported in 1970 for the first time the existence of diacetyl reductase in a variety of animal species and tissues and studied its intracellular distribution in beef and pigeon liver; more recently Gabriel *et al.*[8] confirmed the existence of diacetyl reductase in rat liver.

This paper describes a method for purification of diacetyl reductase from beef liver and some properties of this enzyme, and provides evidence that diacetyl reductase and butyleneglycol dehydrogenase activities are not associated to the same enzyme in this organ.

MATERIAL AND METHODS

NAD^+, NADH and NADPH were obtained from Boehringer. Acetoin and diacetyl were supplied by B.D.H.; acetoin was purified by washing it with peroxyde-free ether as recommended by Westerfeld[9] to remove diacetyl traces. Acetone, $(NH_4)_2SO_4$ and all other chemicals used were of high purity.

Protein determinations

Protein concentration was generally determined by the biuret method described by Chance and Redfearn[10] and for purified preparations with low protein concentration by the absorbance at 280 and 260 nm (Warburg and Christian[11]).

Enzyme assays

Diacetyl reductase activities were determined using a Beckman DBGT spectrophotometer with thermostatized cells following the procedure already published by the authors[7].

Butyleneglycol dehydrogenase was spectrophotometrically determined under the following conditions: Phosphate buffer pH 6.1, 0.15 mmole; acetoin, 12 μmoles; NADH, 0.6 μmole; total volume, 3 ml.

Units are defined as the amount of enzyme that reduces 1 nmole of substrate per min at 25 °C in the standard assay conditions.

RESULTS

Isolation of diacetyl reductase

A large number of standard methods for protein purification were applied to

water extracts of beef liver for diacetyl reductase isolation. Some resulted in a substantial loss of both total and specific activities (precipitation by acetic acid), others in a negligible increase in specific activities (precipitation by HCl, protamine sulfate and $(NH_4)_2SO_4$).

TABLE I

PURIFICATION OF DIACETYL REDUCTASE FROM BEEF LIVER

Starting liver fresh weight, 1 kg.

Step	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification (-fold)**	*Yield (%)*
1. Aqueous extract of first acetone cake	36 000	200 000	5.5	2.2	100
2. Acetone (1–1.3 vol.) precipitate	260	44 000	150	60	22
3. Calcium phosphate gel (unadsorbed)	200	42 000	210	84	21
4. Water extract of lyophilized powder	160	38 500	240	96	19
5. DEAE-22 column eluate	11	8 250	750	300	4

* Referred to a water extract obtained by homogenizing the liver with 10 vol. of glass-distilled water in a M.S.E. blades homogenizer at 0–2 °C and centrifuging at 12 000 × *g* for 10 min.

The following procedure (summarized in Table I) was finally developed (all operations were performed at 0–3 °C): Fresh beef liver, obtained from the slaughter house, was decapsulated and homogenized in a M.S.E. blade homogenizer with 2 vol. of acetone at −15 °C. The acetone suspension was added to 8 vol. of acetone at −15 °C, stirred, and filtered through a porous filter paper under reduced pressure on a Büchner funnel. The acetone powder was now homogenized by the same procedure with 10 vol. of acetone at −15 °C, stirred and filtered as before. The acetone cake was dried by suction on the Büchner funnel and pressed by hand among several layers of filter paper. The diacetyl reductase activity in the resulting cake is stable for at least three months at −15°C.

The acetone cake was extracted by stirring it in 4 vol. of glass-distilled water at 0 °C for 30 min. The water suspension was centrifuged in a refrigerated centrifuge at 12 000 × *g* for 10 min and the pellet discarded. At this step the ratio of activities butyleneglycol dehydrogenase/diacetyl reductase equals 0.1.

One volume of acetone at −15 °C was added dropwise, with continual stirring, to the water extract. The precipitate was removed by centrifugation and discarded. The supernatant was filtered through two layers of filter paper and another 0.3 vol. of acetone was added as before and the suspension equally centrifuged. The pellet was redissolved in a few ml of glass-distilled water. The enzyme preparation at this step lacks any butyleneglycol dehydrogenase activity.

