THE REACTION BETWEEN THE ENZYME TYROSINASE AND ITS SPECIFIC ANTIBODY

By MARK H. ADAMS, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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Tyrosinase is the enzyme which catalyses the aerobic oxidation of monohydric phenols to derivatives of catechol, and catechol derivatives to the corresponding quinones. For example, pressor substances such as tyramine and adrenalin are oxidised in the presence of tyrosinase to colored products which are physiologically inactive. Since this reaction occurs *in vitro* it was thought that tyrosinase might have a similar effect *in vivo*, and might conceivably affect experimental hypertension in animals (1) and essential hypertension in human beings (2). Because it was planned to administer tyrosinase parenterally to test this possibility two problems were presented immediately; first, is the enzyme antigenic, and second, do the antibodies, if produced, affect the activity of the enzyme? This work is an attempt to answer these questions.

Previous attempts to demonstrate the production of antibodies to the enzyme tyrosinase (3–5) have failed because of the crudeness of the enzyme preparations available, and because the tests employed to indicate the production of specific antibodies were based upon the assumption that an antibody to an enzyme must neutralize its catalytic activity. Recognized immunological reactions were not used to demonstrate that antibodies had been produced. In the present work purified preparations of tyrosinase were used to immunize rabbits, and the production of specific precipitins for the enzyme tyrosinase was demonstrated.

EXPERIMENTAL

Materials and Methods.—The tyrosinase used in these experiments was prepared from the domestic mushroom, *Psalliota campestris*, in the laboratory of Professor J. M. Nelson of Columbia University (6). The preparations, which closely approached the highest purity thus far obtained for this enzyme, contained about 500 catechol-hydroquinone units (7) per mg. dry weight. The copper content was 0.13 per cent of the dry weight of the enzyme. Preparations available contained 3,600 catechol-hydroquinone units per cc. of aqueous solution. The QO_2 value using the catechol-hydroquinone substrate system was 300,000.

Immunization.—Three adult male rabbits were bled from the ear, about 10 cc. of blood being taken from each for the preparation of normal control sera. These rabbits were then injected intravenously with a solution of tyrosinase in saline sterilized by passage through a Berkefeld filter. Each rabbit received 1 mg. of tyrosinase daily for 6 days, then was allowed to rest for a week. This course of treatment was

followed by two additional similar courses of injections, each rabbit thus receiving a total of 18 mg. of tyrosinase over a period of 5 weeks. Ten days after the final injection of tyrosinase the rabbits were bled again and the sera tested for precipitins.

Test for Precipitation.—The rabbit sera, normal and immune, were diluted with 1.5 volumes of saline. The tyrosinase at an initial concentration of 5 mg. per cc. or 1:200 was diluted serially with saline. Equal volumes of diluted serum and tyrosinase

TABLE I

Precipitin Titer of Normal and Immune Rabbit Sera

Rabbit serum	Concentration of tyrosinase in the mixture					
	1/400	1/1,600	1/6,400	1/25,000	1/100,000	1/400,000
Normal sera 9-93, 9-94, 9-95	−	−	−	−	−	−
Immune serum 9-93	++±	+++	++++	++++	++	±
Immune serum 9-94	+++	+++±	+++	+++	+±	trace
Immune serum 9-95	++±	+++±	++++	+++	++	±

++++ = pronounced precipitation with clear supernatant.
± = faint precipitation.
− = no precipitation.

TABLE II

Precipitation of Anti-Psalliota Tyrosinase Rabbit Serum by Psalliota and Lactarius Tyrosinases As Antigens

Antigen used	Concentration of antigen in the mixture					
	1/1,000	1/2,000	1/4,000	1/8,000	1/16,000	1/32,000
Psalliota tyrosinase	+++	+++±	++++	++++	++++	++++
Lactarius tyrosinase	−	−	−	−	−	−

++++ = pronounced precipitation with clear supernatant.
− = no precipitation.
The serum used was immune serum 9-93. Experimental conditions as in Table I.

solution were mixed, incubated at 37° for 2 hours, and placed in the refrigerator overnight before reading. The results are given in Table I.

It is evident that the sera of rabbits injected with a preparation of the enzyme tyrosinase contained precipitins for the antigen used, and that such precipitins were absent from the sera of these same rabbits before immunization.

