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PURIFICATION AND SOME PROPERTIES OF HEN'S MUSCLE BUTYLENEGLYCOL DEHYDROGENASE

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INTRODUCTION

The piridin-nucleotide coupled reduction of diacetyl and/or acetoin to butyleneglycol by bacteria and other microorganisms is known since the work of DE MOSS *et al.*[1] and STRECKER and HARAY[2] in the early fifties. Four different enzymes catalyzing these reactions have been described in bacteria: two of them are able to reduce only diacetyl, one using as coenzyme exclusively NADH[2] and the other NADPH[3]; the third is specific for butyleneglycol and NADH[2] and the fourth specifically depends on NADH but reduces both diacetyl and acetoin[4].

Knowledge of the ability of animal tissues to perform these reactions is much more recent. The authors group (5-7) reported a few years ago the presence of diacetyl reductase in beef and pigeon liver; GABRIEL *et al.*[8] presented evidence indicating that, in rat and rabbit liver, separate enzymes catalyze the piridin-nucleotide coupled reductions of acetoin and diacetyl. While some of the animal diacetyl reductases have been fairly well studied, (5-7, 9-11) untill now no attention has been paid to the enzymes catalyzing acetoin reduction.

In the present paper we describe a purification procedure which allows to obtain from hens muscle electrophoretically pure preparations of two forms of an enzyme able to reduce both acetoin and diacetyl accepting as hydrogen donor cither NADH or NADPH (being therefore different from all diacetyl reductases and butyleneglycol dehydrogenases untill now described) and report some of its properties.

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MATERIAL AND METHODS

NADH and NADPH were supplied by Boehringer. NAD^+ by Sigma. Acetoin (BHD) was purified by fractional distillation through a Hempel column collecting the material distilling in the range 88-89°C at normal pressure. Calcium pshophate gel was prepared as described by KEILIN and HARTREE[12]. Sephadex, activated Sepharose 4B and blue dextran were supplied by Pharmacia Fine Chemicals. DEAE-23 cellulose and CM-22 cellulose by Whatman; ampholites by LKB; acrylamide and bisacrylamide by BDH; bovine serum albumin by Calbiochem; egg albumin by Schuchardt; myoglobin and ß-lactoglobulin by Sigma, chymotrypsin by Merck and hydroxyapatite by Bio Rad. All other chemicals used were of analytical degree either from Merck or BDH.

Animals used in this work, Leghorn fowls of around 2 kg of weight provided by a local provisioner, were killed by craneal punction; leg muscles were disected and free or surface fat and conective tissue before enzyme extraction.

Electrofocusing was performed along 72 hrs in a 440 ml LKB Svensson and Vesterberg column; the system was refrigerated with cold water (0-0.5°C).

Disc electrophoresis in 7.5 % poliacrylamide gels (bisacrylamide/acrylamide 1/37.5) was performed at 0-4°C and pH 7.8 in 0.1 M sodium-potasium phosphate buffer; 8 mA/tube (6 mm i.d.) were applied untill the bromophenol blue used as trazer reached the end of the tube (12 cm). Protein was stained with Coomassie Blue following for qualitative analysis the ORTEC procedure[13] and that of FENNER *et al.*[14] for quantitative determinations.

Diacetyl/acetoin reductase activity was revealed as described[7] for diacetyl reductase except for the molarity of the incubation buffer (0.5 M) and NADH (1 mM), the introduction between incubation with substrates and staining of the residual coenzyme a 30 min period of rest in the dry at room temperature to exhaust the reduced piridin nucleotide in the enzyme position, and for the NADH staining buffer: pH 9, 0.5 M tris-boric, instead of 0.5 M pH 7 sodium-potassium phosphate.

Protein concentrations were generally determined by the biuret method, as described by GORNALL *et al.*[15], but when protein concentration was low the absorbance at 280 and 260 nm method was used. In samples from electrofocusing experiences, in which some ampholites are allways present, an aliquot was submitted to acrylamide disc electrophoresis and protein was quantitatively determined by the method of FENNER *et al.*[14] using bovine serum albumin as standard protein.