A sufficient amount of calcium phosphate gel was added to the solution to bring about maximum adsorption of inert protein without adsorption of enzyme activity (about 2 mg of dry gel per ml of solution). After stirring for 15 min the suspension was centrifuged and the gel discarded. The supernatant was lyophilized and the powder so obtained was kept at −15 °C; under these conditions diacetyl reductase activity of the powder is stable for at least one month.

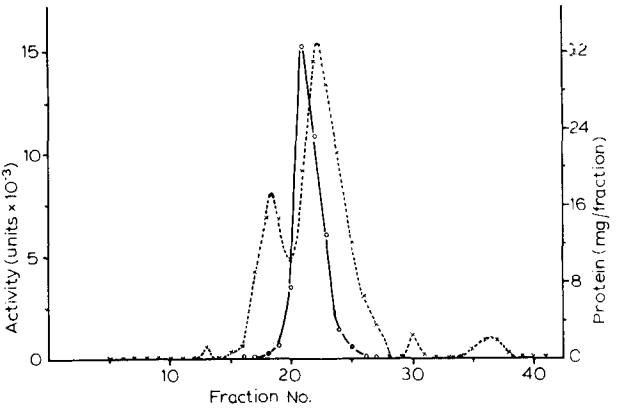


Fig. 1. DEAE-22 chromatography of a beef liver extract purified by acetone precipitation and calcium phosphate gel adsorption. The column (40 cm × 1.5 cm) was equilibrated with 25 mM phosphate buffer, pH 6.3. Enzyme solution was applied in the same buffer, used as well for elution (45 ml/h). Further elution with phosphate buffer up to 0.5 M did not draw out more additional diacetyl reductase activity. Fractions of 2.5 ml were collected. Figures in Table I refer to fraction No. 21. ×---×, protein; ○—○, activity.

For futher purification the lyophilized powder was extracted with 10 vol. of 25 mM sodium–potassium phosphate buffer, pH 6.3. A basically inactive sediment remained unextracted and was removed by centrifugation (20 min at 32 000 × *g*). The supernatant was fractioned by chromatography on DEAE-22 (Fig. 1). At this stage the enzyme can be lyophilized and kept several months at −15 °C without noticeable loss of activity.

After the acetone (1–1.3 vol.) fractionation the preparations are butyleneglycol dehydrogenase and NADH dehydrogenase free and were suitable for kinetics and other studies. Unless otherwise specified, preparations used for the experiments here described were redissolved lyophilized powder of the acetone precipitate (1–1.3 vol.).

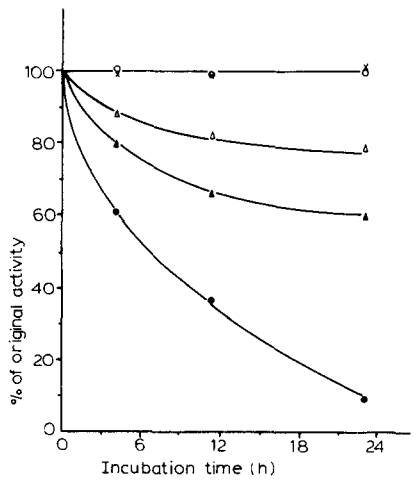


Fig. 2. Stability of diacetyl reductase at 0 °C Solvent: ▲—▲, water; ○—○, 1.5 M sucrose; △—△, 1 M sodium–potassium phosphate buffer, pH 6.1; ×—×, 0.5 M sodium–potassium phosphate buffer, pH 6.1; ●—●, 0.02 M diacetyl.

Stability of the enzyme

Aqueous solutions of enzyme preparations quickly lose diacetyl reductase activity. Its stability at 0 °C in several dilution systems is shown in Fig. 2.

