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IMMUNOCHEMICAL STUDIES ON MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP)

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SUMMARY

1. Malate dehydrogenase (decarboxylating) (NADP) (L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was purified from the supernatant fraction of rat liver.

2. Antibody against this purified enzyme was prepared. This antibody inhibited the activity of the enzyme and precipitated it.

3. Malate dehydrogenase (decarboxylating) (NADP) in supernatant fractions from kidney, heart, brain and adipose tissue of rats reacted with the antibody in the same manner as that of the ra tliver enzyme in the inhibition tests. Supernatant malate dehydrogenases from all these tissues except adipose tissue formed single precipitin bands with the antibody on Ouchterlony double-diffusion analysis which fused with each other.

4. The enzyme from the livers of different species differed immunologically from that of rat liver.

5. Mitochondrial enzyme was immunologically quite distinct from supernatant enzyme.

6. Increase and decrease in enzyme activities in the cytoplasm of liver under various dietary and hormonal conditions were accompanied by proportional changes in the quantity of immunologically reactive protein.

INTRODUCTION

Malate dehydrogenase (decarboxylating) (NADP) (L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was first demonstrated by MOULDER *et al.*[1], and subsequently purified and characterized from pigeon liver by OCHOA *et al.*[2]. Crystalline enzyme was obtained from the supernatant fraction of pigeon liver by HSU AND LARDY[3]. This enzyme has also been found in other animal tissues, plant tissues and microorganisms[4–6]. UTTER[7] demonstrated that this enzyme is located almost entirely in the supernatant fraction of a chicken liver homogenate, and PANDE *et al.*[8] reported a similar finding in rat liver. HÜLSMANN[9], however, found that sonication releases malate dehydrogenase (decarboxylating) (NADP) from sucrose-

washed beef heart sarcosomes, and later HENDERSON[10] showed the existence of two isozymic forms of the enzyme in mouse tissue. SALGANICOFF AND KOEPPE[11] determined the amount and subcellular distribution of the enzyme in rat brain, and reported that 25% of this enzyme is present in the supernatant fraction and 75% in the mitochondrial fraction. Three malate dehydrogenase (decarboxylating) (NADP) isoenzymes, *viz.* a mitochondrial form, a chloroplast form, and a soluble or non-particulate form, were separated from the stem tissue of Opuntia by MUKERJI AND TING[12]. SIMPSON AND ESTABROOK[13] observed the enzyme activity in mitochondria from bovine adrenal cortex and discussed its role.

This paper reports differences in the immunochemical properties of malate dehydrogenase (decarboxylating) (NADP) isoenzymes in the supernatant and in the mitochondria, and the immunochemical specificities of these enzymes in various rat organs and in the livers from several animals. Immunological studies on changes in supernatant malate dehydrogenase activity under different dietary and hormonal conditions are also described.

MATERIALS AND METHODS

Animals and their treatment

Male Sprague–Dawley rats, weighing 200–250 g, were used. Treated rats were starved for 3 days and then given a high carbohydrate, low fat diet[14] containing 1% thyroid powder for 3 days to induce the enzyme before sacrifice[15].

Enzyme assay

Enzymic activity to convert malate to pyruvate was assayed essentially by the method of OCHOA *et al.*[2]. The reaction mixture consisted of 100 mM Tris–HCl (pH 7.4), 1 mM $MnCl_2$, 0.25 mM $NADP^+$, 1.5 mM potassium L-malate and enzyme in a total volume of 1.0 ml. The conversion of pyruvate to malate by the purified enzyme was determined by a modification of the method of WISE AND BALL[15]. The reaction mixture contained 100 mM Tris–HCl (pH 7.4), 1 mM $MnCl_2$, 0.1 mM NADPH, 50 mM sodium bicarbonate (pH 7.4), 60 mM sodium pyruvate (pH 7.4) and enzyme in a total volume of 1.0 ml. The rate of NADPH formation or oxidation was recorded at 340 nm and 25°. Protein was determined by the method of LOWRY *et al.*[16]. With purified preparations protein was determined at 280–260 nm by the method of LAYNE[17]. One unit of enzyme is defined as the amount catalyzing the reaction of 1 μmole of substrate per min under the conditions of assay[3].