Acetoin and diacetyl reductase activities were spectrophotometrically measured in a Perkin Elmer-Hitachi spectrophotometer with thermostatized cells, as described[6] except for the pH 7 and molariy (0.1 M) of the phosphate buffer. The purification procedure was monotored through the NADPH acetoin

reductase reaction; units are defined as the amount of enzyme that reduces 1 nmole of acetoin for min at 25°C under assay conditions.

Diacetyl was determined by the procedure of OWADES and JACOVACK[16]; acetoin and butyleneglycol by a method developed in this laboratory[17] based in a combination of the procedure or HAPPOLD and SPENCER[18] for acetoin and butyleneglycol oxidation to diacetyl and a semimicro version of the diaceyl distillation technique described by PACK *et al.*[19], introducing before the oxidative steps a very deep deproteinization in three stages, first with 0.25 N $Zn(OH)_2$, then with 1/10 vol of saturated $Pb(CH_3COO)_2$, and finally with trichloroacetic acid (10 %).

RESULTS

Extraction

Several extraction procedures, including acetone treatment, were tried before finally adopting the following one: after finely chopping the muscles, the mince was homogenized with 5 vols of cold distilled water by means of a Sorvall-Omnimixer operating at 14,000 rpm in 3 periods of 30 sec with equal lenght of rest in between; the homogenized was afterwards filtrered through two cheese-cloth layers and the filtrate centrifuged for 10 min at 1,500 × g to free it from fat and insoluble particles.

About 4-4.5 g of protein/100 g of tissue with a specific butyleneglycol dehydrogenase activity between 0.5-1.2 is so obtained.

Calcium phosphate adsorption of inespecific protein

Calcium phosphate gel was added to the crude extract free of fat and non soluble particles untill 10-15 % of the enzyme activity was bound (usually about 0.5 vols of gel with 2.8 g of dried weight/100 ml). This resulted in the adsorption of about 80 % of the protein.

Gel filtrations and molecular weight determination

The supernatant was liophilized and the powder, which retains its full activity for no less than one month at −18°C, was afterwards dissolved in 3 ml/g of 3 mM sodium-potassium phosphate buffer and submitted to gel filtration through Sephadex G-100. As Fig. 1 shows acetoin reductase activity is eluted as a single peak after the main protein band. By collecting all tubes with specific activity over 50-60 units/mg protein and rechromatographying under the same conditions those of specific activity between 20 and 50-60 units/mg of protein an enrichment factor of 80-90 can be reached with a yield of 65-70 % of the original activity.

Gel filtration of the lyophilized of the material selected in the previous

Fig. 1

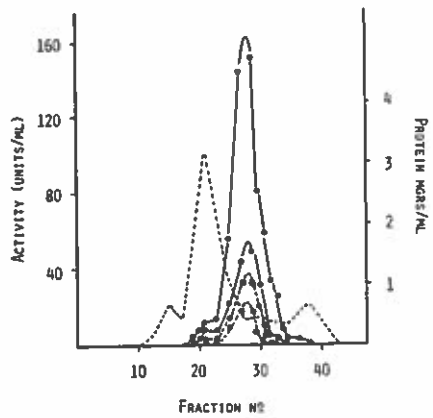

Fig. 1.–Chromatography on Sephadex G-100 of a hen muscle extract after adsorption of part of the inespecific protein on calcium phosphate gel. Eluant 3 mM sodium-potassium phosphate buffer pH 7. Column dimensions 2.5 × 36.5 cm. Fraction vol 3.6 ml.
- - - protein; ● —— ● NADH dependent and o —— o NADPH dependent diacetyl reductase activities. ● - - - ● NADH and o - - - - o NADPH dependent acetoin reductase activities.