A preparation of tyrosinase from the mushroom *Lactarius piperatus* of equivalent strength and purity (8) was tested against the antiserum to the *Psalliota campestris* tyrosinase under the conditions specified above. The results of this experiment are included in Table II.

The tyrosinase from the mushroom *Lactarius piperatus* failed to show any

cross precipitin reaction with the antiserum to the *Psalliota campestris* tyrosinase.

A preparation of *Psalliota* tyrosinase which had been inactivated with regard to catalytic activity by heating at 80° for 10 minutes was also tested in the same way for precipitation with antiserum to the *Psalliota* tyrosinase. The results (Table III) indicate that a temperature sufficient to destroy the catalytic activity of the enzyme affected but little its ability to function as a specific antigen in the precipitin reaction.

Human Sera Reacting with Tyrosinase.—In connection with a study on hypertension undertaken in the Hospital of The Rockefeller Institute by Dr. Schroeder (2) patients were treated by injections of tyrosinase. The tyrosinase, dissolved in physiological saline, was passed through a Berkefeld filter and proven bacteriologically sterile before injection. Blood specimens were ob-

TABLE III

Precipitation of Heat-Inactivated Tyrosinase in Anti-Tyrosinase Rabbit Serum

Antigen used	Concentration of antigen in the mixture			
	1/5,000	1/25,000	1/125,000	1/625,000
Active *Psalliota* tyrosinase	++++	++++	++±	±
Heat-inactivated *Psalliota* tyrosinase	++++	+++±	++	±

++++ = pronounced precipitation with clear supernatant.
± = faint precipitation.
The serum used was immune serum 9-93. Experimental conditions as in Table I.

tained from some of these treated patients, as well as from normal untreated human beings, and sera were prepared. These sera were tested for tyrosinase precipitins in the manner described above for rabbit sera (Table IV).

The treatment with tyrosinase including total dosage, period of treatment, and interval between final injection and removal of blood samples, is summarized in Table V. Included in this table are observations by Dr. Schroeder regarding the severity of local or systemic reactions. It is evident from Table V that there is no correlation between the quantity of antigen administered and the serological antibody response. There is also a lack of correlation between the antibody response and the sensitivity of the patient to injections of the enzyme. The patient sometimes experienced a maximum reaction at the first injection, obviously before any sensitization could take place. The reaction of the patients to injections of the enzyme will be discussed in greater detail in a paper to be published by Dr. Schroeder.

Effect of Immune Rabbit Serum on the Catalytic Activity of Psalliota campestris Tyrosinase.—Since the immune rabbit sera gave precipitin reactions with highly purified preparations of the enzyme tyrosinase at an antigen concentration of

TABLE IV

Precipitin Titers of Sera from Patients Treated with Tyrosinase and from Untreated Human Beings

Serum	Concentration of tyrosinase in the mixture			
	1/5,000	1/15,000	1/50,000	1/500,000
G.P.	+	+++	+++	±
J.P.	+	+	++	−
L.G.	++	+	±	−
J.R.	++	+	±	−
H.H.	+	±	trace	−
J.F.	±	±	−	−
A.Z.	±	±	−	−
J.S.	−	−	−	−
R.L.	−	−	−	−
A.L. normal	−	−	−	−
C.M. normal	−	−	−	−
Rabbit 9-93 (immunized)	+++±	++++	++++	±

++++ = pronounced precipitation with clear supernatant.
± = faint precipitation.
− = no precipitation.

TABLE V

Administration of Tyrosinase to Patients

Patient	Total tyrosinase administered	Route of administration	Period of treatment	Interval between injection and blood sampling	Antibodies in blood sample	Local or systemic reaction
	mg.		*days*	*days*		
G.P.	75	Subcutaneous	23	47	+++	None
J.P.	40	"	7	11 28	++	Swelling and tenderness, slight to moderate at site of injection
L.G.	5	Intramuscular	5	30	+	Shock reaction, not anaphylactic in origin
J.R.	250	Subcutaneous	100			
	25	"	9	6	+	None
H.H.	18	"	4	8	±	No local reaction but high fever
J.F.	55	"	20	45	±	Slight local reactions
A.Z.	44	"	30	None	±	Slight local reaction
J.S.	56	Intravenous	22			
	58	Subcutaneous	15	46	−	Moderate local reaction
R.L.	83	Intravenous	14			
	24	Subcutaneous	5	15	−	None

1/500,000 it was thought that the antisera might neutralize or inhibit the catalytic activity of the enzyme. To test this possibility the mixture of tyrosinase and rabbit antiserum after the precipitin reaction had taken place was titered for enzyme activity.

