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Diacetyl reductase in animal tissues and its intracellular distribution

Diacetyl reductase (acetoin:NAD^+ oxidoreductase, EC 1.1.1.5) was first observed by STRECKER AND HARARY[1] in *Staphylococcus aureus* and *Aerobacter aerogenes*. Since then it has been shown to be widely distributed among bacteria, having been detected in Pseudomonas, Alcaligenes, Streptococcus and Leuconostoc[2], but until now it has never been reported in animal tissues.

In the course of purifying glucose dehydrogenase (EC 1.1.1.47) following the method of STRECKER AND KORKES[3], we accidentally observed diacetyl reductase activity in beef liver. This paper deals with its presence in several animal species and different tissues, and the intracellular distribution and some properties of the animal enzyme.

For diacetyl reductase assays in animal tissues, water extracts were prepared by mincing 1 vol. of tissue with 5 vol. of glass-distilled water in an M.S.E. homogenizer at 0–2° and centrifuging at 5000 × *g* for 15 min. The supernatant was used for the enzyme assays. Partially purified enzyme preparations, devoid of butylene glycol dehydrogenase and NADH oxidase, were obtained from beef liver by collecting the protein fraction of a water extract which precipitates with acetone in the concentration range 1–1.3 vol. of acetone to 1 vol. of water extract.

The identity of the product and the stoicheiometry of the reaction were established as follows: an enzyme preparation devoid of butylene glycol dehydrogenase, from beef liver, was incubated at 25° (water bath) with diacetyl and NADH, in a flask fitted with an air-tight rubber plug easy to perforate; by means of a precision syringe, samples were periodically taken to determine NADH (spectrophotometrically) and diacetyl (by the method of OWADES AND JAKOVAC[4]). After 2 h of incubation, when no diacetyl could be detected, acetoin was determined by the method of WESTER-

TABLE I

REDUCTION OF DIACETYL TO ACETOIN AND OXIDATION OF NADH

Reaction mixture: diacetyl, 8.7 μmoles; NADH, 8.7 μmoles; enzyme preparation, total activity (as determined by the spectrophotometric method described) 2000 nmoles of diacetyl reduced/min; phosphate buffer, pH 6.1, 2.2 mmoles; total volume, 44 ml. Acetoin was not determined at different times during the experiment because diacetyl also gives a positive WESTERFELD[5] reaction. Although it is possible to calculate acetoin and diacetyl concentrations in a mixture of both from the results obtained by the methods of WESTERFELD[5] and OWADES AND JAKOVAC[4], experiments in our laboratory have demonstrated that the estimates so obtained are not entirely reliable unless very sophisticated and tedious experimental procedures are used. Acetate and acetaldehyde give a negative WESTERFELD[5] reaction.

Time (min)	*Diacetyl (μmoles)*	*NADH (μmoles)*	*Acetoin (μmoles)*
0	8.70	8.70	—
5	7.70	7.60	—
10	6.75	6.70	—
20	4.85	4.85	—
40	2.70	2.80	—
80	0.70	0.70	—
120	0.00	0.00	8.5

FELD[5]. Results of one of these experiments are shown in Table I; they prove that: (1) diacetyl is transformed into acetoin mole to mole; (2) for each mole of diacetyl reduced to acetoin, 1 mole of NADH is oxidized to NAD^+.

Cellular fractions were prepared following essentially the procedure outlined by MAHLER AND CORDES[6], but the mitochondria and lysosomes were obtained according to the method of WANG[7]. Minced tissues were homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 0.32 M sucrose containing 0.02 M Tris (pH 7.6) and 3 mM $MgCl_2$, the homogenized tissues being filtered through three layers of cheesecloth. Every 12.5 ml of filtrate was diluted to 20 ml with 0.25 M sucrose and submitted to the following centrifugation pattern in a Spinco L-50 ultracentrifuge: 10 min, 700 × *g* (twice) (nuclei and cell debris); 5000 × *g* for 20 min (mitochondria); 16 500 × *g* for 20 min (lysosomes); 105 000 × *g* for 100 min (microsomes); 105 000 × *g* for 600 min (ribosomes). The supernatant of the last centrifugation was labelled the soluble fraction.

All pellets were resuspended in 0.25 M sucrose before the assays were carried out. For the studies of enzyme distribution in different tissues, protein was determined by the biuret method described by CHANCE AND REDFEARN[8], but for the intracellular distribution studies, protein was calculated from total nitrogen determinations following the micromethod of JOHNSON[9].