Fig. 2

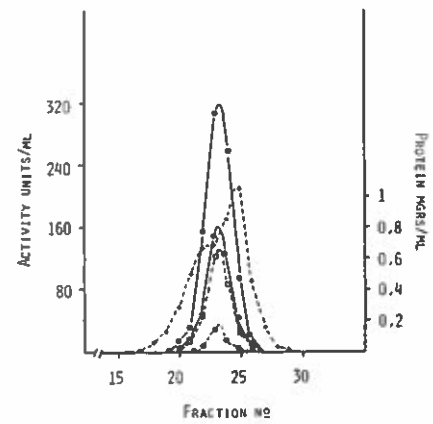

Fig. 2.–Chromatography on Sephadex G-75 Superfine of a hen muscle extract purified by adsorption of part of the inespecific protein on calcium phosphate gel and filtration trough Sephadex G-100. Colum dimensions 2.5 × 38 cm. Fractions vol 3.6 ml.
x - - - - x protein; ● —— ● NADH and o —— o NADPH dependent diacetyl reductase activities. ● - - - ● NADH and o - - - o NADPH dependent acetoin reductase activities.

stage, dissolved in water (1 ml/50 mg of protein), through a bed of Sephadex G-75 Superfine (Fig. 2) allows to reach an enrichment factor of 170-190 with a recovery of 50-55 % of the total original activity. Molecular shieving through Sephadex G-75 Superfine allowed as well to estimate the molecular weight of the enzyme, which resulted to be (Fig. 3) 28,000.

Hydroxyapatite chromatography

Several procedures of eluting the enzyme adsorbed in hydroxyapatite beds were tried before finally adopting as part of the purification procedure the following discontinuous molarity gradient of sodium-potassium, pH 7, phosphate buffer: 3 mM (3 bed vol), 10 mM (4 bed vol), 30 mM (6 bed vol); most of the contaminant protein remains adsorbed and the buffer concentration must be increased to 90 mM to remove significant quantities of it (Fig. 4). With the 30 mM fraction 44-48 % of the activity originally present in the crude extracts with 500-570 units/mg of protein are recovered.

Electrofocusing and multiple forms

Fig. 5a shows the result of electrofocusing (pH range 3-10) these partially purified preparations; several enzyme forms capable of catalyzing acetoin

Fig. 3

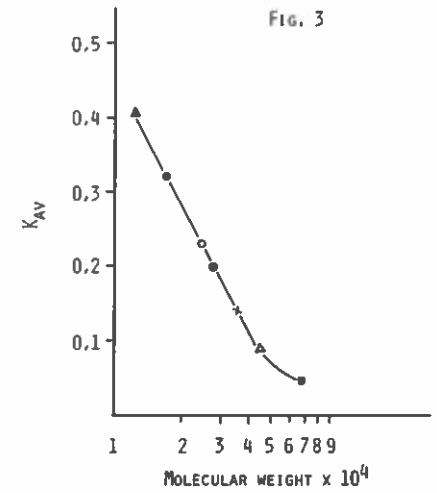

Fig. 3.–Molecular weight estimation of hen muscle diacetyl/acetoin reductase by Sephadex G-75 Superfine filtration. Standards proteins: (▲) cytochrome C (Mr 12500); (●) myoglobin (Mr 17000); (o) chymotripsin (Mr 24500); (⊙) butyleneglycol dehydrogenase; (×) ß-lactoglobulin (Mr 35000); (△) egg albumin (Mr 45000); (■) serum albumin (Mr 65000).

Fig. 4

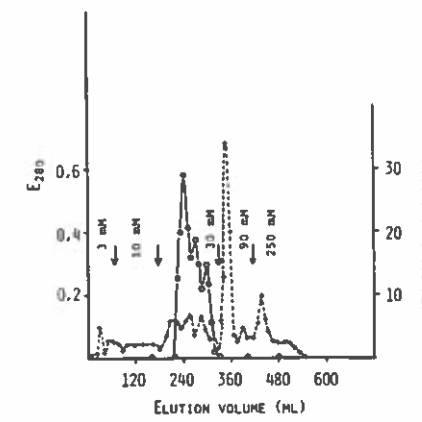

Fig. 4.–Behaviour on hydroxyapatite chromatography of an extract purified by inactive protein adsorption on calcium phosphate gel and Sephadex G-100 and G-75 Superfine filtrations. Column dimensions 1.5 × 15 cm. Fraction vol 3.8 ml. Eluant pH 7 sodium-potassium phosphate buffer 3, 10, 30, 90 y 250 mM. Buffer changes are indicated by arrows.
x - - - - x E_{280}; o —— o NADPH dependent acetoin reductase activity.

