Brit. J. Pharmacol. (1954), **9**, 182.

THE EFFECT OF A SPECIFIC ANTISERUM ON THE ACTIVITIES OF LACTIC DEHYDROGENASE OF MAMMALIAN MUSCLE AND OF *SCHISTOSOMA MANSONI**

BY

TAG E. MANSOUR, ERNEST BUEDING, AND ABRAM B. STAVITSKY

From the Department of Pharmacology and the Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, Ohio, U.S.A.

(RECEIVED DECEMBER 14, 1953)

In a previous study the reaction kinetics of lactic dehydrogenase of *Schistosoma mansoni* were compared with those of lactic dehydrogenase of rabbit muscle (Mansour and Bueding, 1953). Our observations suggested that the enzyme of the parasite may not be identical with that of the mammalian host although both enzymes catalyse the same reaction. More conclusive evidence about this problem could be obtained by the use of an antibody against either one of these enzymes. If the latter are not identical, differences in their interaction with such antibodies would reveal differences in their nature. On the other hand, if the two enzymes are identical then they should exhibit similar behaviour toward specific antibodies.

After repeated injections of rabbit muscle lactic dehydrogenase into roosters their sera inhibited the activity of this enzyme. These sera did not affect the activity of lactic dehydrogenase of *Schistosoma mansoni* and of *S. japonicum*. However, they did inhibit the activity of rat muscle lactic dehydrogenase. In addition, information was obtained about the mechanism by which these antisera inhibit enzymatic activity.

METHODS

Enzyme Preparations.—Lactic dehydrogenase of rat muscle was purified by an adaptation of the method used by Kornberg and Pricer (1951) for the enzyme of rabbit muscle. After decapitation the rat was skinned and the carcass placed in cracked ice. All subsequent operations were carried out at temperatures varying between 0 and 4° C. The muscles were excised, passed through a meat grinder and extracted twice with 2 volumes of 0.03N-KOH for 10 minutes with occasional stirring. The extract was separated from the insoluble portion by straining through cheese cloth. The combined extracts, when assayed according to Kornberg and Pricer (1951), contained 37.5 units per ml. and 3.6 units per mg. of protein. To 154 ml. of this extract (obtained from 86 g. of muscles) was added 175 ml. of a solution of ammonium sulphate (saturated at room temperature; adjusted to *p*H 7.6). The resulting precipitate was centrifuged and dissolved in distilled water (77 ml.; 125 u./ml. and 5.2 u./mg. of protein). To 72 ml. of this fraction 27.3 ml. of the saturated ammonium sulphate solution was added and the mixture was centrifuged. To the supernatant fluid (72 ml.) 30 ml. of the saturated ammonium sulphate solution was added, the resulting precipitate was centrifuged and dissolved in distilled water (22 ml.; 330 u./ml., 20 u./mg. of protein). A solution of ammonium sulphate saturated at 2° C. (*p*H not adjusted) was added gradually in small portions until the first appearance of a turbidity. The mixture was then allowed to stand at room temperature for 10 minutes. The precipitate was centrifuged and dissolved in cold water (9 ml.; 500 u./ml., 75 u./mg. of protein). To 8.5 ml. of this solution 34 ml. of cold distilled water and 8.5 ml. of calcium phosphate gel (dry weight 14 mg./ml.) were added. After centrifugation the residue was washed successively with 8.5 ml. of 0.05 M and 8.5 ml. of 0.15 M glycylglycine buffer (*p*H 7.5). Elution of the enzyme was carried out with 8.5 ml. of 0.5 M glycylglycine buffer (*p*H 7.5). The final eluate contained 223 u./ml. and its specific activity amounted to 223 u./mg. of protein.

Measurements of enzymatic activity and preparations of lactic dehydrogenase of rabbit muscle and of schistosomes were carried out by the same procedures as those used previously (Mansour and Bueding, 1953).

Immunological Procedures.—To three roosters, each weighing approximately 5 lb., alum-precipitated rabbit lactic dehydrogenase was administered. This was prepared as follows: To 0.6 ml. of the enzyme solution containing 10 mg. of protein 0 05 ml. of an

* This investigation was supported by grants from the United States Office of Naval Research and from the Division of Research Grants and Fellowships, National Institute of Health, United States Public Health Service.

alum potassium sulphate solution (1% w/v) was added. After adjustment of the *p*H tc 6.4 the total volume of the suspension was brought to 5 ml. with saline (0.9% NaCl). One course consisted in three intravenous injections of 0.5 ml. of the suspension (equivalent to 1 mg. of protein) administered every other day. Four to five days after the last injection the inhibitory effect of the serum was tested. One week thereafter another similar course was given. Four additional courses were administered after intervals each lasting between one to two weeks. To a second group of three roosters the enzyme was injected in a solution of saline instead of in an alum suspension. A single dose consisted of 1.5 mg. of protein in a volume of 0.5 ml. The schedules and courses were the same as in the first group.