Crude extracts

Livers were homogenized in 4 vol. of medium containing 0.25 M sucrose, 5 mM $MgCl_2$ and 1 mM dithiothreitol at pH 7.4, and the homogenate was centrifuged at 10 000 $\times$ *g* for 10 min. The resulting supernatant was centrifuged at 105 000 $\times$ *g* for 1 h. Supernatant fractions from kidney, heart, brain and epididymal adipose tissue of rats and the livers of mice, pigs and pigeons were prepared in the same way. The mitochondrial fractions from rat liver, kidney, heart and brain were prepared as described in the literature[18–20], and subjected to ultrasonic disruption in 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and 1 mM dithiothreitol. Then the

disrupted mitochondria were centrifuged at 10 000 × *g* for 30 min to obtain the clear supernatant.

Purification of malate dehydrogenase (decarboxylating) (NADP)

In this work, supernatant malate dehydrogenase was purified from the supernatant fraction of rat liver by a modification (Table I) of the method of HSU AND LARDY[3] for that from pigeon liver, because we obtained a low yield of purified enzyme and sometimes impure product using the method without modification.

TABLE I

PURIFICATION OF RAT LIVER MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP)

Fraction	*Vol. (ml)*	*Activity (units/ml)*	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Purification (-fold)*	*Total activity (units)*	*Yield (%)*
Supernatant of rat liver	950	1.74	20.96	0.08	1.0	1530	100
Acid and heat treated	940	1.40	11.55	0.12	1.5	1300	85
$(NH_4)_2SO_4$ fractionated	15	54.5	14.9	3.66	46	818	54
Sephadex G-200 eluate	67.5	11.1	2.25	5.03	63	750	49
Ethanol fractionated	5.0	80.5	6.30	12.8	160	403	26.7
DEAE-cellulose eluate, concentrated	2.0	126.5	2.29	54.7	682	253	16.5

Preparation of crude extract. The livers (315 g) from induced rats were homogenized with 3 vol. of medium (pH 7.4), containing 0.25 M sucrose, 5 mM $MgCl_2$ and 1 mM EDTA. The homogenate was centrifuged at 10 000 × *g* for 15 min. The resulting supernatant fluid was centrifuged at 35 900 × *g* for 2 h. Enzyme activity was almost all recovered in this supernatant fraction.

Heat treatment. Magnesium acetate was added to a concentration of 0.1 M and the mixture was acidified to pH 5.5 with ice-cold 1 M acetic acid. Aliquots of 300 ml of the solution were heated to 50° and maintained for 60 sec. The denatured protein was removed by centrifugation.

$(NH_4)_2SO_4$ fractionation. The solution was adjusted to 65% saturation of ammonium sulfate and centrifuged at 10 000 × *g* for 10 min. The clear supernatant was brought to 75% saturation of $(NH_4)_2SO_4$ and centrifuged. The precipitate was dissolved in a minimum volume of 0.1 M Tris–HCl buffer (pH 7.4) containing 2 mM 2-mercaptoethanol and 5 mM $MgCl_2$.

Sephadex G-200 chromatography. The sample was applied to a Sephadex column (G-200, super-fine) (2.5 cm × 45 cm) equilibrated with buffer (pH 7.4) containing 20 mM EDTA and 2 mM 2-mercaptoethanol. The column was then eluted with the buffer at a flow rate of 11 ml/h. The enzyme activity was eluted in the void volume.

Ethanol fractionation. This procedure was carried out according to the method of HSU AND LARDY[3] except ethanol concentration. The fraction from 30 to 40% was collected.

DEAE-cellulose chromatography. A column of DEAE-cellulose (2 cm × 30 cm) was equilibrated with 0.03 M Tris–HCl buffer (pH 7.4) containing 2 mM 2-mercaptoethanol. The dialyzed sample was applied to the column and washed with the same Tris–HCl buffer. The enzyme was eluted with buffer containing 0.03 M Tris–HCl

(pH 7.4), 2 mM 2-mercaptoethanol and 5 mM $MgCl_2$. The fractions in the peak showed almost identical specific activity, so they were combined and concentrated.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method by DAVIS[21]. A gel concentration of 5% was routinely used. Samples containing 0.2 mg protein per tube in 10% sucrose were applied. Protein on a gel cylinder was visualized with Amido black 10B.

