CCXX. A STUDY OF THE IMMUNOLOGICAL ACTION OF TYROSINASE FROM MEALWORMS.

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THE problem of the production of melanin in mammals has of recent years been regarded mainly, in consequence of the work of Bruno Bloch, as the problem of determining the conditions of conversion of dihydroxyphenylalanine into melanin by a dopa-oxidase. The actual presence of free dihydroxyphenylalanine in human skin has, however, of late been considered doubtful; and on the other hand Schmalfuss *et al.* [1933] have established, by isolation and chemical identification, the normal occurrence of *l*-tyrosine in a free state in human skin, even before any *post mortem* autolysis. The significance of tyrosine and of the tyrosinase requisite to convert the amino-acid into a black pigment appears therefore to be enhanced as a possible explanation of the formation of melanin. The presence of tyrosinase in mammals is, as is well known, not easy to demonstrate. Even in lower animals, as for example in mealworms, the presence of some inhibitory factor along with the tyrosinase is evident. Mealworms, when freshly dissected, are not to any degree darkened, and injury to the tissues is necessary before the powerful melanogenic activity of the cells when ground up is revealed. Knowledge of any factor inhibitory to tyrosinase would consequently appear to be of some importance in the problem of the production of melanin. In order to study such factors, we have attempted to produce by immunisation specific antibodies to mealworm tyrosinase.

The attempt to produce an antityrosinase is not new; Gessard [1901], many years ago, reported success using tyrosinases of plant origin. Von Fürth [1912], on the other hand, reported negative results. We describe here our own observations.

EXPERIMENTAL.

Tyrosinase was prepared by grinding up 100 g. of mealworms in a litre of phosphate buffer mixture, p_H 8, and centrifuging the crude extract. The supernatant liquid was discarded so as to avoid the presence of somewhat toxic dissolved substances. The deposit was again shaken with the buffer mixture. Tyrosinase from the deposit was found to go into solution and give active preparations. This solution was then centrifuged and kept with thymol until required. Immediately before injection the solution was centrifuged and filtered through filter-paper to remove fat and suspended particles. After adjustment to make the solution approximately isotonic it was injected a few ml. at a time, four or five times, at intervals of four days. The injections were made slowly, and no appreciable disturbance was noted among the animals. Ten days after the last injection, blood was drawn and allowed to clot for the collection of serum.

It was noticed first of all, in the preliminary controls, that any fresh serum from an unprepared rabbit has in itself some inhibitory effect on the production of melanin when added to an active tyrosinase from mealworms. But unquestionably the serum from a prepared animal is more inhibitory for an active tyrosinase than any unprepared serum.

To the same quantity of tyrosinase (0.5 ml.) in a number of test-tubes increasing quantities of the sera under examination from 0.005 ml. to 0.5 ml. were added. The volume in all the tubes was equalised by addition of phosphate buffer, p_H 8, and the mixtures were left to stand for ten to twelve hours at room temperature with some thymol to prevent infection. Ten ml. of a solution of tyrosine in phosphate buffer, p_H 8, were then added to every tube and mixed. Under these conditions the production of melanin was very slow, spread over many days even in the controls, an effect which was found convenient. The differences were easily noted, and the slow uniform darkening was readily estimated quantitatively with the aid of a colorimeter, the pigment remaining in suspension in a completely colloidal state. The intermediate red colour was found not to interfere at all in these experiments. The intensity of the production of melanin could be plotted in arbitrary colorimetric units by comparison with the colour of the tube in which enzyme and tyrosine are present in phosphate buffer in the same concentrations but with no serum whatever. This colour was called arbitrarily 100 units. The results of such experiments are given in Table I. It was of interest to compare albino with coloured animals in this connection. Any difference between grey and albino rabbits appeared to be fortuitous. Similarly in a number of other experiments albinos gave no exceptional result. The purity of an albino strain is however difficult to ascertain with certainty.

Table I. *Showing the inhibition produced by normal serum and a serum of an animal (albino) injected with active tyrosinase.*

Both sera are inhibitory for an active tyrosinase but the normal in a lesser degree for equal concentrations.

Serum used	Amount of serum added to 0.5 ml. of tyrosinase ml.	Melanin produced in arbitrary units	Remarks
Normal	0	100	Tyrosinase active
	0.0054	97	? Inhibition
	0.012	101	No change
	0.0236	91	Inhibition
	0.059	79	Marked inhibition
Of an animal injected with active tyrosinase	0	100	Tyrosinase active
	0.0018	118	Slight activation
	0.0054	86	Inhibition
	0.0236	64	Marked inhibition
	0.059	77	Inhibition less marked when the quantity of added serum becomes excessive

The above results were those obtained when an active tyrosinase was injected into rabbits, and the sera were tested on an active tyrosinase.