Before assay the sera were heated for 30 minutes at 55° C. In addition to the inactivation of complement this procedure considerably reduced the "blank" lactic dehydrogenase activity of the serum, but produced no reduction in the inhibitory effect of sera on lactic dehydrogenase. The enzyme, diluted in 0.1 M-NaCl, was incubated with the serum for 10 minutes at room temperature. The total volume was 0.08 ml. The concentration of serum in this solution never exceeded 25% because occasionally higher concentrations of control sera (from untreated animals) had an inhibitory effect on the activity of the rabbit enzyme. At the end of the incubation period the other constituents of the reaction mixture were added. Unless stated otherwise DPN* or $DPNH_2$* were added last. The volume was 1 ml.

On a number of occasions the precipitating and agglutinating properties of anti-enzyme sera were tested. The precipitin tests were performed by the ring reaction (Boyd, 1947). Salt concentrations varying from 0.9 to 8.0% were employed for the ring reaction (Goodman, Wolfe, and Norton, 1951). The agglutination reaction was carried out by a modification of the tannic acid method of Boyden (1951) as developed by one of us (Stavitsky, 1954). Sheep red blood cells were treated with tannic acid, which modified their surface so that it reacted with proteins. Such cells were then agglutinated by the specific anti-protein sera. By this method it has been found that exceedingly small amounts of antibody and antigen can be estimated (Stavitsky, 1954). Ordinarily, the method is far more sensitive than the ring reaction. Because of the small amounts of antigen (enzyme) and antiserum available the complement fixation reaction was not employed.

* In this paper the following abbreviations are used: DPN= oxidized diphosphopyridine nucleotide; $DPNH_2$=reduced diphosphopyridine nucleotide.

RESULTS

Sera from roosters which had received the enzyme in either form began to exhibit an inhibitory effect on the activity of rabbit lactic dehydrogenase after the third course of injections. Inhibition of enzymatic activity invariably was greater with the sera of roosters to which the alum precipitated enzyme was administered, although the dose of the enzyme was lower. After a fourth course of injections the degree of inhibition reached its maximum. This inhibitory effect remained unchanged after exhaustive dialysis of the sera against isotonic solutions of sodium chloride. Control experiments revealed that at optimal *p*H and substrate concentrations sera of untreated roosters produced no inhibitory effect when used in concentrations of 25% or less. In fact, there was a slight stimulation of enzymatic activity, presumably because of the protective action of serum proteins on the enzyme (Table I).

Incubation of the rabbit enzyme with immune sera resulted in a marked reduction of enzymatic activity. The degree of inhibition was lowered as the concentration of the serum was decreased (Fig. 1). The immune serum against the rabbit enzyme significantly affected the activity of lactic dehydrogenase of rat muscle. The inhibitory effect of equal concentrations of immune sera on the activity of the rat enzyme was about half as great as on the enzyme from rabbit muscle (Fig. 1).

TABLE I

EFFECT OF CONTROL AND OF IMMUNE SERA ON THE ACTIVITIES OF LACTIC DEHYDROGENASES FROM RABBIT AND RAT MUSCLE AND FROM SCHISTOSOMES

All activities are expressed in millimicromoles of DPN, oxidized in one minute or reduced in two minutes. E: Activity of enzyme. S: Activity of serum. Concentration of sera during pre-incubation: 25%.

Substrates	Serum	Rabbit Muscle Lactic Dehydrogenase			Rat Muscle Lactic Dehydrogenase			*Schistosoma mansoni* Lactic Dehydrogenase			*Schistosoma japonicum* Lactic Dehydrogenase		
		E+S†			E+S†			E+S†			E+S†		
		Calc.	Found	%Inhib.	Calc.	Found	%Inhib.	Calc.	Found	%Inhib.	Calc.	Found	%Inhib.
Pyruvate + $DPNH_2$	Control	15·0	16·5	0	14·4	14·8	0	15·3	16·1	0	15·6	15·8	0
	Immune	15·5	6·1	68	16·4	11·6	39	16·6	18·0	0	14·9	14·8	0
Lactate + DPN	Control	25·0	32·2	0	35·9	44·0	0	22·8	26·3	0	26·9	30	0
	Immune	24·7	13·7	56	35·6	29·0	21	23·3	26·8	0	27	29·4	0

† In these experiments the activity of the serum did not exceed 20% of that of the enzyme.