Substrate specificity

It was studied in both acetone-fractionated powder and DEAE eluate. Diacetyl cannot be replaced as a hydrogen acceptor by any of the following compounds: acetone, pentane-3-one, pentane-2,4-dione, hexane-2,5-dione, pyruvate, oxaloacetate, α-ketoglutarate or acetylmethylcarbinol. No activity was detected with any of the above substances at concentrations up to 4 mM.

NADPH can be substituted for NADH as a hydrogen donnor; both nucleotides are equally effective in this role.

Enzyme concentration and time course of the reaction

Under the assay conditions described in Methods there was a linear relationship between the rate of NADH oxidation and the enzyme concentration (Fig. 3). Fig. 4 shows the time course of the reaction under standard assay conditions.

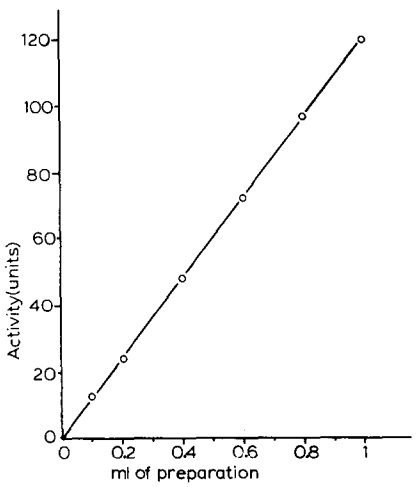


Fig. 3. Relationship between enzyme concentration and initial rate of NADH oxidation under standard assay conditions.

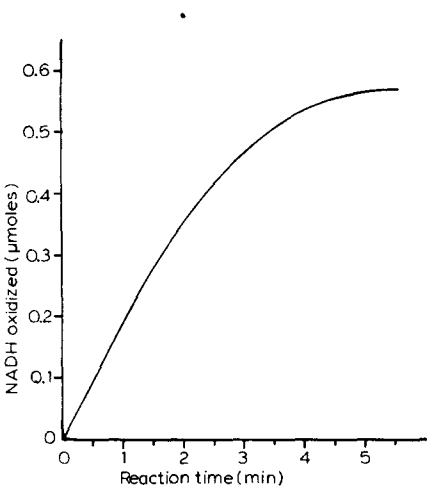


Fig. 4. Time course of the diacetyl reductase reaction under standard assay conditions.

Metallic ions effects

EDTA, sodium pyrophosphate, oxalic acid, cupferron or thiourea at 1 mM show no substantial effects on diacetyl reductase activity (Table II), but salicylic acid at this concentration reduces the activity to 60%.

Dialysis at 0 °C against 20 vol. of water for 4 h followed by a change of water and another 20 h of dialysis against 100 vol. of water resulted in a loss of 40% of the activity which cannot be recovered by addition of the following substances: concentrate dialysis medium, Zn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+} or Fe^{3+}.

TABLE II

THE EFFECTS OF SEVERAL CHELATING AGENTS ON DIACETYL REDUCTASE ACTIVITY

All chelating agents were at a concentration of 1 mM.

Chelating agent	*Activity*	
	Units	*% of control*
None	69.5	—
EDTA	70	100.7
Salicylic acid	42.5	61.1
Sodium pyrophosphate	65.3	94.1
Oxalic acid	67.6	97.1
Cupferron	73.7	106
Thiourea	72.2	103.8

There is no loss of activity if dialysis is performed against 1.5 M sucrose or 0.5 M sodium–potassium phosphate buffer, pH 6.1 .

Activity at various pH values

The activity of the enzyme was measured in sodium–potassium phosphate buffer (0.15 mmole per 3 ml of reaction mixture) at various pH values. Maximal activity of the enzyme was obtained at pH 6.1 (Fig. 5). A second peak of activity is observed at pH 6.8 in the pH profiles of all states of purification. The ratio activity at pH 6.8 to activity at pH 6.1 remains constant (0.9) throughout the whole purification procedure.