Immunochemical methods

For preparation of antibody against supernatant malate dehydrogenase of rat liver, rabbits were injected three times at 10 days intervals with 1 mg of purified enzyme (specific activity, 54 units/mg of protein) incomplete Freund's adjuvant. Control animals were injected with saline and Freund's adjuvant. The animals were bled on the 10th day after the last injection. The blood was allowed to clot and the resulting serum was used. The γ-globulin fraction was purified by fractionation with $(NH_4)_2SO_4$ (ref. 22) and then DEAE-cellulose chromatography[23]. Immunoelectrophoresis was carried out in 1.5% Difco Noble agar containing 0.025 M veronal buffer at pH 8.6 on a standard microscope slide at 50 V for 1 h in 0.05 M veronal buffer (pH 8.6). Ouchterlony double-diffusion tests were performed as described by KABAT AND MAYER[24]. For neutralizing enzyme activity, completion of the antigen–antibody reaction usually took 10 min at 25°. The enzyme solution contained 5 mM $MgCl_2$ and 1 mM dithiothreitol besides 0.03 M Tris–HCl (pH 7.4) to prevent loss of enzyme activity. These compounds did not cause any detectable interference with the enzyme–antibody reaction.

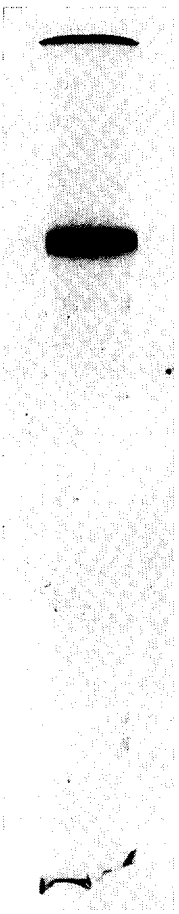
RESULTS

Purity of malate dehydrogenase (decarboxylating) (NADP) from rat liver and its antibody

The purified enzyme gave a single band on polyacrylamide gel electrophoresis (Fig. 1). When examined by immunoelectrophoresis, antiserum against rat liver supernatant malate dehydrogenase gave only one precipitin band with the purified enzyme (Fig. 2) and in the Ouchterlony double-diffusion system it also reacted with the purified and the crude enzymes to give a single fused precipitin band. Under the same conditions, control serum gave no precipitin band with the purified enzyme.

Inhibition of the enzyme activity by antibody

The effect of this antiserum on the activities of purified supernatant malate dehydrogenase in conversion of malate to pyruvate and of pyruvate to malate was examined. As shown in Fig. 3A, when the enzyme was treated with the antiserum, the pyruvate forming and malate forming activities were inhibited to the same degree. An inhibition rate of almost 100% was reached at a ratio of 0.013 unit of enzyme to 0.2 ml of antiserum. Control serum had no effect on the activities. Fig. 3A also shows the inhibitory effect of antiserum on the enzyme activity of the supernatant fraction of a liver homogenate. The activity of the crude enzyme was inhibited by the antiserum like that of the purified enzyme. The γ-globulin from the antiserum also inhibited the activity of the crude enzyme. as shown in Fig. 3B.



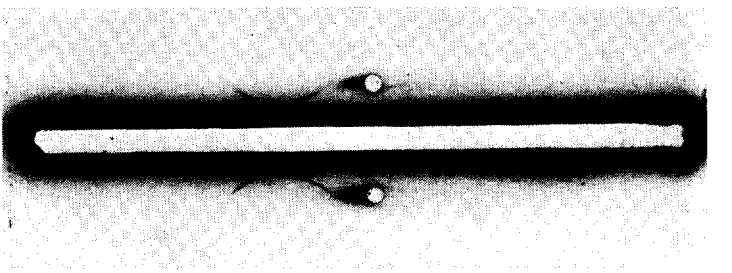


Fig. 1. Polyacrylamide gel electrophoresis of the purified enzyme. 0.2 mg of protein was used per tube. Other conditions are described in the text.