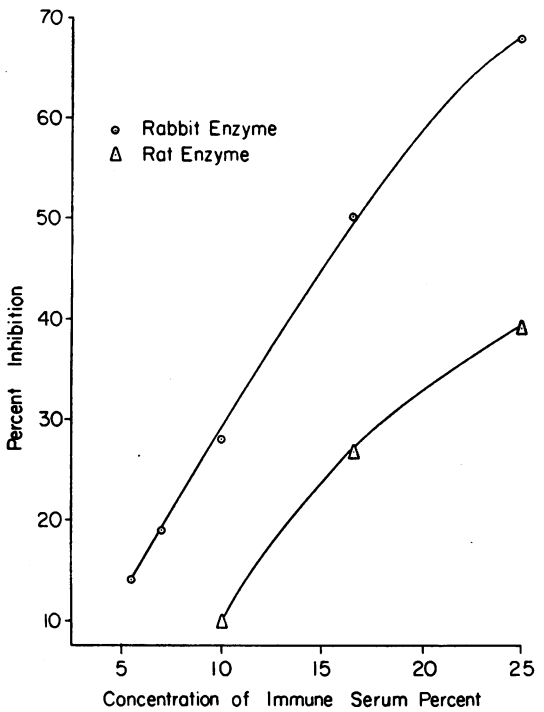

FIG. 1.—Effect of the concentration of immune serum on the activity of lactic dehydrogenase of rabbit and of rat muscle. Abscissa: concentration of immune serum %. Ordinate: % inhibition.

The sera from roosters immunized against the rabbit enzyme did not affect the activity of the lactic dehydrogenases of *Schistosoma mansoni* or of *Schistosoma japonicum* (Table I). It should be noted here that essentially identical results were obtained regardless of the direction in which the catalytic activities of the enzymes were measured —that is, whether by the reduction of pyruvate to lactate or the oxidation of lactate to pyruvate. There was an exception to this rule; the stimulatory or protective effects of normal sera on the schistosome enzymes were noticeable only during the oxidation of lactate, but not during the reduction of pyruvate.

The inhibitory effect of the immune serum on the rabbit enzyme was not altered by the presence of the lactic dehydrogenase preparation of *Schistosoma mansoni* (Table II).* Therefore, the lack of an inhibitory effect of the immune serum on the enzyme of schistosomes was not due to the destruction of the antibody by the enzyme preparation of the worms.

Incubation of the immune serum with three other glycolytic enzymes resulted in no change in their activities. The enzymes tested were phosphohexose isomerase, aldolase, and phosphoglyceraldehyde dehydrogenase. They were prepared and assayed (with and without the serum) according to Cori *et al.* (Cori, Slein, and Cori, 1948; Taylor, Green, and Cori, 1948; Slein, Cori, and Cori, 1950). Furthermore, incubation of crystalline phosphoglyceraldehyde isomerase of rabbit muscle with the immune serum did not alter the inhibitory effect of the latter on mammalian lactic dehydrogenase.

If an anti-enzyme serum inhibits enzymatic activity, it is generally assumed that this occurs by an attachment of the antibody at the site where com-

* The activity of a mixture of the two enzymes was slightly lower (approx. 10%) than the sum of the activities of each enzyme (see Table II, columns 3b and 3c). Therefore, it was necessary to make a corresponding correction in the calculation of the activity of the mammalian enzyme in this mixture (see Table II, columns 7b and 7c). Without this correction the inhibitory effect of the antiserum would have been greater in the presence of the worm enzyme than in its absence. This would appear highly improbable.

TABLE II

EFFECT OF IMMUNE SERA ON THE ACTIVITY OF LACTIC DEHYDROGENASE OF RABBIT MUSCLE IN THE PRESENCE OF A LACTIC DEHYDROGENASE PREPARATION OF *S. MANSONI*

RE=activity of rabbit enzyme, WE=activity of worm enzyme, S=activity of immune serum. Enzymatic activities are expressed in the same manner as in Table I.