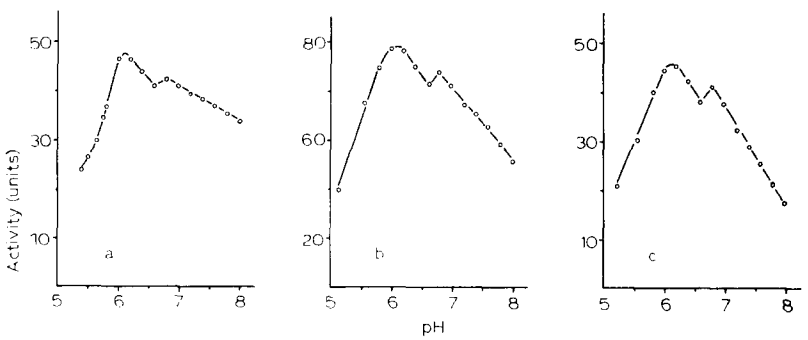


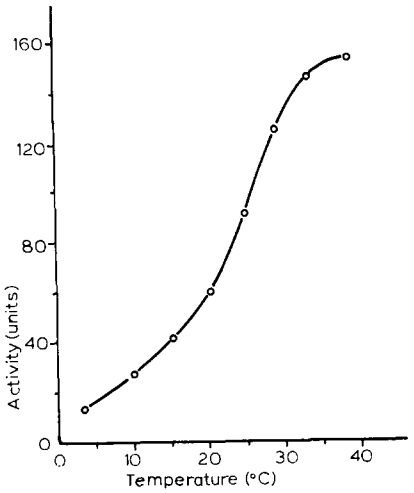
Fig. 5. Effects of pH on enzyme activity in 0.05 M phosphate buffer. Enzyme preparation: (a) Acetone cake aqueous extract. (b) Acetone precipitate (1–1.3 vol. of acetone per vol. of medium; Step II of described purification procedure). (c) DEAE eluate.

Temperature effect on enzyme activity and activation energy

Enzyme activity rises between 5 and 25 °C with a Q_{10} of 2.5 (Fig. 6). Arrhenius's law is followed at least between 3.5 and 28–30 °C. Activation energy was calculated from Fig. 7 as 14 400 cal/mole.

Reversibility

No activity could be spectrophotometrically detected in the acetoin–diacetyl direction, even with acetoin concentrations up to 0.24 M and NAD^+ up to 0.01 M. Furthermore, no diacetyl could be detected by the Owades and Jakovac method[12]



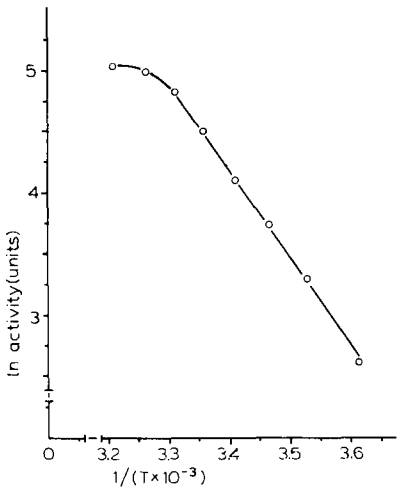


Fig. 6. Effects of temperature on enzyme activity.

Fig. 7. Arrhenius plot of the changes of diacetyl reductase activity with temperature.

after incubating an enzyme preparation with a total activity of 130 units in the forward direction for 12 h at 25 °C in the presence of the previously stated maximal concentrations of acetoin and NAD^+.

Molecular weight estimation by Sephadex gel filtration

Sephadex gel filtration of the enzyme preparations was performed on a 46.5 cm × 1.5 cm column equilibrated with a sodium–potassium phosphate buffer, pH 6.3. Buffer molarity was varied as shown in Fig. 8. Two different peaks of activity