Fig. 5

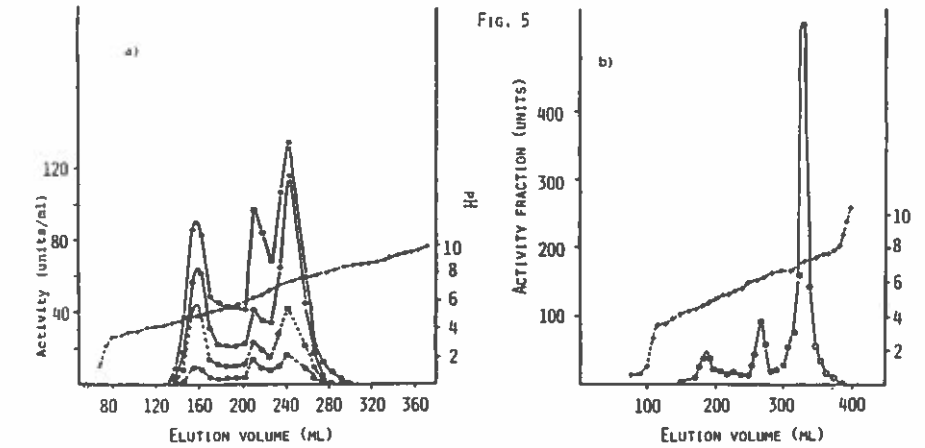

Fig. 5.–Multiple forms of the enzyme.
a) Electrofocusing of an extract purified by calcium phosphate gel adsorption and Sephadex G-100 filtration on an ampholites 3-10 pH range.
x - - - - x pH gradient; ● —— ● NADH dependent and o —— o NADPH dependent diacetyl reductase activities. ● - - - ● NADH and o - - - o NADPH dependent acetoin reductase activities.
b) Electrofocusing (ampholites pH range 4-8) of an extract previously purified by calcium phosphate gel adsorption and Sephadex G-100 and G-75 Superfine filtration.
x - - - - x pH gradient; o —— o NADPH dependent acetoin reductase activity.

reduction coupled to NADPH oxidation are detected, those of pI 4.8, 6.2 and 7.2 being the major ones. Their relative proportions widely varied in different experiment, as Fig. 5 (a and b) proves, but the total recovered activity, around 35-38 % of that present in the crude muscle extracts is usually distributed as follows: pI 7.2: pI 6.2: pI 4.8 = 10: 2: 1.3.

The material of each of these three main bands was bulked and free of sucrose and ampholites by double filtration (with a freeze drying concentration stage in between) through short and wide columns (2.6 × 24.5 cm) of Sephadex G-25 Coarse.

Table I summarizes the purification procedure.

Electrophoresis of the final preparations

Fig. 6 shows the purity of the final preparations; as can be seen the forms of pI 7.2 and 6.2 behave as electrophoretically pure and that of pI 4.8 gives wide and difuse bands both of protein and activity; all three show significantly equal migration.