Diacetyl reductase activities were determined at 25° by a spectrophotometric method based on NADH oxidation during diacetyl reduction, using a Beckman DB spectrophotometer with automatic recorder. In partially purified fractions the assays were performed as follows: *Reference solution*: phosphate buffer, pH 6.1, 0.15 mmole; enzyme preparation; NADH, 0.6 μmole; total volume, 3 ml. *Test solution*: the same as the reference solution *plus* diacetyl (10 μmoles).

In tissue extracts and cellular fractions it was necessary to avoid interference from NADH oxidase and butylene glycol dehydrogenase, thus acetoin (10 μmoles) was added to the test and reference cells. The acetoin (British Drug Houses) was washed with peroxide-free and dry ether to remove traces of diacetyl.

Partially purified enzyme preparations with a specific activity of 200 nmoles diacetyl reduced/min per mg protein did not show any measurable rate of appearance of NADH when acetoin and NAD^+ replaced NADH and diacetyl in the assay method. For all practical purposes the reaction is irreversible. This agrees with the reported behaviour of the bacterial enzyme[1].

Diacetyl reductase from animal tissues is not inhibited by acetoin or butylene glycol. The rate of reaction with purified preparations was not modified by addition of acetoin or butylene glycol in concentrations even 10 times higher than the diacetyl.

Diacetyl reductase activities have been observed in beef liver and in kidney, heart, brain and breast muscle of hen and pigeon. Pigeon is the animal in which we have detected maximum activities. Specific activities of water extracts of liver were as follows (average of three animals): pigeon, 184 nmoles of diacetyl reduced/min per mg protein; hen, 25.3; beef, 3.

Specific activities were always higher in liver and kidneys. Averaging the three assays in pigeon gave the following results: liver, 184 nmoles of diacetyl reduced/min per mg protein; kidney, 98.3; brain, 2.8; heart, 3.4; breast muscle, 8.1.

The intracellular distribution of the enzyme was studied in beef and pigeon

TABLE II

INTRACELLULAR DISTRIBUTION OF DIACETYL REDUCTASE IN PIGEON LIVER

Activities expressed in nmoles of diacetyl reduced/min per mg protein.

Fraction	*Activity*			*Distribution (%)*		
	*Expt. 1**	*Expt. 2*	*Expt. 3*	*Expt. 1**	*Expt. 2*	*Expt. 3*
Nuclei and cell debris	0	0.40	0.32	0	0.34	0.53
Mitochondria	0.41	1.90	0.73	0.40	1.60	1.30
Lysomes		1.40	2.00		0.31	0.51
Microsomes	1.03	0.00	0.00	0.21	0.00	0.00
Ribosomes	75.90	24.60	8.13	12.90	7.30	6.20
Soluble	91.02	99.30	66.30	86.40	90.40	91.50
Whole homogenate	24.00	26.80	14.50	100.00	100.00	100.00

* No attempt was made to separate mitochondria and lysosomes.

liver. Table II shows the distribution of the diacetyl reductase in various cellular fractions of pigeons.

Similar experiments with beef liver showed higher diacetyl reductase activities in the particulate fraction compared to the soluble fraction than pigeon liver. The average of three experiments gave the following diacetyl reductase distribution: nuclei and cell debris, 6.7%; mitochondria, 17.5%; lysosomes, 12.3%; microsomes, 12.9%; ribosomes, 6.3%; soluble fraction, 44.2%.

Diacetyl reductase seems to be an enzyme more widely distributed in nature than originally thought. It is not, however, the only enzyme that catalyses the transformation of diacetyl into acetoin. GREEN *et al.*[10] described a diacetylmutase which catalyses the reaction $2CH_3 \cdot CO \cdot CO \cdot CH_3 \rightarrow 2CH_3 \cdot COOH + CH_3 \cdot CHOH \cdot CO \cdot CH_3$ and which is especially abundant in pigeon breast muscle and liver and has no NADH requirements; it requires diphosphothiamine instead.

It is quite obvious that this enzyme can never be mistaken for diacetyl reductase in our analytical procedure.

Diacetyl reductase appears to be a soluble enzyme in pigeon liver. Beef liver has, in contrast, about 50% of the enzyme activity bound to particles.

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