Reaction	Serum	1 RE	2 WE	3 WE+RE			4 S	5 RE+S	6 WE+S	7 RE+WE+S			Inhibition %
				Found a.	Calc. b.†	Diff. c.‡				Found a.	Calc. b.§	RE c.‖	
Pyruvate→ Lactate	Immune	12·0					2·6	7·7					57
	Immune		17·2				2·6		19·7				—
	Immune	9·5	9·2	17·1	18·7	1·6	2·6			14·3	15·9	4·1	57
	Control	12·2	10·8	21·4	23·0	1·6	2·6			25	26·6	13·2	—
Lactate→ Pyruvate	Immune	22					3·4	17·5					36
	Immune		14				3·4		20·0				—
	Immune	20·0	18·0	37	38	1·0	3·6			32·5	33·5	11·9	40
	Control	22·6	13·4	35·4	36	0·6	5·6			40	40·6	21·6	4

† Sum of activities of worm enzyme and of rabbit enzyme (Columns 1 and 2).
‡ Calculated sum of activities of each enzyme alone (Column 3b) minus observed activity of mixture of both enzymes (Column 3a).
§ Observed activity (Column 7a) plus difference between calculated sum of activities of each enzyme alone minus observed activity of mixture of both enzymes (Column 3c).
‖ Calculated activity of mixture (Column 7b) minus activity of worm enzyme (Column 2) minus activity of serum (Column 4).

bination of the enzyme with the substrate or coenzyme occurs. Under these conditions the antibody would prevent the substrate (or coenzyme) from reacting with the enzyme, resulting in an inhibition of enzymatic activity. If competition for the same point of attachment to the enzyme, between antibody on the one hand, and substrate or coenzyme on the other, took place, the inhibitory effect of the antibody on the enzymatic activity might be reduced when the enzyme and its antibody are incubated in the presence of substrate or the coenzyme. Conversely, inhibition of the activity would be increased when the concentration of the competing substrate is reduced. However, pre-incubation of rabbit muscle lactic dehydrogenase with pyruvate, lactate, $DPNH_2$ or DPN did not affect the inhibitory action of the immune serum. Furthermore, a decrease in the concentration of either substrate or coenzyme resulted in a lower rather than in a higher inhibition of enymatic activity.

All attempts to demonstrate precipitation of the enzyme and agglutination of enzyme-treated red cells with the antisera were consistently negative.

Discussion

It should be noted that relatively small amounts of antigen have been employed in this study for the production of antibodies to lactic dehydrogenase. A relatively large quantity of injected soluble proteins is ordinarily degraded and lost from the body before stimulating antibody production, and it appears that the use of alum-precipitated proteins results in a more efficient utilization of the protein. On the other hand, it is known that the injection of excessive amounts of protein may lead to the phenomenon of "immunological paralysis," in which the animal fails to produce antibody (Dixon and Maurer, 1953).

The detection of antibody by the measurement of specific inhibition of enzymatic activity in the absence of visible serological activity was not expected. These observations demonstrate, at least in this instance, the superiority of the enzymatic inhibition method over serological procedures. Furthermore, unlike the serological procedures, this method does not depend on the use of highly purified antigens and antibodies because the specificity is associated with the substrate specific for the enzyme.

Kubowitz and Ott (1943) have shown that the rabbit antibody against lactic dehydrogenase of rat muscle competes with pyruvate for the active centre of the enzyme. Krebs and Najjar (1948) were able to protect phosophoglyceraldehyde dehydrogenase of rabbit muscle from inhibition by the antibody by pre-incubating the enzyme with high concentrations of DPN. Zamecnik and Lipmann (1947) found that lecithin interfered with the reaction of lecithinase of *Cl. welchii* (alpha toxin) and the antibody. In these instances the antibodies competed specifically with substrate or coenzyme for combination with the enzyme. On the other hand, there are examples where the antibody combines with the enzyme and precipitates it without inhibition of enzymatic activity (Campbell and Fourt, 1939). Furthermore, according to a hypothesis proposed by Cinader (1953), the antibody does not necessarily exert its inhibitory effect by combining with the same site on the enzyme as the substrate or coenzyme. Our observations appear to support this view because neither pyruvate nor DPN protected lactic dehydrogenase from inhibition by the antibody. Therefore, the antibody could have exerted its inhibition by combining with sites other than the active centre of the enzyme. Further study is required to provide a definitive answer regarding the nature of this combination.

In the present study it was observed that an antiserum against rabbit muscle lactic dehydrogenase inhibited the activity of this enzyme, but had no effect on lactic dehydrogenase of *Schistosoma mansoni* and of *Schistosoma japonicum*. Since inactivation or destruction of the antibody by the enzyme preparation of the worms has been ruled out it is concluded that lactic dehydrogenase of rabbit muscle is not identical with lactic dehydrogenase of schistosomes although the enzymes catalyse the same reactions. This conclusion is supported also by differences in the kinetics of the enzymes (Mansour and Bueding, 1953). The nature of these differences remains to be determined.

