XIII. IRON IN RELATION TO TYROSINASE.

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By some authors prominent rôles have been ascribed to certain metals in connection with the action of respiratory enzymes. Bertrand [1897] examined preparations of laccase, which he found to contain manganese in proportion to their oxidase activities. He found that addition of a manganese salt to a feebly active preparation increased its activity, and that manganese salts, especially of organic acids, were able to accelerate oxidation of quinol. He concluded that the enzyme laccase consisted essentially of manganese in organic combination, probably with protein. A laccase has, however, been prepared by Suminokura [1930] the action of which was not affected by manganese salts; and the oxidase of Wieland and Fischer [1926] from *Lactarius vellereus*, which had properties similar to those of laccase, contained no manganese.

Iron is another metal which acts as an activator in oxidative processes, and its importance in connection with the action of glutathione has been established by Harrison [1924] and others. Because ferrous salts will bring about an activation of hydrogen peroxide, and because haemoglobin will act as a peroxidase, it has often been thought that peroxidases necessarily contain iron. Willstätter and Pollinger [1923], however, have obtained a preparation of peroxidase which was very active yet contained only 0.04 % of iron; and the activity of their preparations was not proportional to the iron content. Shibata and Shibata [1930] have shown that a number of complex salts of metals such as cobalt, nickel, copper, zinc and cadmium imitate in certain respects the action of oxidases, and have put forward the view that oxidising enzymes are complex compounds containing metals such as these.

The rôle of iron in connection with tyrosinase is specially interesting. The distinctive action of tyrosinase is to convert certain monohydric to *o*-dihydric phenols, since its action on *o*-dihydric phenols can be readily imitated by other enzymic oxidative systems. It has, however, been shown by Martinon [1885] that hydrogen peroxide in conjunction with ferrous salt is able to oxidise phenol to catechol, and Raper (private communication) has now shown that tyrosine itself can be oxidised to 3 : 4-dihydroxyphenylalanine by similar means. Now, hydrogen peroxide is probably produced when tyrosinase brings about oxidation of *o*-dihydric phenols, and it was shown by Pugh [1929] that the action of tyrosinase on monohydric phenols is subject to an initial lag. The

possibility therefore presents itself that the action of tyrosinase preparations on monohydric phenols may be due to the presence of iron as an impurity, or, failing this, that tyrosinase itself contains iron as an essential constituent. Pinhey [1930] has already suggested that tyrosinase necessarily contains iron to account for the inhibition of its action by reagents such as cyanide.

In order to throw more light on these questions, tyrosinase preparations have been made from mealworms, and their properties investigated, using reagents free from detectable quantities of iron. Preparations have also been made from *Lactarius* fungi. The amount of iron in these various preparations has been estimated. The effect of addition of salts of various metals has also been investigated.

Narayanamurti and Ayyar [1929] found that the activity of tyrosinase preparations from *Dolichos lablab*, the field bean, was not decreased by electro-dialysis or ultrafiltration, and concluded that the enzyme does not depend for its action on iron or manganese salts. Tyrosinase from potatoes was found by Raper and Wormall [1923], and from mealworms by Pugh [1930], to lose considerably in activity when subjected to ordinary dialysis, but still retained some activity. As part of the present work the cause of loss in activity on ordinary dialysis has therefore been investigated.

EXPERIMENTAL.

Throughout the present work, the following purified reagents were used: freshly glass-distilled water, "iron-free" phosphate buffer as described by Elvehjem [1930], *p*-cresol twice distilled in carbon dioxide, and tyrosine recrystallised twice from glass-distilled water.

Iron estimations.

The estimations of iron were carried out as follows, using the method of Mariott and Wolf [1906] and of Smirk [1928] for the colorimetric estimation. A platinum crucible was used in a first series of experiments, and porcelain in the remainder, without any observable difference. 10 cc. of the enzyme preparation were evaporated to dryness, and ignited to constant weight, 1 cc. nitric acid (50 cc. concentrated nitric acid per litre) was then added, and evaporated to dryness. The residue was dissolved in 1 cc. 10 % HCl, filtered with 5–10 cc. water through a small disc of filter-paper, and made up to a given volume, usually 25 cc. In the later (*Lactarius*) estimations, filtration was omitted, because it was found that ammonium thiocyanate solution caused coloration of even best quality "ashless" filter-paper. The standard iron solution for comparison was made as follows. 0.7 g. ferrous sulphate was dissolved in water, 1.1 cc. pure concentrated sulphuric acid and 0.1 cc. Merck "perhydrol" added, the liquid boiled to remove excess hydrogen peroxide, cooled, and made up to 100 cc. This standard was suitably diluted, usually 1 in 200, for estimations. To 2 cc. liquid to be estimated were added 2 cc. distilled acetone

and 1 cc. 24 % ammonium thiocyanate solution, with shaking and cooling. The colours were compared in a Briggs colorimeter.