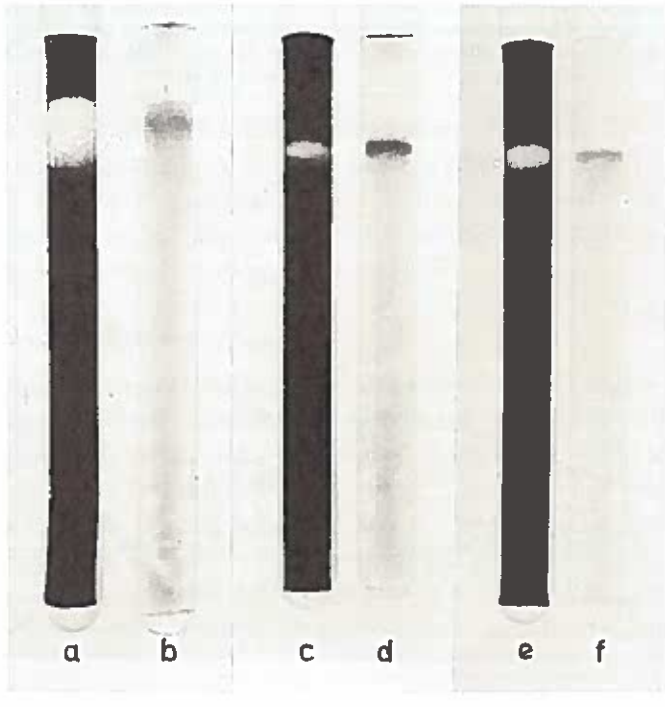

Fig. 6.–Electrophoresis on polyacrylamide gel (pH 7.8) of the three forms of the enzyme obtained by electrofocusing. Anode at the bottom.
a) form of pI 4.8, c) form of pI 6.2, and e) form of pI 7.2 stained for diacetyl reductase activity and b, d and f the same stained for protein.

TABLE I

Purification of diacetyl/reductase from hens muscle, starting with 1 Kg of tissue (about 10 fowls)

	Step	Vol (liters)	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Purification (fold)	Yield (%)
1	Aqueous extract (homogenization with 5 vol of water)	5	40,000-45,000	0.5-1.2	20,000-45,000		
2	Calcium phosphate gel (unadsorbed)	5.7	13,000-13,500	2-3.5	26,000-47,000	3-4	90
3	Sephadex G-100 eluate	0.1	400-450	60-70	24,000-32,000	80-90	65-70
4	Sephadex G-75 Superfine eluate	0.02	100-140	130-150	12,000-20,000	170-190	50-55
5	Hydroxyapatite eluate with 30 mM pH 7 sodium-potassium phosphate buffer	0.15	30-45	400-450	12,000-20,000	500-570	44-48
6	Electrofocusing						
	form of pI 7.2	0.02	2-4	2,900	6,000-11,000	4,000	26.3-28.5
	form of pI 6.2	0.02	0.5-1	2,200	1,200-2,200	3,000	5.2-5.7
	form of pI 4.8	0.02	2-3.5	400	800-1,500	500	3.5-3.8
							35-38

A single enzyme catalyzes NAD(P)H diacetyl and acetoin reduction

Diacetyl and acetoin reductase activities with both NADH and NADPH show the same elution profile in gel filtration (see Figs. 1 and 2) and electrofocusing experiments (see Fig. 5 a). Furthermore, a partially purified preparation (treated with calcium phosphate gel and filtered through Sephadex G-100 and G-75) was submitted to activity tests with equimolar mixtures of both substrates (or coenzymes) obtaining (Table II) intermediate activities between those reached with single substrates (or coenzymes). Finally, when the forms of pI 7.2, 6.2 and 4.8 were submitted to electrophoresis and the discs of acrylamide gel were stained for protein and NADH dependent diacetyl reductase activity, single coincidental bands of protein and activity were obtained; since the whole purification procedure was monitored through the acetoin reductase activity the electrophoresis experiments are a further evidence in favour that there is a single enzyme catalyzing the diacetyl and acetoin NAD(P)H dependent reductase reactions. It would have been desirable to stain the gels as well for the other three activities but, in our hands, the enzyme stains very poorly with acetoin and NADPH.

Stoichiometry

The stoichiometry of the reaction was studied by incubating samples of the enzyme forms of pI 7.2, 6.2 and 4.8 at 25 ± 0.1°C (in a water bath) with diacetyl and NADPH, periodically removing aliquots and measuring diacetyl, acetoin, butyleneglycol and NADPH. As shown in Table 3 for the form of pI 7.2, 1 mole of NADPH is consumed for mole of acetoin measured and 2 for each mole of butyleneglycol formed. Equal results were obtained with the other two forms.

TABLE II
Enzymatic activities of partially purified samples (step 4 included) with equimolar mixtures of single substrates (4 mM) and coenzymes (0.2 mM). Mean of two observations ± s. d.

