CATECHOLASE (TYROSINASE):* REVERSIBLE INACTIVATION AND REACTIVATION†

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Studies of the chemical properties of the enzyme, catecholase, have shown that this polyphenoloxidase consists of a copper-protein complex (1–3). Indications have also been obtained that the enzymic activity of catecholase is associated with the copper part of the molecule, since it has been found that the addition of certain reagents known to react with cupric ions will inhibit the enzymic activity of catecholase preparations (1, 2, 4, 5). We were interested to find out whether the enzyme, so inactivated, could be reactivated by the addition of certain metal ions. Cupric, ferric, cobaltous, and manganous ions were tested. In the following we wish to report on our findings.

EXPERIMENTAL

Preparation of Enzyme—The catecholase preparation, used in the inactivation studies, was obtained from the common mushroom, *Psalliota campestris*, according to the method of Tenenbaum and Jensen (6). The preparation had an activity of about 250 Adams and Nelson units per mg. of dry organic weight, a unit being defined as that amount of enzyme which will cause an oxygen uptake of 10 c.mm. per minute when the catechol-hydroquinone substrate (7) is used.

Inactivation Experiments—The first series of experiments was carried out in order to determine the effect of time on the inactivation of the enzyme by the addition of the reagents, potassium cyanide, sodium diethyldithiocarbamate, and potassium ethyl xanthate, and also the degree of reactivation following the addition of cupric ions to the inactivated enzyme solution.

The following solutions were prepared.

Solution 1—This was a solution containing enzyme, buffer (phosphate-citrate, 0.4 M, pH 6.5), and gelatin, made up to contain 1 ml. of the buffer and 5 mg. of gelatin for every 1.3 units of enzyme in the solution.

* The term catecholase is preferable to tyrosinase, but the latter, however, is still in general use.

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Solution 2—This solution prepared as described for Solution 1 contained in addition 0.1 mg. of inactivating agent for every 1.3 units of enzyme.

Solution 3—This solution was prepared as described for Solution 2, but contained in addition 0.5 mg. of cupric ion (added as a solution of cupric chloride) for every 1.3 units of enzyme. The copper salt was added immediately after the addition of the inactivating agent.

In determination of the enzymic activity, an aliquot containing 1.3 units of enzyme was withdrawn from the desired solution for the test.

After the solutions were prepared, they were allowed to stand at room temperature for 10 minutes and then tested. They were then placed in the refrigerator and tested 24 and 72 hours afterwards. The solution containing the active enzyme (Solution 1) was run as a control to determine the changes in the enzymic activity which occur on standing. The solution containing the enzyme and inactivating agent (Solution 2) was tested to determine the loss in potency due to inactivation. An equivalent portion of Solution 2 was tested at the same time in the presence of 0.5 mg. of cupric ion (added just before the determination) to determine the amount of reactivation which could be obtained after the enzyme had been in contact with the reagent for the given length of time. Solution 3, which contained enzyme, inactivating agent, and cupric ion, was tested to determine the protective influence of the cupric ion when it was added immediately after the addition of the inactivating agent.

A second series of experiments was carried out to ascertain the effect of other metal ions on the degree of reactivation of a previously inactivated enzyme solution. The metal ions besides cupric used in these experiments were ferric, manganous, and cobaltous and were added as solutions of their corresponding chlorides.

For this test, the enzyme solutions were prepared as described for Solution 2 and allowed to stand for 10 minutes. At the end of the 10 minute period 0.5 mg. of the desired ion (cupric, ferric, manganous, or cobaltous) was added and the solution tested. The oxygen uptake given by the inactivated enzyme solution to which the metal ion had been added was compared with the uptake obtained with an inactivated enzyme solution to which no metal ion had been added. A similar series of experiments was undertaken in which a 0.4 M acetate buffer (pH 5.9) was used in place of the phosphate-citrate buffer. Controls were run to determine whether the metal ions, themselves, would cause an oxygen uptake; none was observed when the quantities mentioned above were used.

DISCUSSION

As can be seen from Table I, it was found in agreement with other investigators (1, 2, 4, 5) that potassium cyanide, sodium diethyldithio-

carbamate, and potassium ethyl xanthate will inhibit catecholase activity. The addition of cupric ions will restore enzymic activity, the degree of reactivation apparently depending upon the time which elapses between the addition of the inactivating agent to the enzyme solution and the addition of the cupric ion. When cupric ions were added immediately following the addition of the inactivating agent, a small loss in activity occurred, which, however, did not increase upon standing. Apparently the addition of cupric ions prevents any further inactivation from taking place (see the last two columns in Table I). It appears that longer contact

TABLE I

Effect of Time on Reactivation of Inactivated Catecholase by Cupric Ions

0.1 mg. of the agent and 0.5 mg. of copper were used in these experiments. The oxygen uptake is given in c.mm. for a run of a half hour duration. The per cent reactivation is calculated on the basis of the amount of activity which still remains after inactivation, and not on the total amount of activity.