Fig. 2. Immunoelectrophoresis of the purified enzyme. The upper well contained 5.7 μg and the lower well 10.4 μg of purified enzyme. Antiserum was present in the center trough. Other conditions are as described in the text. The photograph was taken after staining with Amido black 10B.

Specificities of malate dehydrogenase (decarboxylating) (NADP) from various organs of rat

The antiserum against supernatant malate dehydrogenase of rat liver reacted with supernatant malate dehydrogenases of liver, heart, kidney and brain to give single precipitin bands which fused with each other on Ouchterlony double-diffusion plates (Fig. 4). In contrast, under similar conditions no precipitin band could be observed between the antiserum and supernatant malate dehydrogenase in the crude supernatant fraction of adipose tissue. The enzyme activity was detected in the mitochondrial fractions of heart, kidney and brain, but there was scarcely any in that of liver. These enzymes gave no precipitin band with antiserum against supernatant malate dehydrogenase. The immunological specificities of the enzymes in various rat organs were studied by neutralizing the enzyme activity with antibody (γ-globulin)

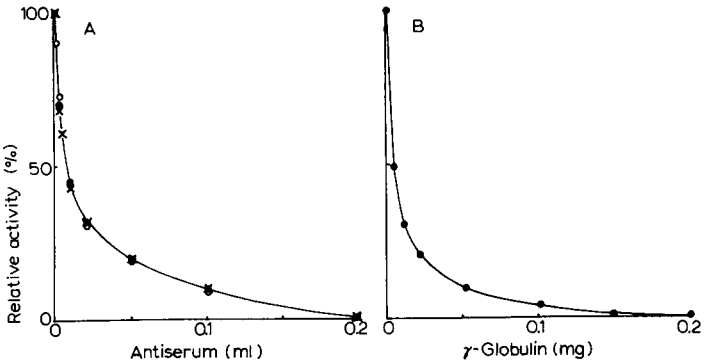


Fig. 3. Inhibitory effects of antiserum (A) and γ-globulin (B) from it on malate dehydrogenase (decarboxylating) (NADP). Purified (○, ×) and crude (●) enzymes (0.013 unit) were preincubated with antiserum (γ-globulin) or normal serum (γ-globulin) at 25° for 15 min and then the enzyme activity causing conversion of malate to pyruvate (○, ●) or pyruvate to malate (×) was determined as described in the text.

$$\text{Relative activity} = \frac{\text{activity in the presence of antiserum } (\gamma\text{-globulin})}{\text{activity in the presence of normal serum } (\gamma\text{-globulin})} \times 100$$

