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# PIGEON LIVER DIACETYL REDUCTASE: PURIFICATION AND SOME PROPERTIES

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## SUMMARY

1. Diacetyl reductase (Acetoin:$NAD^+$ oxidoreductase, EC 1.1.1.5) from pigeon liver has been purified about 400 fold by a method including aqueous extraction, acetone precipitation and Sephadex G-100 chromatography.

2. The purified preparations catalyze the (for all practical purposes) irreversible reduction of diacetyl to acetoin, using as hydrogen donors either NADH or NADPH. One single enzyme is responsible for the NADH- and NADPH-dependent reactions. Thus, the enzyme is different from that described by entry EC 1.1.1.5 in Enzyme Nomenclature (1972).

3. Pigeon liver diacetyl reductase is highly specific with respect to hydrogen acceptors: it does not reduce acetoin, monoketones or nonvicinal diketones.

4. The pH profiles show one single broad peak in the range 5.6–6.2. At pH 6.1 and 0.2 mM NADH, the apparent $K_m$ for diacetyl has been estimated to be 3.5 mM. The enzyme does not require metallic activators for maximal activity.

5. The molecular weight, as calculated by Sephadex gel filtration, was found to be about 110 000.

## INTRODUCTION

Diacetyl reductase (Acetoin:$NAD^+$ oxidoreductase, EC 1.1.1.5) seems to be a rather ubiquitous enzyme; the enzymatic transformation of diacetyl to acetoin coupled to NADH oxidation has been reported in a wide variety of biological systems (bacteria [1–8], mammalian [9–12] and bird tissues [9]), but the reaction has only been characterized in a few cases and the enzyme comparatively well studied in *Aerobacter aerogenes* [1, 5–8] and beef liver [9, 11, 12].

The properties of diacetyl reductase from various origins differ considerably with regard to substrate specificity [1, 2, 4, 5, 9–11], molecular weight [7, 11] and affinity for diacetyl [4, 8, 10, 12].

Very little attention has been paid to diacetyl reductase from birds, although it has been shown that the level attained by this enzymatic activity in these animals is higher than in mammals. Early experiments [9] showed also that, while in beef liver about a 55% of the diacetyl reductase activity was bound to particles, in pigeon liver no less than 90% of the total activity is found in the soluble fraction. Other significant

differences in the behaviour of both animal enzymes were observed in previous experiments in our laboratory. Therefore, it appeared justifiable to investigate the properties of diacetyl reductase from pigeon liver. As the purification procedure described for the beef liver enzyme [11] was not successful when applied to this material, a new one was developed.

It has been reported that animal diacetyl reductase preparations oxidize in the presence of diacetyl NADPH as well as NADH [10, 11] but neither the stoichiometry of the reaction nor the possible identity of the enzymes catalyzing both reactions were studied. The present report describes a purification procedure for pigeon liver diacetyl reductase and some properties of the enzyme; it provides as well conclusive evidence of the presence of NADPH-dependent diacetyl reductase activity and that one single enzyme catalyzes the reaction with either nicotinamide adenine dinucleotide.

## MATERIALS AND METHODS

Reagent solutions and buffers were prepared in deionized glass-distilled water. Coenzymes were obtained from Boehringer. Diacetyl, acetoin, hexane-2,5-dione, acetone, acetate, α-ketoglutarate, oxaloacetate, pentane-3-one, pentane-2,4-dione and all other chemicals used were purchased from Merck, B.D.H., Koch–Light or Sigma. Acetoin was purified as already described [12].

### *Analytical procedures*

Diacetyl was determined by the method of Owades and Jakovac [13] and acetoin by the procedure of Westerfeld [14]. Protein content was measured by either the Biuret method as published by Chance and Redfearn [15], or by the absorbance at 280–260 nm [16] when the concentration was too low or the sample had to be recovered.