Inactivating agent	Time	Catecholase alone	Catecholase + inactivating agent		Catecholase + inactivating agent + cupric ions added just before test		Catecholase + inactivating agent + cupric ions added immediately after agent had been added	
		Oxygen uptake	Oxygen uptake	Per cent inactivation	Oxygen uptake	Per cent reactivation	Oxygen uptake	Per cent reactivation
Potassium	10 min.	424	3.5	99	304	72	335	79
cyanide	24 hrs.	420	5.0	98	236	56	342	81
	72 "	408	3.8	99	178	44	328	80
Sodium diethyl-	10 min.	402	11.4	97	280	70	375	93
dithio-	24 hrs.	396	0	100	100	25	358	90
carbamate	72 "	384	0	100	45	12	350	91
Potassium ethyl	10 min.	411	55	87	379	79	402	98
xanthate	24 hrs.	406	48	88	271	55	388	96
	72 "	392	44	89	228	47	370	94

of the inactivating reagent with the enzyme in the absence of cupric ion produces some irreversible inactivation.

The presence of gelatin in the reaction mixture did not exert any qualitative influence on the degree of inactivation and reactivation; however, the oxygen uptake was less in the absence of gelatin.

As can be seen from Table II, addition of cupric ions gives considerably more reactivation than any of the other metal ions employed. Ferric and cobaltous ions cause little reactivation except in the case in which the enzyme was inactivated by sodium diethyldithiocarbamate. Manganous ions produced some reactivation when the buffer medium was phosphate-citrate, but in the acetate buffer no reactivation was observed.

Potassium ethyl xanthate and sodium diethyldithiocarbamate gave about the same amount of inactivation in the presence of either phosphate-citrate or acetate buffer; however, potassium cyanide did not produce sufficient inactivation in the presence of acetate buffer and a larger quantity (0.3 mg.

TABLE II

Reactivation of Inactivated Catecholase by Various Metal Ions

E. = enzyme (catecholase), A. = inactivating agent. The oxygen uptake is given in c.mm. for a run of a half hour duration. The per cent reactivation is calculated on the basis of the amount of activity which still remains after inactivation, and not on the total amount of activity. When no reactivation or further inactivation occurs upon the addition of a metal ion, the per cent reactivation is given as zero.

Inactivating agent	Solution tested	In phosphate-citrate buffer			In acetate buffer		
		Oxygen uptake	Per cent inactivation	Per cent reactivation	Oxygen uptake	Per cent inactivation	Per cent reactivation
Sodium diethyldithiocarbamate, 0.1 mg.	E.	390			373		
	“ + A.	8	98		0	100	
	“ + “ + Cu^{++}	265		68	242		63
	“ + “ + Fe^{+++}	98		25	60		16
	“ + “ + Co^{++}	172		44	149		40
	“ + “ + Mn^{++}	209		54	0		0
Potassium ethyl xanthate, 0.1 mg.	“	393			349		
	“ + A.	51	87		35	90	
	“ + “ + Cu^{++}	323		69	252		62
	“ + “ + Fe^{+++}	51		0	16		0
	“ + “ + Co^{++}	45		0	103		19
	“ + “ + Mn^{++}	194		36	31		0
Potassium cyanide, 0.1 mg.	“	387			370		
	“ + A.	38	90		227	39	
	“ + “ + Cu^{++}	307		71	270		12
	“ + “ + Fe^{+++}	60		6	179		0
	“ + “ + Co^{++}	69		8	268		11
	“ + “ + Mn^{++}	172		35	261		9
Potassium cyanide, 0.3 mg.	“				368		
	“ + A.				27	93	
	“ + “ + Cu^{++}				261		64
	“ + “ + Fe^{+++}				74		13
	“ + “ + Co^{++}				85		16
	“ + “ + Mn^{++}				33		0

in place of 0.1 mg.) of potassium cyanide had to be used in the presence of that buffer. This is probably a pH effect (the pH of acetate buffer is 5.9 and that of phosphate-citrate is 6.5), since it was observed that less inactivation was produced by potassium cyanide when a phosphate-citrate buffer of lower pH was used.

From the observations reported in this paper and those of other investigators, it appears that the tyrosinase molecule may be considered as being composed of a more or less separable metallic component, copper, which is bound presumably by coordinate bonds to a protein of specific nature. The copper part of the molecule is probably the chief "anchoring" group. The "fixing" of the free bonds of the copper part of the enzyme by the addition of certain reagents leads to inactivation. The liberation of these bonds by the addition of certain metal ions, especially cupric, leads to reactivation. It is probable that the linkages between cupric ion and these reagents are less dissociable than the linkages between these reagents and the other metal ions. It is unlikely that any loss in activity and its reversal are due to first reduction and then oxidation of the cupric part of the molecule. The irreversible inactivation which appears on standing may be caused by the action of the reagents on the protein portion of the molecule.

SUMMARY

Potassium cyanide, sodium diethyldithiocarbamate, and potassium ethyl xanthate were found to inhibit catecholase activity.

Addition of various metal ions indicated that cupric ions gave the greatest percentage of reactivation.

The longer the period of time which elapses between the addition of inactivating agent and addition of cupric ions, the smaller the amount of reactivation.

The significance of these findings in relation to the chemical structure of the enzyme, catecholase, has been discussed.

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