Control estimations were carried out, using the usual procedure and reagents, but without any enzyme preparation; filtered through ordinary filter-paper, 0.0044 calculated as mg. Fe per cc. preparation; through "ashless" filter-paper, 0.0059; unfiltered, 0.002.

A preparation of tyrosinase from mealworms was made by the centrifuge method described by Pugh [1930], except that contamination with extraneous iron was carefully avoided, and the mealworms were first of all starved for a few days¹. Iron was estimated in the preparation both before dialysis and after dialysis for 2 days through a collodion sac into 25 volumes of water, the iron in the outer liquid being also estimated. For each experiment 10 cc. preparation were used.

Whole preparation	0.045; 0.047	mg. Fe per cc. preparation
Dialysed residue	0.03; 0.035	" " "
Dialysed outer liquid	0.01; 0.01	" " "

The outer liquids contained organic matter which had apparently passed through the collodion sac. The preparation had been so well washed that it is improbable that the iron in the outer liquid on dialysis represents altogether inorganic iron salts. The residue, however, definitely contains non-diffusible iron; whether this is a component of organic molecules or is adsorbed inorganic iron is as yet undetermined.

Lactarius rufus fungi (Cannock Chase) were crushed, pressed between porcelain plates, and the juice collected and allowed to stand overnight, after which the sediment was centrifuged off. To 30 cc. juice was added alcohol to 40 %. After a few hours the precipitate was centrifuged off, dissolved in 15 cc. water, and 10 cc. of the solution were taken for iron estimation (*A*). To the alcoholic liquid was added alcohol to 60 %, and the precipitate similarly treated (*B*). Alcohol was then added to 80 % similarly (*C*). Iron was also estimated in the final 80 % alcoholic liquid, and in whole juice. Preparation of *A* was carried out a second time.

Whole juice	...	0.021	mg. Fe per cc. juice
<i>A</i>	...	0.0031	" " "
<i>B</i>	...	0.0044	" " "
<i>C</i>	...	0.0096	" " "
Alcoholic liquid		0.010	" " "
Second <i>A</i>	...	0.0028	" " "

Bertrand and Muttermilch [1907] showed that tyrosinase acts on tyrosine but not on guaiacol, while laccase acts on guaiacol but not on tyrosine. The tyrosinase and laccase activities of the *Lactarius* preparations were therefore determined by noting the time required for first appearance of colour when

¹ Note on starved mealworms. The action on catechol of a preparation made from starved mealworms was found to be retarded and never accelerated by hydrogen peroxide. The slight initial acceleration noted by Pugh [1930] was apparently due to a trace of peroxidase, which is present in the foodstuff. Acceleration of action on tyrosine and *p*-cresol was unaltered.

0.5 cc. *A*, *B* or *C* was added to (1) 2 cc. phosphate buffer p_H 6.5, (2) 2 cc. saturated tyrosine in phosphate buffer p_H 6.5, (3) 2 cc. 0.2 % guaiacol in phosphate buffer p_H 6.5.

	<i>A</i>	<i>B</i>	<i>C</i>
(2)	10 mins.	20 mins.	Slight next day
(3)	4 „	Slight next day	Slight next day

The amount of iron in these *Lactarius* preparations is thus very small, and no direct relationship to oxidase activity is apparent.

Lactarius rufus fungi (Delamere) and *Lactarius turpis* fungi (Delamere) were treated similarly, and similar iron estimations carried out. 40 cc. *rufus* juice were used, 60 cc. *turpis* juice. A fresh set of reagents was used, and a fresh control estimation carried out, which yielded the figure 0.0005.

<i>L. rufus</i>				<i>L. turpis</i>			
Whole juice ...	0.010 mg. Fe per cc. juice			Whole juice ...	0.012 mg. Fe per cc. juice		
<i>A</i>	0.005 „ „ „			<i>A</i>	0.006 „ „ „		
<i>B</i>	0.0025 „ „ „			<i>B</i>	0.003 „ „ „		
<i>C</i>	0.0025 „ „ „			<i>C</i>	0.005 „ „ „		
Alcoholic liquid	0.003 „ „ „			Alcoholic liquid	0.005 „ „ „		

The tyrosinase and laccase activities of the preparations were determined as before.