### *Enzyme assays*

Diacetyl reductase activities were spectrophotometrically determined at 25 °C and pH 6.1 (unless otherwise stated) in the presence of sodium–potassium phosphate buffer, 0.15 mmole; NADH or NADPH, 0.6 μmole; diacetyl, 30 μmoles; total volume, 3 ml. One unit is defined as the amount of enzyme that oxidizes 1 nmole of NADH per min under these conditions.

Substrate specificity tests were performed substituting the compound under test for diacetyl, at the same concentration; other assay conditions (pH, temperature and coenzyme concentration) were as above.

### *Polyacrylamide gel electrophoresis*

Disc electrophoresis in 7.7% polyacrylamide gels (bisacrylamide versus acrylamide, 2.7%) was performed at 0–4 °C and pH 8.5 in 0.05 M Tris–glycine buffer. 3 mA/tube (0.9 cm internal diameter) were applied for 6 h. Protein was stained with Amidoschwartz (0.5 g in 45 ml methanol$^+$ 45 ml water$^+$ 10 ml acetic acid). For activity staining, the gels were first incubated in 12 mM diacetyl and 3 mM NADH or NADPH in 0.05 M sodium–potassium phosphate buffer, at pH 7, for 15–20 min; after incubation, they were transferred to a freshly prepared solution of 25 mg of nitroblue tetrazolium and 12.5 mg of phenazine methosulphate per 100 ml of 0.05 M sodium–potassium phosphate buffer, at pH 7. Diacetyl reductase bands appear as colourless zones on a dark purple background.

RESULTS

*Purification of diacetyl reductase*

Several extraction (water, acetone, 0.25 M sucrose) and protein fractionation procedures (acid, protamine sulphate, lead acetate, acetone, ethanol and ammonium sulphate precipitations, calcium phosphate gel adsorption, ion exchange and Sephadex G-100 and G-200 chromatography in various elution systems) were tried. The following purification procedure, which resulted in a 400-fold purification with about a 8% yield, was finally adopted:

After slaughtering the pigeons, the livers were quickly removed, chilled in ice-cold distilled water, decapsulated, cut into small pieces and homogenized with 4 vols of distilled water (0–2 °C) in either a M.S.E. blades homogenizer or an all-glass Potter–Elvehjem. The homogenate was filtered throught 4 layers of cheese cloth; the filtrate was centrifuged for 100 min at 105 000 × *g* in a refrigerated centrifuge and 1.2 vols of acetone at −15 °C were added dropwise, with continual stirring, to the supernatant. The precipitate was discarded by centrifugation (12 000 × *g* for 10 min at −5 °C) and 1.3 more vols of acetone at −15 °C were added, as before, to the supernatant. After standing for 10 min, the suspension was centrifuged; the pellet was collected in a few ml of ice-cold distilled water and lyophilized. At this stage, the preparations can be kept several months at −15 °C with a high retention of activity; lyophilization of the acetone precipitate is also advantageous to remove acetone traces, which we have observed inhibit the reaction.

The lyophilized powder was extracted with 10 vols of 1 M sodium–potassium phosphate buffer, pH 6.1. An inactive sediment which remained in suspension was removed by centrifugation at 105 000 × *g* for 15 min. The supernatant was fractionated by chromatography on Sephadex G-100 (Fig. 1). The maximal specific activity