The immune serum from rabbit 9-93 was utilized since it had the highest precipitin titer. As a matter of convenience the normal serum of rabbit 9-94 was utilized as the control. The tyrosinase was so diluted with saline that the final concentration of tyrosinase in the mixture with serum was 1/20,000, the concentration at which maximum precipitation occurred as indicated in Table I. The sera employed were diluted with 1.5 volumes of saline. To 1 cc. of diluted serum was added 1 cc. of tyrosinase in saline at a concentration of 1/10,000, an amount corresponding to 45 units of catecholase activity. The serum-antigen mixtures were incubated 2 hours at 37°C. and kept overnight in the refrigerator. Three centrifuge tubes were set up as follows:—

Tube 1. 1 cc. normal serum 9-94 + 1 cc. of tyrosinase 1/10,000.

Tubes 2 and 3. 1 cc. immune serum 9-93 + 1 cc. of tyrosinase 1/10,000.

TABLE VI

Effect of Homologous Antibody on the Catalytic Activity of Tyrosinase

Rabbit serum plus tyrosinase	Tyrosinase activity in catecholase units per cc. of final dilution	
Normal serum 9-94, tube 1	0.45	0.45
Immune serum 9-93, tube 2	0.05	0.05
Immune serum 9-93, tube 3	0.46	0.45

No precipitate developed in tube 1 containing the normal serum, while tubes 2 and 3 contained a flocculent precipitate. An aliquot of tube 1 was diluted 1:50 with saline. Tube 2 was centrifuged to bring down the precipitate and an aliquot of the clear supernatant was diluted 1:50 with saline. Tube 3 was well stirred to distribute the precipitate uniformly and an aliquot was diluted 1:50. No precipitate was noted in this case after dilution. The enzyme in each case had been diluted to one one-hundredth of its initial concentration and hence should have had an activity of 0.45 units per cc. The catecholase activities were determined in the Warburg apparatus in the usual manner (7). The results of duplicate determinations are given in Table VI.

It is evident from this experiment that near the optimum concentration of enzyme and antibody for the precipitin reaction, as previously determined by the experiment of Table I, neither normal nor immune rabbit serum had any effect on the catalytic action of tyrosinase. If, however, the immune precipitate was removed by centrifugation, nine-tenths of the enzymatic activity was likewise removed.

In another experiment the ratio of antibody to antigen was increased 100-fold by mixing undiluted immune serum with 0.9 unit of enzyme and incubating samples of the mixture at 37° for various intervals from 15 minutes to 2

hours, after which the enzyme activities were determined. In no case did this large excess of antibody have any effect on the catalytic activity of the enzyme in the mixture.

We can conclude then, that it is valid to determine the activity of tyrosinase in the presence of antibody, and, furthermore, that the activity so determined is a correct indication of the amount of tyrosinase present in the sample under investigation.

TABLE VII

Quantitative Titration of the Precipitin Reaction between Tyrosinase and the Homologous Immune Rabbit and Human Sera

Experiment No.	Enzyme added		Enzyme in supernatant	Enzyme in precipitate	Percentage of added enzyme in precipitate	Enzyme in precipitate	Total precipitate	Antibody N in precipitate	Ratio of Antibody N / Antigen N in precipitate
	mg. of N	*units*	*units*	*units*		*mg. of N*	*mg. of N*	*mg.*	
				Rabbit serum					
1	0.0125	30	0.4	30	100	0.0125	0.094	0.080	6.4
2	0.0125	30	0.4	30			0.092		
3	0.025	59	0.5	59	100	0.025	0.158	0.132	5.28
4	0.025	59	—	59			0.155		
5	0.050	118	0.5	118	100	0.050	0.258	0.206	4.12
6	0.050	118	—	118			0.253		
7	0.10	235	34	201	82	0.082	0.276	0.190	2.32
8	0.10	235	51	184			0.268		
				Human serum					
9	0.01	28	0.4	28	100	0.010	0.079	0.069	6.9
10	0.02	57	2.0	55	98	0.020	0.130	0.110	5.5
11	0.03	85	6.0	79	93	0.028	0.151	0.123	4.4
12	0.04	113	14.0	99	88	0.035	0.157	0.122	3.5
13	0.05	142	36.0	106	75	0.037	0.159	0.122	3.3
14	0.10	284	150.0	134	47	0.047	0.126	0.079	1.7