The specificity of the enzyme–antibody reaction is illustrated by the observations that the immune serum did not affect the activities of three other glycolytic enzymes of rabbit muscle—phosphohexose isomerase, aldolase, and phosphoglyceraldehyde dehydrogenase. Since incubation of the last enzyme did not affect the inhibitory activity of the serum against mammalian lactic dehydrogenase it appears that the antibody reacts with specific groupings common to lactic dehydrogenase of both rabbit and rat muscle.

Further evidence that the nature of the schistosome enzyme differs from that of the host was supplied by the use of sera obtained by immunizing roosters against lactic dehydrogenase of *Schistosoma mansoni*. These sera markedly inhi-

bited the activities of lactic dehydrogenase of *Schistosoma mansoni* and of *Schistosoma japonicum* but had no effect on the rabbit or rat muscle enzymes. Moreover, in contrast to the mammalian enzyme–antibody system, pre-incubation of the enzyme with $DPNH_2$ markedly reduced the inhibition of the worm enzyme by its antibody (Henion, Mansour, and Bueding, unpublished observations) indicating that the latter may interact with the active centre of the worm enzyme. This observation and the fact that this antiserum does not affect the activity of mammalian lactic dehydrogenase suggest that the active centre of the worm enzyme differs from those of lactic dehydrogenase of rabbit and of rat muscle. The antiserum against the lactic dehydrogenase of schistosomes had no effect on the activity of phosphohexose isomerase of the same parasite; conversely, an antiserum against the latter enzyme did not inhibit the activity of lactic dehydrogenase of schistosomes (Bueding, MacKinnon, and Henion, unpublished observations). Therefore, it appears that these two antisera do not react with groupings common to all schistosome proteins but with sites specific for a particular enzyme of the parasite.

The antiserum against lactic dehydrogenase of rabbit muscle significantly inhibits the activity of lactic dehydrogenase of rat muscle, but to a lesser degree than that from rabbit muscle. The enzymes of these two mammalian species are probably similar to, but not identical with, each other.

Species differences in the nature of enzymes catalysing the same reaction might be of interest for the development of chemotherapeutic agents because they may afford opportunities to select specific inhibitors against the enzyme of the parasite without affecting that of the host.

Summary

1. Sera from roosters which had been immunized against lactic dehydrogenase from rabbit muscle markedly inhibited the activity of this enzyme but did not affect those of the lactic dehydrogenases of *Schistosoma mansoni* and of *Schistosoma japonicum*.

2. It is concluded that lactic dehydrogenase of schistosomes is not identical with that of mammalian tissues although these enzymes catalyse the same reaction in the parasite and in the host.

References

Boyd, W. C. (1947). *Fundamentals of Immunology*, 2nd ed., p. 421. New York: Interscience Publishers.

Boyden, S. V. (1951). *J. exp. Med.*, **93**, 107.

Campbell, D. H., and Fourt, L. (1939). *J. biol. Chem.*, **129**, 385.

Cinader, B. (1953). *Biochemical Society Symposium No.* 10, p. 16.

Cori, G. T. Slein, M. W., and Cori, C. F. (1948). *J. biol. Chem.*, **173**, 605.

Dixon, F. J., and Maurer, P. H. (1953). *Fed. Proc.*, **12**, 441.

Goodman, M., Wolfe, H. R., and Norton, S. (1951). *J. Immunol.*, **66**, 225.

Kornberg, A., and Pricer, W. E. (1951). *J. biol. Chem.*, **193**, 481.

Krebs, E. G., and Najjar, V. A. (1948). *J. exp. Med.*, **88**, 568.

Kubowitz, F., and Ott, P. (1943). *Biochem. Z.*, **314**, 94.

Mansour, T. E., and Bueding, E. (1953). *Brit. J. Pharmacol.*, **8**, 567.

Slein, M. W., Cori, G. T., and Cori, C. F. (1950). *J. biol. Chem.*, **186**, 763.

Stavitsky, A. B. (1954). *J. Immunol.*, in press.

Taylor, J. F., Green, A. A., and Cori, G. T. (1948). *J. biol. Chem.*, **173**, 591.

Zamecnik, P. C., and Lipmann, F. (1947). *J. exp. Med.*, **85**, 395.