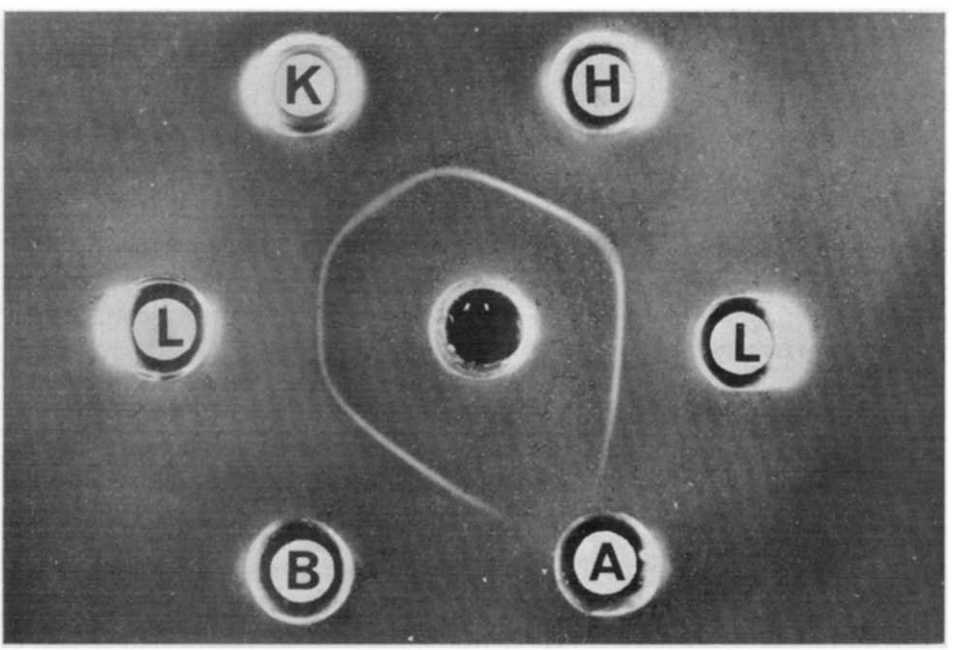


Fig. 4. Ouchterlony double-diffusion analysis of malate dehydrogenase (decarboxylating) (NADP) in the supernatant fractions of various organs. The enzymes (0.08 unit) in supernatant fractions of liver (L), heart (H), kidney (K), brain (B) and epididymal adipose tissue (A) were put in the outer wells and antiserum (20 μl) in the center well. The photograph was taken after development for 18 h.

TABLE II

INHIBITION OF MALATE DEHYDROGENASES (DECARBOXYLATING) (NADP) OF VARIOUS RAT ORGANS BY ANTIBODY

Each malate dehydrogenase (0.013 unit) was incubated with 0.1 mg of γ-globulin from antiserum or control serum for 15 min at 25°. Experimental and assay conditions were as described for Fig. 3 and in the text.

Enzyme source	*Fraction*	*Inhibition (%)*
Liver	Supernatant	92.5
	Mitochondrial	*
Kidney	Supernatant	90.8
	Mitochondrial	0
Heart	Supernatant	27.4
	Mitochondrial	0
Brain	Supernatant	91.1
	Mitochondrial	0
Adipose tissue	Supernatant	92.6
	Mitochondrial	**

* Scarcely any enzyme activity was found.
** Not tested.

against supernatant malate dehydrogenase from liver. Crude extracts of liver, kidney, heart, brain and adipose tissue containing similar amounts of the enzyme activity were each treated with a fixed amount of antibody, and the inhibition ratios were determined (Table II). The antibody inhibited the enzyme activities of kidney, brain and adipose tissue to the same extent as that of liver. Results were variable with malate dehydrogenase in the heart supernatant, but its activity seemed to be inhibited less than that of supernatant malate dehydrogenase from liver. Mitochondrial malate dehydrogenase from heart, kidney and brain were not inhibited by the antibody against supernatant malate dehydrogenase from liver (Table II).

Differences between the supernatant and the mitochondrial enzymes of rat heart

As described above in Ouchterlony double-diffusion tests, malate dehydrogenase in the supernatant fraction from heart reacted with antiserum against supernatant malate dehydrogenase from liver in the same manner as that in the supernatant from liver (Fig. 4). In inhibition tests, however, malate dehydrogenase activity of the supernatant fraction of heart was inhibited less by the antibody than that of the liver supernatant (Table II). In preparation of the supernatant fraction from heart, on very gentle homogenization of cardiac muscles, the inhibition rate increased to nearly that of the supernatant enzyme from liver. Therefore, the low inhibition rate of malate dehydrogenase in the heart supernatant might be due to contamination of the heart mitochondrial malate dehydrogenase that was not inhibited by the antibody. This possibility was supported by results of inhibition tests on two malate dehydrogenases from a sonicated homogenate of heart which were separated from each other by DEAE-cellulose column chromatography. Heart was homogenized in a Waring blender with 0.02 M Tris–HCl buffer (pH 7.4) containing 0.02 M EDTA and 2 mM 2-mercaptoethanol, and the homogenate was subjected to ultrasonication. The material was then centrifuged at 105 000 $\times$ *g* for 60 min and the supernatant was treated with ammonium sulfate. Material precipitating between 30 and 90% saturation (about