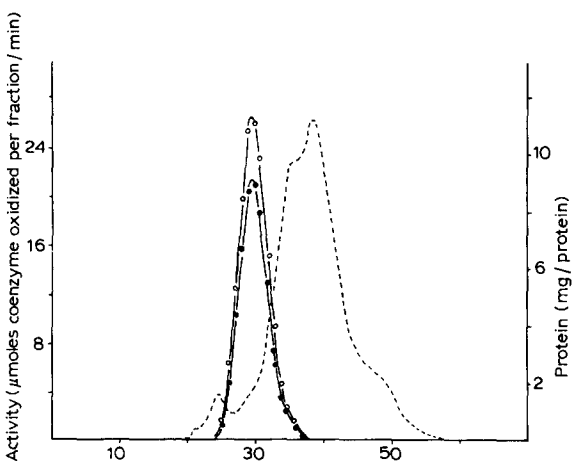


Fig. 1. Chromatography on Sephadex G-100 of a pigeon liver extract purified by acetone precipitation. The column (38·2.5 cm) was equilibrated with 1 M sodium–potassium phosphate buffer, at pH 6.1. The enzyme solution was applied in the same buffer, which was also used for elution (10 ml/h). Fractions of 3.2 ml were collected. ---------, protein; ○—○, NADPH-dependent activity; ●—●, NADH-dependent activity.

TABLE I

PURIFICATION OF DIACETYL REDUCTASE FROM PIGEON LIVER

Starting with liver of fresh weight, 200 g. NADH/NADPH is the ratio of the activities of NADH-dependent/NADPH-dependent.

| Step | Total protein, (mg) | Total activity, (units) | Specific activity, (units/mg protein) | Purification, (-fold) | Yield, (%) | NADH/ NADPH activity |
|---|---|---|---|---|---|---|
| 1. Water homogenized | 30 000 | 900 000 | 30 | — | — | 1.06 |
| 2. Supernatant 100 min at 105 000 × g | 8 500 | 870 000 | 100 | 3.5 | 97 | 1.06 |
| 3. Water extract of lyophilized acetone (1.2–2.5 vol.) precipitate | 320 | 250 000 | 800 | 24 | 28 | 0.77 |
| 4. Sephadex G-100 column eluate | 6 | 70 000 | 12 000 | 400 | 8 | 0.77 |

fractions were collected, lyophilized and stored at −15 °C; under these conditions, preparations can be kept for at least six months with partial retention of the diacetyl reductase activity.

Table I summarizes the data for a typical preparation. The purity of the final preparation was estimated to be about 40% on the basis of polyacrylamide gel electrophoresis (Fig. 2).

The enzyme preparations as obtained in Steps 3 and 4 are devoid of butyleneglycol dehydrogenase and nonspecific NADH-oxidase activities, and show under

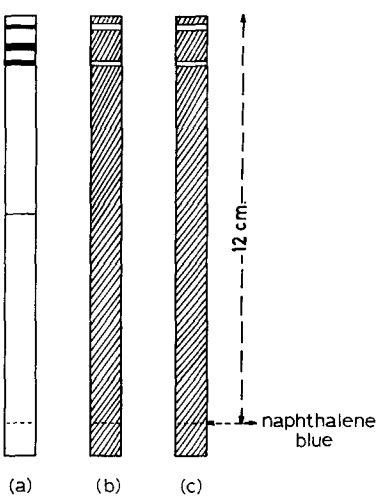


Fig. 2. Disc electrophoresis in polyacrylamide gel of a purified sample (Step 4) of pigeon liver diacetyl reductase. Anode at the bottom. (a) Stained for protein; (b) stained for NADH-dependent diacetyl reductase activity; (c) stained for NADPH-dependent diacetyl reductase activity. For experimental details, see Materials and Methods.

the standard assay method a linear relationship between initial diacetyl reductase activity and enzyme concentration (Fig. 3).

Storage of water solutions of the enzyme preparations at stages 3 and 4 of the purification procedure results in a loss of activity of about a 75% in 24 h at 0 °C. There is no noticeable inactivation in 1 M sodium–potassium phosphate buffer, at pH 6.1 or 1.5 M sucrose for the same time under identical temperature of storage.