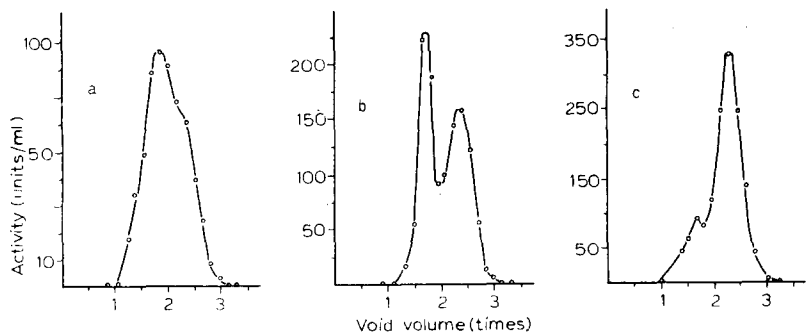


Fig. 8. Elution pattern of diacetyl reductase from a Sephadex G-100 column (46.5 cm × 1.5 cm). Temp., 0–2 °C; hydrostatic pressure, 10 cm; eluant, phosphate buffer, pH 6.3; molarities: (a) 0.05 M; (b) 0.5 M; (c) 1 M.

were observed at high buffer molarities. From comparing their K_{av} with those of known standard proteins, their molecular weights were found to be about 76 000 and 26 000 (Fig. 9). At low molarity (0.05 M) only the peak of molecular weight 76 000

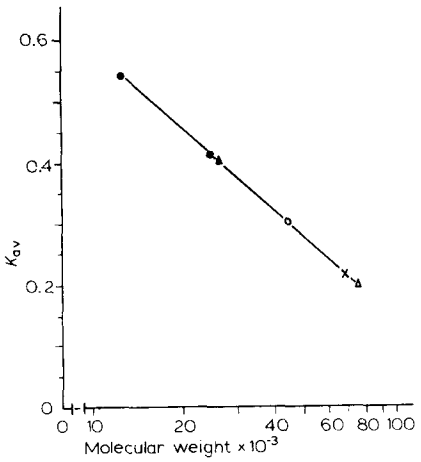


Fig. 9. Estimation of the molecular weight of diacetyl reductase by Sephadex gel filtration. The following protein standards were included: ×, haemoglobin (M_r 68 000); ○, egg albumin (M_r 45 000); ●, chymotrypsin (M_r 24 500); ⊗, cytocrome *c* (M_r 12 500). △, diacetyl reductase fast moving and ▲, slow moving species.

can be clearly observed; the slow moving peak is only reflected as a shoulder in the elution pattern.

DISCUSSION

Diacetyl reductase has been purified about 300-fold from beef liver. Water extraction of the acetone cake is fully effective: it brings out as much activity as water extraction of an equal amount of liver. The stability of the enzyme against the acetone treatment is remarkable: diacetyl reductase precipitates in the acetone concentration range of 0.4–1.5 vol. of acetone per vol. of medium. Estimation of the activities of the precipitates in this range gives a 100% yield. By taking advantage of the fact that most of the contaminant protein precipitates in the range of acetone concentration 0.25–1 vol. per vol. of medium, a substantial purification can be obtained collecting the precipitate in the range 1–1.3 vol. of acetone. Some batches of preparation give at this step non linear plots of initial activities against enzyme concentration, showing the presence of an internal inhibitor that compels, for kinetics studies, performance of a linearity test in all batches and the discarding or further purification of those with non-linear plots.

Phosphate gel adsorption requires either a previous determination of the appropriate quantity of gel to be added or a gradual gel addition with continuous monitoring of the supernatant activity. Once adsorbed, diacetyl reductase is extremely difficult to remove from the gel.

Fibrous forms of DEAE are preferable to microgranular ones for diacetyl reductase purification because of the low stability of the enzyme at the low buffer molarity used for elution.

The results delineated in this work demonstrate that diacetyl reductase and butyleneglycol dehydrogenase are two different enzyme species in beef liver. The

same conclusion was reached recently by Gabriel *et al.*[8] on rat liver. This is in contrast with the data reported by Bryn *et al.*[3] for *A. aerogenes* systems and by Juni and Heym[2], but agrees with the former work of Strecker and Harary[1] in *A. aerogenes* and *S. aureus*.

The enzyme responsible for diacetyl reduction to acetoin in beef liver is similar to the majority of dehydrogenases in regard to substrate specificity. Structural substrate requirements are stricter than the presence of a ketonic group in the nearest neighbourhood of a methylic one or the existence of two ketonic groups in the same molecule with one or more methylene groups in between.