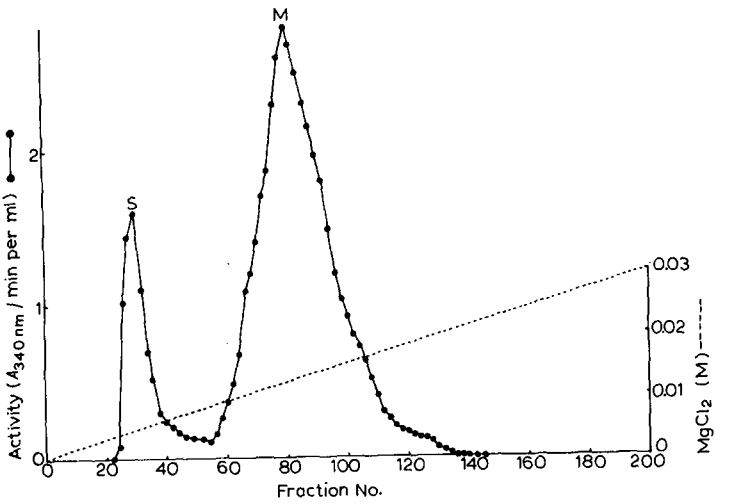


Fig. 5. DEAE-cellulose chromatography of malate dehydrogenase (decarboxylating) (NADP) in the supernatant of a sonicated rat heart homogenate. Experimental conditions are described in the RESULTS.

95% recovery) was suspended in 0.03 M Tris–HCl buffer (pH 7.4) containing 2 mM 2-mercaptoethanol and dialyzed against the same buffer. The dialyzed sample was placed on a DEAE-cellulose column (1.6 cm × 20 cm) equilibrated with 0.03 M Tris–HCl buffer (pH 7.4) containing 2 mM 2-mercaptoethanol and eluted with a linear gradient of 0 to 30 mM $MgCl_2$. As shown in Fig. 5, two peaks with malate dehydrogenase activity were eluted from the DEAE-cellulose column. Peak I (S) (about one-seventh of the total malate dehydrogenase activity) showed the same behavior as that of the liver supernatant malate dehydrogenase on DEAE-cellulose chromatography, and the same rate of inhibition by antibody. Peak II (M) enzyme was eluted with a higher concentration of $MgCl_2$ and corresponded to the malate dehydrogenase activity in the mitochondrial fraction separated from a rat heart homogenate by differential centrifugation. Malate dehydrogenase in Peak II, like that in mitochondria, was not inhibited by the antibody and gave no precipitin band with antiserum against supernatant malate dehydrogenase from the liver.

Species specificity of liver malate dehydrogenase (decarboxylating) (NADP)

Sprague–Dawley rats, Donryu rats, *ddo* mice, pigs and pigeons were used in this experiment. The effects of the antibody against liver supernatant malate dehydrogenase of Sprague–Dawley rats on the enzyme activities of liver supernatants of these animals were examined. The inhibitory effects of the antibody on the liver supernatant malate dehydrogenases of the Sprague–Dawley and the Donryu strain were similar. The enzyme of mouse liver was partially inhibited by the antibody. The enzymes of pig liver and pigeon liver were scarcely inhibited by the antibody.

Immunochemical determination of malate dehydrogenase (decarboxylating) (NADP) in liver extracts of rats under various dietary and hormonal conditions

The level of supernatant malate dehydrogenase activity in the liver and adipose tissue of rats has been found to vary under different dietary conditions. High activity

is associated with conditions favoring fatty acid synthesis[8,15,25-26], and, conversely, the activity decreases during fasting[8]. It was of interest, therefore, to see whether these changes in enzyme activity were accompanied by proportionate changes in the quantity of immunologically reactive protein. For this purpose, liver extracts were prepared from normal and starved rats and those in which enzyme had been induced. Fig. 6 shows the results of inhibition tests with the antibody. Crude liver extracts of these rats containing similar supernatant malate dehydrogenase activity were added to increasing amounts of antibody for supernatant malate dehydrogenase purified from the livers of rats in which enzyme had been induced. On plotting the inhibition rate against the antibody concentration, the three curves for normal and starved rats and those in which enzyme had been induced coincided with each other. On Ouchterlony double-diffusion analysis, supernatant malate dehydrogenase from normal and starved rats and those in which enzyme had been induced gave single precipitin bands with antibody which fused with each other.