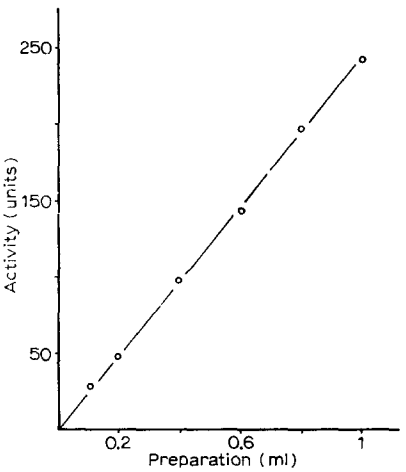


Fig. 3. Relationship between enzyme concentration and initial rate of NADH oxidation under the standard assay conditions.

*Coenzyme specificity*

Enzyme preparations at any stage of purification oxidize NADH and NADPH in the presence of diacetyl. Table I shows the ratio of the activity NADH-dependent/NADPH-dependent.

The elution patterns of the NADH and NADPH activities from Sephadex G-100 columns are identical (Fig. 1). Both activities also show the same migration pattern in polyacrylamide gel electrophoresis at pH 8.5 in 0.05 M Tris–glycine buffer (Fig. 2).

Activity tests with equimolar mixtures of both coenzymes were performed as follows: The activity of a solution of the enzyme at the highest purity attained in this work was measured at standard assay conditions with: (a) 0.2 mM NADH; (b) 0.2 mM NADPH; and (c) 0.2 mM NADH plus 0.2 mM NADPH. The averages of four determinations in each case, were as follows: (a) 50.6 ($\pm$ 1.6) nmoles of NADH oxidized/min per 0.1 ml of preparation; (b) 66.2 ($\pm$ 1.7) nmoles of NADPH oxidized/min per 0.1 ml; (c) 61.3 ($\pm$ 2.2) nmoles of coenzyme oxidized/min per 0.1 ml.

*Substrate specificity*

With the most purified preparations diacetyl cannot be replaced as substrate, with either coenzyme, by any of the following compounds: acetone, acetoin, pentane-3-one, pentane-2,4-dione, hexane-2,5-dione, acetate or $\alpha$-ketoglutarate. All enzyme preparations show some $NAD^+$-dependent L-malate dehydrogenase activity when assayed at the pH, temperature and substrate (oxaloacetate) and coenzyme (NADH)

concentrations used for the diacetyl reductase determinations, but the ratio of activities of diacetyl reductase/malate dehydrogenase progressively increases during the purification procedure, being about 100 times higher in the final preparation than in the original water extracts.

*Reversibility*

No diacetyl production could be detected by the Owades and Jakovac method [13] after incubating in an air-tight flask an enzyme preparation with 240 units in the forward direction for 12 h at 25 °C in the presence of up to 0.24 M acetoin and up to 0.01 M $NAD^+$ or $NADP^+$. No activity could be spectrophotometrically detected either.

An identical enzyme preparation was equally incubated at 25 °C for 12 h with 12 μmoles of acetoin, 15 μmoles of $NAD^+$, 1 mg of nitroblue tetrazolium and 0.5 mg of phenazine methosulphate in 4 ml of 0.5 M sodium pyrophosphate buffer at pH 9. A blank without enzyme was treated in the same way. At the end of the incubation period, 0.49 μmole of diacetyl was detected in the test sample and 0.19 in the blank.

*Stoichiometry of the reactions*

Purified enzyme preparations were incubated at 25 °C with diacetyl and NADH or NADPH in a flask fitted with an air-tight rubber plug. Samples were periodically removed by means of a precision syringe and NADH or NADPH and diacetyl were determined. When no diacetyl could be detected, the acetoin concentration was measured. Results, proving that for each mole of diacetyl reduced one mole of coenzyme is oxidized and one mole of acetoin is produced, and further experimental data are given in Table II.