It was noticed that the preparations of tyrosinase in phosphate buffer, p_H 8, prepared as described above were not very stable. Their activity decreased more quickly than that of the usual extracts in dilute ammonia, although chloroform or thymol was used as antiseptic. When, after an interval of six days, the experiments described in Table I were repeated using the same tyrosinase and sera, the tyrosinase in the controls was found to be very much weaker. The

results on addition of sera were however surprisingly different. The sera initially used which had proved to be inhibitory for strongly active tyrosinase preparations, at the same concentrations and under the same conditions, were now powerful activators of the feebly active tyrosinase preparations. There was, in fact, a complete inversion of the previous phenomenon. An example of this effect is recorded quantitatively in Table II.

Table II. *Showing the activation of a tyrosinase which has lost very much of its original activity when sera are added.*

The serum of an albino prepared by injections of active tyrosinase exhibits a greater power of reactivation for equal doses than a normal serum.

Serum used	Amount of serum added to 0.5 ml. of tyrosinase ml.	Melanin produced in arbitrary units	Remarks
Normal (albino)	0	100	Tyrosinase of a very poor activity
	0.0018	110	Tyrosinase somewhat activated
	0.0054	125	Activation
	0.012	127	Activation
	0.0236	170	Activation
	0.059	201	Marked activation
Normal (dark rabbit)	0	100	Tyrosinase of a very poor activity
	0.0018	100	No activation
	0.0054	114	Slight activation
	0.012	139	Activation
	0.0236	166	Marked activation
	0.059	140	Activation less pronounced when the quantity of serum is too large
Serum of an albino injected with active tyrosinase	0	100	Very poorly active tyrosinase
	0.0018	158	Considerable reactivation even with minute traces of serum
	0.0054	198	Very marked activation
	0.012	228	Very marked activation
	0.0236	252	Very marked activation
	0.059	229	Activation somewhat decreased when the amount of added serum is too large

The fact that the inversion was due simply to the feebleness of the tyrosinase was readily established by testing the same sera again with an active tyrosinase. Inhibition of the production of melanin was observed as previously. Repetition with a feebly active tyrosinase gave on the other hand an activation of the enzyme.

A preparation of tyrosinase (in the usual phosphate buffer, p_H 8), the activity of which had become so feeble that it might be considered practically inactive, was then injected into other rabbits. This preparation of tyrosinase had been kept with the antiseptics mentioned and in the concentrations described for the first experiment. It gave hardly a trace of melanin after two weeks. The serum prepared by use of a feebly active tyrosinase, however, appeared to be a powerful activating agent for any feeble tyrosinase; melanin was produced after four or five days and continued to accumulate. On the other hand the serum had no appreciable inhibitory effect on an active tyrosinase, such as is exercised by a normal serum, as already stated.

The reactivation of a practically inactive tyrosinase by a serum of a rabbit injected with a practically inactive tyrosinase is illustrated in Table III.

Table III. *Showing the reactivation of an inactive tyrosinase by a serum of an animal prepared by injections of the inactive enzyme.*

The inactivity of the enzyme was due to ageing.

Serum added	Amount of serum added to 0.5 ml. of tyrosinase ml.	Melanin produced (arbitrary units)	Remarks
Normal	Various quantities of normal serum could not reactivate the solution of tyrosinase which was practically inactive		
Serum of an animal prepared by injections of inactive tyrosinase	0.025	102	No reactivation
	0.05	107	Trace of activation
	0.1	113	Trace of activation
	0.2	158	Marked reactivation
	0.4	180	Marked reactivation

A final experiment was made with two rabbits which were injected with the following preparations in the order given: (1) a fairly active tyrosinase, (2) a feebly active tyrosinase, (3) and (4) a tyrosinase with activity between (1) and (2). The effects of the sera thus prepared varied with the amount added to the tyrosinase-tyrosine mixture. On testing with a feebly active enzyme the latter was activated by small or intermediate amounts of the serum but inhibited by larger amounts. This inhibition is probably due solely to the large amount of serum added and occurs with any serum, normal or prepared.

Certain additional observations were made. Heating the prepared sera to 55° for about a quarter of an hour was found to change their properties. The activating effect on a feebly active tyrosinase was diminished but the inhibitory effect of high concentrations of the serum was abolished (see Table IV). This inhibitory effect likewise disappeared when the serum was kept with thymol for some months, the reactivating power on feeble tyrosinase preparations, however, remaining unaffected.

Table IV. *Differences in the reactivations of a poorly active tyrosinase by a serum of an animal prepared by injections of active tyrosinase when the serum has been heated to 55° for a quarter of an hour.*

Amount of serum to 0.5 ml. of tyrosinase ml.	Melanin produced	
	A. Serum unheated	B. Serum heated to 55°
0.0018	158	102
0.054	198	113
0.012	228	129
0.0236	252	151
0.059	229	203

DISCUSSION.