Substrate(s)	Coenzyme(s)	Activity
Acetoin	NADH	5.76 ± 0.68
»	NADPH	111.36 ± 2.94
»	NADH + NADPH	100.80 ± 5.45
Diacetyl	NADH	35.52 ± 1.36
»	NADPH	286.80 ± 15.30
»	NADH + NADPH	285.60 ± 3.40
Acetoin + Diacetyl	NADH	34.10 ± 0.68
»	NADPH	319.20 ± 3.40

TABLE III
Reduction of diacetyl to butyleneglycol and oxidation of NADPH

Time (min)	Reduced diacetyl (μmoles)	Produced acetoin (μmoles)	Produced butyleneglycol (μmoles)	Oxidized NADPH (μmoles)
0	0	0	0	0
5	7.19	*	*	7.26
10	10.88	9.71	*	11.12
90	11.78	8.07	2.64	13.29
390	11.78	4.47	6.75	19.11

* No determinated.

pH profiles and enzyme stability

The activity of the three isolated forms of the enzyme was measured at different pH values on 0.1 M sodium-potasium phosphate buffer. A plateau extending from pH 5 to pH 6.5-7, and a descending branch at higher pH was observed. The enzyme is at all purification stages very stable in either water or low molarity buffers of pH 6, provided that solutions are mantaineed at low temperature, but is easily inactivated in high molarity buffers.

DISCUSION

The enzyme here described differss from the two animal enzymes untill now well described which are able to catalyze the piridin nucleotide coupled diacetyl reduction (beef and pigeon liver diacetyl reductases) in substrate specifity since it accepts as oxidized substrate both diacetyl and acetoin; shows as well minor differences respect to both diacetyl reductases in pH profile and molecular weight. GABRIEL *et al.*[8] did not purified sufficiently enough their preparations to obtain deffinite conclusions with regard to oxidized substrate specifity but they presented evidence wich only can be interpreted as proof of the existence, in rat liver, of two enzymes: one accepting as oxidized substrate only diacetyl and the other specific for acetoin, both of them using the two reduced piridin nucleotides, and NADH more efficiently than NADPH. The enzyme to which this paper is concerned is therefore clearly different from all diacetyl and acetoin reductases known in the animal kingdon. It differs as well from that purified by Bryn *et al*[4] from *Aerobacter aerogenes*, which is able to reduce both diacetyl and acetoin and named by them diacetyl/acetoin reductase, since the enzyme from hens muscle uses both coenzymes and prefers NADPH, while that from bacterial origin only uses NADH.

The molecular weight found for the enzyme here reported is very closed both to the monomeric form of beef liver diacetyl reductase (26,000)[6] and to the four inactive subunits of which the native diacetyl/acetoin reductase from *Aerobacter aerogenes* is composed of[21].

The purification procedure described, although rather tedious and time-consuming, allows to obtain electrophoretically pure enzyme preparations of at least the two major enzyme forms by using only very mild treatments not likely to alter the native properties of the enzyme and results in a very high final yield. The later seems in part due to the behaviour of impure preparations along the Sephadex filtrations, in which higher than 100 % recoveries are sistematically reached; this suggests the presence in the aqueous muscle extracts of some inhibitor accompaning the enzyme along the first stages of the purification.

In the developpement of this procedure several other usual protein purification techniques were tried: fractional precipitation with hydrochloric or acetic acid, ammonium sulphate, acetone or ethanol were discarded since the enzyme proved to have very scarce stability at acid pH, to precipitate over a wide ammonium sulphate concentration range (from 55 % to 100 % saturation) and to be easily inactivated by organic solvents, even at −20°C. Heat treatment (60°C, 10 min) in the presence of NAPH ($0,4 \times 10^{-4}$M) of the crude extracts allows to multiply the specific activity by a factor of 1,75 with a yield close to 100 % but was discarded as well as purification procedure because of its high cost. Ionic exchange techniques on CM cellulose and DEAE cellulose using both batch and column procedures were unsuccesfully tried using as eluant sodium-potassium phosphate buffers covering the pH range (6-7.5) in which the enzyme is reasonably stable and the 0.003-0.8 M interval. Affinity chromatography over NAD^+ Sepharose 4-B (prepared as described by Mosbach *et al.*[20] and checked by fractionating a mixture of lactate-dehydrogenase and glyceraldehyde 3-P dehydrogenase)[20] proved to be of little value because the enzyme was not bound to the ligand, which suggest very low NAD^+ affinity.