TABLE II

STOICHIOMETRY OF THE CATALYZED REACTIONS

Reaction mixtures: Experiment (a) NADH-dependent reaction: diacetyl, 38.4 μmoles; NADH, 38.4 μmoles; enzyme preparation, total activity 10 000 units; sodium–potassium phosphate buffer, pH 6.1, 1 mmole; total volume, 20 ml. Experiment (b) NADPH-dependent reaction: diacetyl, 26.8 μmoles; NADPH, 31.2 μmoles; enzyme preparation, total activity 6500 units; sodium–potassium phosphate buffer and total volume, as above. Acetoin was not determined in the experiment because diacetyl is also positive in the Westerfeld reaction [14] and can only be reliably measured when all the diacetyl has been consumed unless very tedious experimental procedures are used.

| Time, (min) | Experiment (a) | | | Experiment (b) | | |
|---|---|---|---|---|---|---|
| | Reduced diacetyl (μmoles) | Oxidized NADH (μmoles) | Produced acetoin (μmoles) | Reduced diacetyl (μmoles) | Oxidized NADPH (μmoles) | Produced acetoin (μmoles) |
| 0 | 0 | 0 | — | 0 | 0 | — |
| 3 | 17.8 | 17.8 | — | 10.6 | 10 | — |
| 6 | 23.7 | 25.6 | — | 17.8 | 16.2 | — |
| 12 | 30 | 31 | — | 23.8 | 22.9 | — |
| 20 | 35.1 | 35.2 | — | 25.4 | 25 | — |
| 40 | 37.1 | 37.2 | — | 26.1 | 25.9 | — |
| 60 | 37.8 | 38.2 | — | 26.5 | 26.3 | — |
| 80 | 38.4 | 38.4 | 39.1 | 26.8 | 26.7 | 26.9 |

*Additional cofactors requirements*

Continuous dialysis of enzyme preparations against 1000 vols of 1 M sodium–potassium phosphate buffer, at pH 6.1, for 9–12 h does not result in loss of activity. Incubation with 1 mM EDTA for 30 min at 0 °C has no effect on activity. Furthermore, activity recoveries in gel filtration experiments with 1 M sodium–potassium phosphate buffer at pH 6.1, as eluant, are approximately 100%.

*Molecular weight*

Since diacetyl reductase from beef liver is eluted from Sephadex G-100 near the void volume, where the linearity of the plots of $K_{av}$ versus the log of molecular weights is lost, Sephadex G-200 was used for the estimation of its mol. wt. Comparing the $K_{av}$ of the enzyme with those of known standard proteins (Fig. 4) its molecular weight was found to be about 110 000.

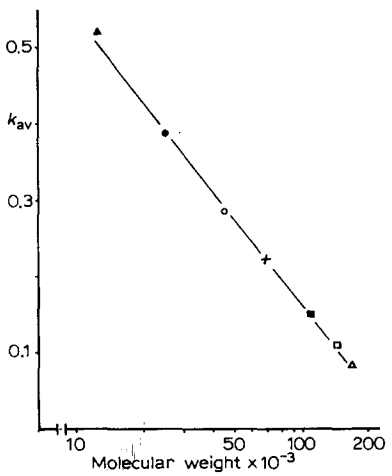


Fig. 4. Estimation of the mol. wt of pigeon liver diacetyl reductase by Sephadex G-200 gel filtration. The following protein standards were included: ▲, cytochrome *c* ($M_r$ 12 500); ●, chymotrypsin ($M_r$ 24 500); ○, egg albumin ($M_r$ 45 000); ×, serum albumin ($M_r$ 65 000); ◊, lactate dehydrogenase ($M_r$ 140 000); △, γ-globulin ($M_r$ 160 000); ◆, diacetyl reductase.

*pH Profiles*

In contrast with data reported for the beef liver diacetyl reductase, the pH versus NADH-dependent activity curve for the pigeon liver enzyme shows, at the standard assay conditions, one single broad peak in the pH range 5.6–6.2. A practically identical pH profile is observed if NADPH is substituted for NADH as coenzyme (Fig. 5).