Quantitative Determination of Precipitins.—Since evidence for the absolute purity of the tyrosinase preparation is lacking it was necessary to learn if the antibody of the rabbit serum was precipitating the tyrosinase itself and not merely reacting with an accompanying contaminant. The test involved a quantitative precipitin titration by the method of Heidelberger and Kendall (9) together with a simultaneous assay for enzymatic activity of the supernatant liquid remaining after removal of the specific precipitate. Since it has been shown above that the presence of immune serum has no effect on the determination of the catalytic activity of tyrosinase, it is legitimate to conclude that any tyrosinase not found in the supernatants must be part of the immune precipitate.

Immune rabbit sera 9-93 and 9-95 which were approximately equal in precipitin titer were pooled, and centrifuged under the conditions of the experiment to remove any sedimentable material. The human serum of highest precipitin titer for tyrosinase available (G.P. in Table IV) was treated in the same manner. The tyrosinase preparation to be used as antigen in the precipitin reactions was centrifuged also to make certain that no sedimentable material was present in the antigen solution. The Kjeldahl nitrogen content of this enzyme solution was determined, and enzyme dilutions in saline were

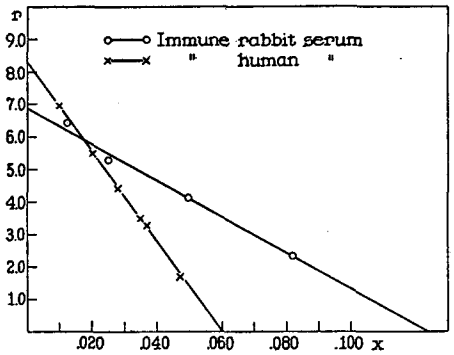

CHART 1. In this chart the variables r and x of the equation of Heidelberger and Kendall are plotted:

$$r = \text{ratio of } \frac{\text{Antibody N}}{\text{Antigen N}} \text{ in the precipitate}$$

and x = milligrams of antigen N in the precipitate.
In the equation of Heidelberger and Kendall the constants as calculated from these curves are for the immune rabbit serum

$R = 3.4$ and $A = 0.212$ mg. of N,

and for the immune human serum

$R = 4.2$ and $A = 0.126$ mg. of N.

made on the basis of nitrogen content. In each experiment 2 cc. of serum were diluted with 3 cc. of saline, and 1 cc. of tyrosinase appropriately diluted with saline was added. After mixing the tubes were incubated 2 hours at 37°C. and overnight in the refrigerator. The precipitates were centrifuged down at 0°C., washed twice with saline at 0°C., dissolved in dilute NaOH, and transferred to Kjeldahl flasks for nitrogen analysis. The clear supernatant fluid, decanted from the precipitate after centrifugation, was titered for tyrosinase activity in the Warburg apparatus. The second saline washings of the precipitates contained no measurable enzyme activity indicating that the immune precipitates did not dissociate appreciably in physiological saline.

Since the nitrogen content of the added tyrosinase is known, and since the

tyrosinase remaining in the supernatants can be determined, the amount of tyrosinase nitrogen present in the specific precipitates can be calculated by difference. The total nitrogen content of the specific precipitate is known from the Kjeldahl determinations. Consequently the amount of antibody nitrogen present in the immune precipitates can be calculated by difference. Then knowing the amounts of both antigen and antibody nitrogen in the immune precipitates it becomes possible to apply the empirical relationship of Heidelberger and Kendall (9) to our results.

The equation of Heidelberger and Kendall is:

$$r = 2R - \frac{R^2 X}{A}$$

in which

r = ratio of $\frac{\text{Antibody N}}{\text{Antigen N}}$ in precipitate

X = milligrams of antigen N in precipitate

R = ratio of $\frac{\text{Antibody N}}{\text{Antigen N}}$ at equivalence point

A = total antibody present in milligrams of N.