As it has been reported for diacetyl reductase from other sources[1,13,14], the reduction of diacetyl catalyzed by the beef liver enzyme is for all practical purposes an irreversible reaction. In the already mentioned work of Gabriel *et al.*[8] some diacetyl was shown to be enzymatically formed from acetoin if methosulphate of phenazine and nitroblue of tetrazolium were included in the reaction system. Theoretically there are not irreversible enzyme reactions, and it is reasonable to assume that dye oxidation of any traces of NADH formed during acetoin–diacetyl transformation shift the equilibrium towards higher diacetyl concentrations; but, on the other hand, the low purity of their enzyme preparations introduce a high factor of uncertainty about the significance of diacetyl reductase in this dye participating reaction.

Diacetyl reductase from beef liver does not require metallic activators or other dialyzable cofactors, as reported for the bacterial enzyme (Seitz *et al.*[13]). Loss of activity during dialysis against water should be imputed to the low enzyme stability in this solvent rather than to a loss of dialyzable factors, since activity remains constant if 1.5 M sucrose or 0.5 M phosphate buffer, pH 6.1, are substituted for water. Inhibition by salicylic acid does not necessarily imply chelating effect since the complexity of their inhibition effects in other enzymes is well known[15]. Furthermore, a chelating mechanism for salicylic acid inhibition would be rather difficult to reconcile with the absolute lack of effects of other complexing agents (cupferron, sodium pyrophosphate, oxalic acid, EDTA, *etc.*).

The pH profile and the elution pattern from Sephadex chromatography could be interpreted as proof of the existence of two different enzyme systems in beef liver. Nevertheless, the relationship between activities at the two peaks of the profile (pH 6.1 and pH 6.8) remains constant along the purification procedure and the distribution of the total activity of the enzyme preparation between the two different molecular weight catalytical species varies with the molarity of the eluent buffer. It seems reasonable to assume that it is a single enzyme with a rather complex pH profile in our standard assay conditions, and that it can adopt two different states of association whose equilibrium is somehow regulated by the ionic strength of the medium. Since the molecular weight of the two states is about 26 000 and 76 000 respectively, this is taken as indicative that the higher molecular weight species is a composite of three units of the lower molecular weight species. This would be in agreement with the results recently reported for rat liver[8] where two fractions are obtained in CM-Sephadex and the same elution pattern is obtained by rechromatography of one of them.

Physiological role of diacetyl reductase

The presence of butyleneglycol dehydrogenase and diacetyl reductase in animal

tissues, first reported by the authors[7] and recently confirmed by another group[8], deserves some comments on their possible physiological role.

Diacetyl synthesis in animal tissues has not been reported yet, but the existence of two different metabolic routes capable of disposing of it, *i.e.* diacetyl reductase-butyleneglycol dehydrogenase and the diacetyl mutase system of Green *et al.*[16] now identified with the pyruvate oxidase system[5,17], suggest that diacetyl must be somehow produced in animals. It is not easy to accept the synthesis of an ubiquitous enzyme system, of such a high specificity as diacetyl reductase appears to be, devoid of all physiological functions.

If diacetyl were produced in animal tissues by condensation of activated acetaldehyde and acetyl-CoA, as proposed by Speckman and Collins[14] for other systems, one molecule of diacetyl would be synthesized from two of pyruvate. The reduction of diacetyl to butyleneglycol would be linked to the oxidation of two molecules of NADH. Since acetyl-CoA production from pyruvate brings about the reduction of a NAD^+ molecule, the net balance of the pathway 2 pyruvate → butyleneglycol would be, in terms of pyridine nucleotides, the oxidation of one molecule of NADH. Under partially anaerobic conditions, as in straining work, that would help the operation of the glycolytic pathway and would avoid lactate and pyruvate accumulation. Intracellular distribution of this enzyme[7] seems the most appropriate for performance of this task.

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