It appears from these experiments that the response of a rabbit to injections of tyrosinase from mealworms is different when the injected enzyme is active and when it is feeble or even practically inactive. When the injected enzyme is very feeble, or inactive, a feebly active tyrosinase is reactivated by the serum, and no appreciable effect is observed when the serum is tried on an active tyrosinase.

When the injected enzyme is active, the effect of the serum is different according as the tyrosinase with which it is tested *in vitro* is active or feeble.

Such serum is inhibitory towards an active tyrosinase and reactivates a feeble one.

In attempting to elucidate the complex phenomena observed, it would appear to be helpful to postulate two factors in the usual solutions of tyrosinase, the enzyme itself and an antagonist to its activity.

Graubard [1933] came across inhibitors to tyrosinase in *Drosophila melanogaster*. He found that extracts of the larvae and pupae of this insect exhibited inhibitory phenomena, but by direct observation did not find the same phenomena with mealworms. We consider that the existence of an inhibitor in mealworm tyrosinase is suggested by our experiments in view of the following considerations.

Assuming the presence of an antagonistic factor in the usual tyrosinase preparation, both the enzyme itself and the antagonist may be able to develop specific antibodies when injected into the circulation of a rabbit. Suppose now the prepared serum be allowed to react with two sorts of tyrosinase, one active and the other feeble. A very active preparation of tyrosinase may be regarded as a mixture in which the amount of enzyme is relatively greater than that of the antagonistic factor. If it be assumed that in contact with the prepared serum, on account of the relatively greater masses of the reactants, the neutralisation of the active part of the enzyme preparation will be more readily achieved than any other combination then a reduction in activity of the enzyme by this neutralisation would be the expected result. This would explain the decrease in melanogenic power of the active enzyme which is illustrated in Table I. It may be noted that when the concentration of serum for the same quantity of tyrosinase increases (more than 0.0236 ml. in the particular experiment quoted) the amount of the second supposed antibody (specific to the inhibitory factor in the active tyrosinase preparation) would seem to be great enough to react with the antagonistic factor and to neutralise it partly, with a resulting decrease in the inhibitory effect on melanin formation observed with smaller concentrations of serum.

A tyrosinase which has become feebly active may be regarded as a solution in which the concentration of the enzyme is now relatively less than that of the antagonistic factor. The neutralisation of the latter by the serum will therefore, on account of the greater masses of the reactants, be more readily achieved than any other reaction. The result will be a development of melanogenic power following from the neutralisation of the antagonistic substance. This would explain the phenomena recorded in Table II.

When a solution of tyrosinase ages and becomes inactive, it would seem that all the enzyme has disappeared by autolysis or has otherwise undergone destruction. The antagonistic factor may nevertheless remain, and when injected, the solution would now be able to produce antibodies to the antagonistic factor only. (Numerous observations have established that such antibodies are the more stable with the lapse of time and are still active when the antibodies specific to the enzyme have become inactive.) This would explain the fact that the serum of an animal injected with inactive tyrosinase is a powerful reactivator of the feeble enzyme preparation, in contrast to the normal inhibitory effect of an unprepared serum on an active tyrosinase.

It is noteworthy that the fresh serum of an unprepared rabbit is a definite though weak inhibitor for an active tyrosinase, and a definite though weak reactivator for a feeble tyrosinase. The preparation of rabbits by injections of tyrosinase enhances greatly these latent properties. But whether this is due to the production of some substance already present in normal serum or to formation of a new inhibitor cannot be settled by these experiments.

Finally, apart from the particular interest attached to tyrosinase itself, it might be of use to test a number of enzymes for the production of effective activators in the way described for tyrosinase. The injection of the inhibitory constituent of an enzyme preparation might be a general method for the activation of an enzyme by means of the antibodies produced.

SUMMARY.

1. The injection of an active tyrosinase preparation into rabbits modifies the properties of the animal's serum.
2. The prepared serum, when the injected tyrosinase is active, has an inhibitory effect on a tyrosinase preparation if this is strongly active, but it reactivates a tyrosinase which is of poor or even very feeble activity.
3. The prepared serum, when the injected tyrosinase is very feeble or practically inactive, has no effect on an active tyrosinase. The same serum is able to reactivate a feeble or practically inactive tyrosinase.
4. A possible interpretation of the facts observed is that two antibodies are generated, specific to the enzyme and to a presumed antagonistic factor in the enzyme preparations; this suggestion is discussed.

REFERENCES.

- Gessard (1901). *Ann. Inst. Pasteur*, **15**, 539.
Graubard (1933). *J. Genetics*, **27**, 199.
Schmalfuss, Heider and Winkelmann (1933). *Biochem. Z.* **263**, 371.
Von Fürth (1912). *Probleme d. physiol. u. pathol. Chemie*, **1**, 537. (Leipzig.)