*Saturation curve for diacetyl*

Fig. 6 compares the saturation curves for the diacetyl from pigeon liver and beef liver diacetyl reductases at 0.2 mM NADH, 25 °C and pH 6.1 in 0.05 M sodium–potassium phosphate buffer. From these saturation curves the apparent $K_m$ of 3.5 mM for the pigeon liver enzyme and 67 μM for the beef liver enzyme were calculated.

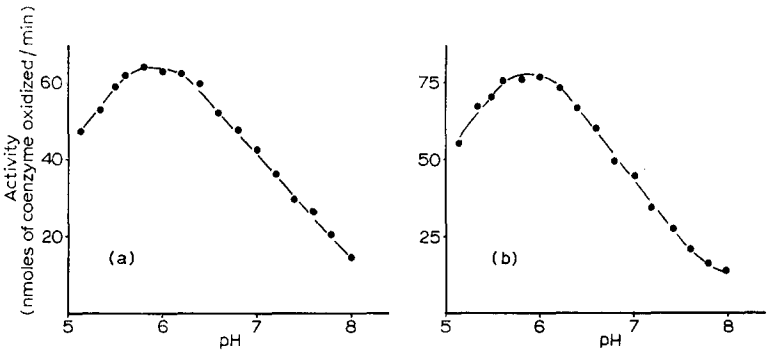


Fig. 5. Effects of pH on the enzyme activities at standard assay conditions. (a), NADH-dependent activity; (b), NADPH-dependent activity.

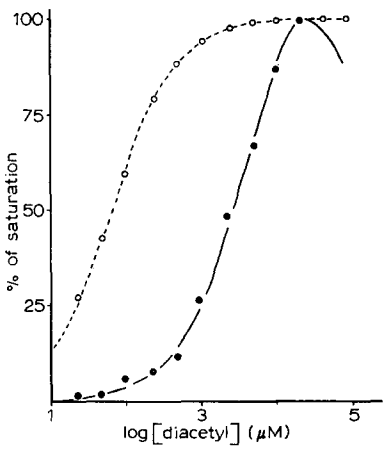


Fig. 6. Saturation curves for diacetyl of beef liver (○---○, calculated from reported data [12]) and pigeon liver (●—●) diacetyl reductases at standard assay conditions.

## DISCUSSION

The results reported above show that pigeon liver diacetyl reductase preparations are able to catalyze the reduction of diacetyl to acetoin coupled to the oxidation of either NADH or NADPH. The Sephadex elution pattern, polyacrylamide electrophoretic pattern and pH profiles are identical for both activities. The total activity in the presence of equimolar mixtures of NADH and NADPH lies between the two velocities obtained with the same concentration of NADH and NADPH separately. Altogether, these observations prove that both activities reside in one single enzyme. The constancy of the ratio of NADH-dependent/NADPH-dependent activities in Steps 3 and 4 of the purification procedure is in accordance with this conclusion; this ratio of activities is higher before the acetone precipitation, but this could be explained assuming the presence in the aqueous extracts of other enzyme(s) exclusively or primarily specific for NADH, which would precipitate in a different range of acetone concentration.

Like that from beef liver [11] the enzyme from pigeon liver has very strict

structural requirements for the substrate and seems to use only diacetyl as hydrogen acceptor. As the beef-liver purified preparations obtained by Burgos and Martín [11] and those of *Lactobacillus casei* by Branen and Keenan [4], the enzyme purified from pigeon liver reported here does not reduce acetoin to butyleneglycol. Although no such unequivocal evidence that diacetyl reductase and butyleneglycol dehydrogenase (2,3-butanediol:$NAD^+$ oxidoreductase, EC 1.1.1.4) are different enzymes was presented, Strecker and Harary [1], in *A. aerogenes* and *Staphylococcus aureus*, and Gabriel et al. [10], in rat liver, reached similar conclusions. Nevertheless, in the *A. aerogenes* Strain 1033 and with two mutants of Strain 35 the presence of an enzyme which catalyzes the reduction of both diacetyl and acetoin has been conclusively proved [5, 17], and a similar conclusion can be drawn from the data reported by Juni and Heym [2] for other microorganisms. It seems clear that the enzymes catalyzing the diacetyl reductase reaction are widely different in their substrate specificity, which must be checked before classifying them.