DISCUSSION

The purified enzyme obtained from the supernatant fraction of rat liver (Table I) was a homogeneous protein as judged by polyacrylamide gel electrophoresis (Fig. 1), immunoelectrophoresis (Fig. 2) and agar immunodiffusion. The final product had about 2-fold higher specific activity than that of the crystalline enzyme from pigeon liver described by HSU AND LARDY[3]. This may be due to a difference between rats and pigeons.

The antibody completely inhibited the enzyme activity (Fig. 3) and precipitated its enzyme. This suggests a close relationship between the catalytic center and the antigenic determinant.

The enzyme in the supernatant fraction from rat heart was inhibited less by the antibody (Table II), although results were sometimes variable. The smaller inhibition rate can be explained by leakage of mitochondrial malate dehydrogenase from the mitochondria during preparation of the enzyme sample (Fig. 5). Therefore, the supernatant enzymes from kidney, heart, brain and adipose tissue were indistinguishable from that of rat liver in inhibition tests with antibody.

In double-diffusion analysis (Fig. 4) supernatant malate dehydrogenase of adipose tissues failed to give a precipitin band, although its catalytic activity was affected by antibody in the same manner as that of the liver enzyme (Table II). Adipose tissue supernatant malate dehydrogenase partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-50 gel filtration also gave no precipitin band. When purified supernatant malate dehydrogenase from liver was mixed with either the supernatant fraction of adipose tissue or the boiled supernatant of this fraction, it formed a precipitin band with the antibody. These results show that there is no inhibitor in the supernatant fraction of adipose tissue preventing supernatant malate dehydrogenase from forming a precipitin band, and that the antigenic determinant groups of supernatant malate dehydrogenase of adipose tissue are a little different from those of other supernatant malate dehydrogenases in the liver, kidney, heart and brain.

The results in Fig. 6 suggest that increase and decrease in enzyme activities in rat livers under various conditions may be due to changes in the amount of enzyme

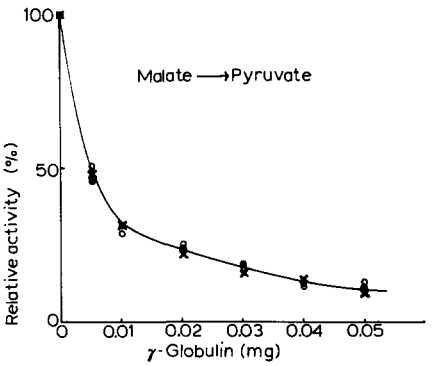


Fig. 6. Immunochemical determination of rat liver malate dehydrogenases (decarboxylating) (NADP) from normal and starved rats and those with induced enzyme. Various amounts of antibody (γ-globulin) were added to fixed amounts (each 0.013 unit) of crude enzyme from normal and starved rats and those with induced enzyme. After completion of the antigen–antibody reaction, the enzyme activity was assayed. Other experimental conditions were as described for Fig. 5 and in the text. ○, normal rats (specific activity, 0.009 unit/mg protein); ●, starved rats (specific activity, 0.005 unit/mg protein); ×, rats with induced enzyme (specific activity, 0.06 unit/mg protein).

protein which is immunologically indistinguishable from that of normal animals.

It has been suggested that supernatant malate dehydrogenase of liver and adipose tissue may participate in fatty acid synthesis[15,25,27–29]. However, an immunologically different form of malate dehydrogenase is present in heart, kidney and brain mitochondria of rats (Table II), and this isoenzyme may have a different role. Recently, SIMPSON AND ESTABROOK[13] reported that mitochondrial malate dehydrogenase in bovine adrenal cortex formed NADPH for steroid hydroxylation. In other organs where there is no formation of steroid, however, the role of mitochondrial malate dehydrogenase is obscure. HÜLSMANN[9] suggested that heart mitochondrial malate dehydrogenase might be involved in the stimulatory effect of malate on fatty acid synthesis by heart mitochondria. Results in this paper (Fig. 5) show that most of the malate dehydrogenase activity in rat heart is in the mitochondria. Further biochemical and physiological studies are now in progress to elucidate the role of malate dehydrogenase in mitochondria.

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