As can be seen from Chart 1 both human and rabbit immune sera satisfy the empirical equation of Heidelberger and Kendall even in the region of antigen excess where the reaction is in the post zone. As can be seen from the constant A of the equation the rabbit serum contained a much greater quantity of antibody than did the human serum.

DISCUSSION

The sera obtained from rabbits injected with tyrosinase precipitate tyrosinase activity from solution quantitatively. Similarly the serum obtained from a human being injected with tyrosinase precipitated this enzyme completely from solution. Since the reaction between these sera and the antigen, tyrosinase, follows the same quantitative equation which has been applied to many other precipitin reactions, it is felt that the production of precipitating antibodies to the enzyme tyrosinase in rabbits and in human beings has been demonstrated. The enzyme tyrosinase is antigenic in rabbits and human beings, but the antibodies produced, though precipitating the enzyme do not neutralize its catalytic activity.

The results of immunization of the three rabbits were very uniform. However, in the case of human beings injected with tyrosinase there was wide variation in antigenic response (Table IV) and no correlation was evident between antibody production and dosage or extent of treatment. There was, moreover,

no correlation between the amount of circulating antibody at the time of blood sampling and the severity of local reaction at the site of injection or of systemic reaction to the injection of tyrosinase.

There is in the literature adequate evidence for the production of antibodies against only a few enzymes. Sumner and Kirk (10) prepared an immune rabbit serum which gave a precipitin reaction with jack bean urease at an antigen concentration of one part in 600,000. Campbell and Fourt (11) prepared an immune rabbit serum which gave a positive ring test at a catalase concentration of one part in 300,000. Seastone and Herriott (13) prepared an immune rabbit serum which gave a positive ring test at a pepsin concentration of one part in 100,000. They also prepared an immune serum which reacted with pepsinogen diluted one part to 1,000,000. During the present work immune rabbit serum has been prepared which gives a precipitin reaction at a tyrosinase concentration of one part in 500,000.

Sumner found that the specific precipitate exhibited 80 per cent of the catalytic activity of the urease present. The 20 per cent loss of activity was ascribed to the lowered degree of dispersion of the enzyme in the immune precipitate. Campbell and Fourt found that the immune precipitate between antibody and catalase was just as active as the enzyme alone would have been. Similar observations cannot be made in the case of pepsin since this enzyme is inactivated at the pH of serum, or in the case of pepsinogen which is an enzyme precursor. In the case of tyrosinase the immune precipitate exhibits all the catalytic activity of the precipitated enzyme.

Kirk found that the rabbit antibody to jack bean urease also was able to precipitate soy bean urease (14). Campbell and Fourt found that serum prepared from rabbits immunized to crystalline beef liver catalase also precipitated horse and dog liver catalase (11). Tria found that a similar rabbit antiserum to beef liver catalase likewise precipitated lamb liver catalase (12). Seastone and Herriott immunized rabbits to swine pepsin and found that the resultant antiserum precipitated beef and guinea pig pepsins as well as swine pepsin, but did not precipitate rabbit, shark, or chicken pepsins. In the present work it was shown that rabbit antiserum against *campestris* tyrosinase failed to precipitate the tyrosinase prepared from the *Lactarius piperatus* mushroom.

It is evident in the cases of urease, catalase, and tyrosinase that the portion of the molecule involved in catalytic reaction with the substrate is not identical with the portion of the molecule involved in the reaction with the serum antibody. The enzyme molecule is capable of reacting simultaneously with two different substances. Similar observations have been reported in the case of antipneumococcus horse serum proteins which can react simultaneously with pneumococcal capsular polysaccharide and with anti-horse chicken serum (15) and also in the case of antigenic antitoxins. We must conclude that when a

physiologically active substance is used as an antigen the production of antibodies must be tested by recognized immunological reactions such as the precipitin reaction, as well as by possible neutralization of the physiological activity of the antigen.

SUMMARY

1. Antibodies to the enzyme tyrosinase, obtained from the mushroom *Psalliota campestris*, have been produced in rabbits and human beings.

2. These antibody preparations, though precipitating the enzyme from solution, do not affect its catalytic activity.

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