The reversibility experiments here reported with preparations more purified than those used by Gabriel et al. [10], confirm their observation that some diacetyl could be produced from acetoin by diacetyl reductase if nitroblue tetrazolium and phenazine methosulphate are introduced into the reaction system. It is extremely doubtful, nevertheless, that this could have any biological significance because of the negligible activity detected and the very unphysiological conditions required. At the standard assay conditions, which are closer to the biological ones, no backward activity can be detected. As far as we know, whenever the reversal of the diacetyl reductase reaction without dye participation has been attempted, with preparations of whatever origin, the trial has failed. Although the work of Sebeck and Randles [18] is sometimes taken as indicative of diacetyl reductase working in the backward direction, it only proves that diacetyl is produced by whole cells of *Pseudomonas fluorescens* from either butyleneglycol or acetoin, but this does not necessarily imply the reversal of the diacetyl reductase reaction. It is worth noting that López and Fortnagel [19] have recently suggested the existence of a different enzyme (acetoin dehydrogenase) catalyzing the oxidation of acetoin to diacetyl in *Bacillus subtilis*.

Stoichiometry and specificity studies demonstrate that the pigeon liver enzyme object of this work catalyzes the following reaction:

$$CH_3\text{–}CO\text{–}CO\text{–}CH_3 + NAD(P)H \rightarrow CH_3\text{–}CO\text{–}CHOH\text{–}CH_3 + NAD(P)^+$$

According to the rules of the Enzyme Commission this enzyme should be systematically named "Acetoin: $NAD(P)^+$ oxidoreductase", the irreversibility of the reaction making it advisable to retain the trivial name of diacetyl reductase. Nevertheless, its final classification and nomenclature must await a wider substrate specificity study which is at present in progress.

Pigeon liver diacetyl reductase has been isolated as a single species of mol. wt of about 110 000. This is in contrast with the diacetyl reductase from beef liver for which no less than two states of association (monomer and trimer) of a basic unit of molecular weight 25 000 have been suggested [11]. The enzyme from pigeon liver is, in this regard, more like that of *A. aerogenes* for which a tetrameric structure of a basic unit of mol. wt 25 000 has been demonstrated as the only active form [7].

Saturation curves demonstrate that the affinity for diacetyl is much lower in

the enzyme from pigeon liver than in that from beef liver. The apparent $K_m^{diacetyl}$ of the pigeon liver diacetyl reductase (3.5 mM) is about two orders of magnitude higher than that of the beef liver enzyme (67 $\mu$M) and close to that reported for *L. casei* preparations [4] and to the value which can be estimated for similar conditions from the data published by Johansen et al. [8] for the enzyme of *A. aerogenes*. At diacetyl concentrations over 20 mM a substrate inhibition effect can be observed (see Fig. 6) which does not allow the maximum rate of the reaction to be reached experimentally.

Pigeon and beef liver diacetyl reductases differ also in their pH profile. The enzyme from pigeon liver shows one single peak at pH about 6, lacking the second one (at about pH 6.8) of the enzyme from beef liver [11]. This may be due to the participation in the reaction of different ionizable groups of the active centre of the enzyme, but differences in the saturation degree for diacetyl could also explain this.

Since neither dialysis nor gel filtration nor EDTA addition result in a loss of activity, it is obvious that pigeon liver diacetyl reductase does not require metallic activators or other dialyzable cofactors, as has been reported for the enzyme from other sources [11, 20].

As shown by polyacrylamide gel electrophoresis, two coenzymes are present in the purified preparations.

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