The enrichment factors in Table I for each of the 3 isolated forms of the enzyme are probably under-estimated since they have been calculated respect to the whole original activity (sum of that from all the enzymatic forms) and not to that of each individual enzymatic species. If the distribution of the three purified forms were in the original tissue the same observed at the end of the purification process, the enrichment factors would be 5,300 (form of pI 7.2) 20,000 (form of pI 6.2) and 5,000 (form of pI 4.8).

The enzyme behaves along the whole preparation procedure as a single species up to the electrofocusing step in which its heterogeneity is revealed; only in the hydroxyapatite chromatoghraphy has a rather anormalous behaviour since it allways gives wide and difuse bands (Fig. 4). The existence of multiple molecular forms of an enzyme is a frequent and well know phenomenon detected in no less than 15 % of the enzymes untill now characterized[21], and seems to be rather common among diacetyl and acetoin reductases: Hetland *et al.*[22] reported that *Aerobacter aerogenes* diacetyl/acetoin reductase migrates as a single band under electrophoresis but can be resolved by electro-

focusing in 4-12 different species. This microheterogeneity is also shown by hen muscle diacetyl/acetoin reductase as evidenced in this work. Several hypotesis can be formulated to account for this properties; in the case of hen muscle diacetyl/acetoin reductase the two most plausible are: differences in hystidine content (highest in the form of pI 7.2 and lowest in that of pI 4.8), since because of its pK the imidazol group will sparingly dissociate at the electrophoresis pH (7.8), or different degrees of deamidation of amide aminoacides (glutamine or asparagine) from the species of pI 7.2. The later has been proved to be the mechanism responsable for microheterogeneity of citochrome C[24] and rabbit muscle aldolase[25] and has been considered[26] a device specifically designed for intracellular proteins turnover regulation.

Gabriel *et al.*[8] showed as well that both diacetyl and acetoin reductase from rat liver could be resolved in two species each by cellulose acetate electrophoresis. The nature of the multiple forms production mechanism seems nevertheless to be quite different in this case: the two species of diacetyl reductase could be isolated by chromatography on CM cellulose Sephadex and both species were evidenced again in each isolated band when they were independently submitted to the same chromatographic procedure, suggesting that equilibrium among forms of different association degree were involved. Beef liver diacetyl reductase presents as well two species of different molecular weght[6] and that from pigeon can be electrophoretically resolved in at least two forms[7].

RESUMEN

En este trabajo se describe la purificación, a partir de tejido muscular del muslo de gallina, de tres formas enzimáticas de peso molecular 28.000, que catalizan la reducción del diacetilo y la acetoína acoplada a la oxidación de NADH o NADPH. Las citadas formas se diferencian en sus puntos isoeléctricos, 7,2 y 6,2 el de las más abundantes y 4,8 el de una tercera, muy minoritaria. Las dos primeras han sido purificadas hasta homogeneidad electroforética y la de pI 4,8 hasta un elevado grado de pureza, con un rendimiento global en torno a un 38 %.

«PURIFICATION AND SOME PROPERTIES OF HEN'S MUSCLE BUTYLENGLICOL DEHIDROGENASE»

SUMMARY

1.–And NAD(P)H dependent enzyme of molecular weight about 28,000, able to catalyze acetoin and diacetyl reduction has been purified to electrophoretical homogeneity and resolved in three main forms of pI 7.2, 6.2 and 4.8 by a procedure including aqueous extraction, inactive protein adsorption on calcium phosphate, gel filtration through Sephadex G-100 and G-75 Superfine, hydroxyapatite chromatography and electrofocusing in the pH range 4-8.

2.–The enzyme differs in substrate and coenzyme specificy and in several minor aspects from any untill now described.

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