<i>L. rufus</i> :	Whole juice	<i>A</i>	<i>B</i>	<i>C</i>
(2)	3 mins.	7 mins.	5 mins.	Overnight
(3)	At once	1 min.	8 mins.	25 mins.
<i>L. turpis</i> :				
(2)	At once	3 mins.	50 mins.	Overnight
(3)	25 mins.	Overnight	Nil in two days	Nil in two days

Note. Whole juice was half as concentrated as the other preparations.

Again no relationship between iron content and oxidase activity is apparent, and it is evident that the oxidases occur in the juices contaminated with impurities which do contain iron.

In order to calculate the percentage content of iron, 10 cc. of each of these preparations were evaporated to dryness on the water-bath, dried in an oven at 110°, and the dry residue weighed. The smallest percentage content of iron was that in the alcoholic liquid residues, which contained only 0.01 % of iron. The smallness of this percentage is accounted for by the presence of substances which neither contain iron nor are precipitated by alcohol. The smallest percentage in an active tyrosinase preparation was 0.07 % in *L. turpis A*.

Effect of metallic salts.

The precautions taken to avoid contamination with extraneous iron in no wise prevented tyrosinase preparations from mealworms or fungi from acting on the monohydric phenols, tyrosine and *p*-cresol. Also, action on these substrates was still hastened by hydrogen peroxide. With precautions to avoid iron, however, the coloration of tyrosine and of *p*-cresol solutions by hydrogen

peroxide without enzyme reported by Pugh [1930] was observed only after many days with *p*-cresol and not at all with tyrosine, and must therefore have been due to contamination with iron salts.

The experiments next to be described were carried out to find the effect of addition of metallic salts on the action of tyrosinase on monohydric phenols in buffered solution. The coloration of tyrosine and of *p*-cresol by tyrosinase was hastened by addition of either ferrous salt or hydrogen peroxide in small quantities. With *p*-cresol, however, the colour in presence of iron was yellow, in presence of hydrogen peroxide, pink. The tyrosine tubes all acquired first of all a reddish colour, but later, when blackening occurred, those containing iron became more blackish than those without it; this is probably due to the direct action of the iron salt on the *o*-dihydric phenol. In each series tube 1 contained 2 cc. phosphate buffer p_H 7.0. In the first series tubes 2–26 contained 2 cc. saturated tyrosine solution in phosphate buffer p_H 7.0. In the second series tubes 2–26 contained 2 cc. 0.2 % *p*-cresol solution in phosphate buffer p_H 7.0. Hydrogen peroxide, in the form of Merck "perhydrol," was added as stated in the following Table, and iron in the form of ferrous ammonium sulphate solution. 0.5 cc. tyrosinase from mealworms, dialysed into distilled water, was added, diluted 1 in 10 for action on *p*-cresol. The contents of each tube were made up to 3 cc. with water. The time of onset of coloration was noted.

Tubes	0.5 cc. "perhydrol" %	Mg. Fe	First series (tyrosine). Time	Second series (<i>p</i> -cresol). Time
2	Nil	Nil	6 hrs.	About 1 hr.
3	0.5	"	15 mins.	<5 mins.
4	0.05	"	2 hrs.	15–20 "
5	0.005	"	4 "	30 "
6	0.0005	"	6 "	Nearly 1 hour
7	Nil	0.5	25 mins.	10 mins.
8	"	0.05	3 hrs.	25 "
9	"	0.005	5 "	40 "
10	"	0.0005	6 "	Nearly 1 hr.
11	0.5	0.05	15 mins.	<5 mins.
12	0.05	"	2 hrs.	15–20 "
13	0.005	"	3 "	20 "
14	0.0005	"	3 "	15 "
15	0.5	0.005	15 mins.	<5 "
16	0.05	"	2 hrs.	15–20 "
17	0.005	"	4 "	30 "
18	0.0005	"	5 "	40 "
19	0.05	0.5	17 mins.	5–10 "
20	"	0.05	2 hrs.	15 "
21	"	0.005	2 "	15 "
22	"	0.0005	2 "	15 "
23	0.005	0.5	20 mins.	10 "
24	"	0.05	3 hrs.	20 "
25	"	0.005	4 "	30 "
26	"	0.0005	4 "	30 "

As controls both series were repeated with omission of tyrosinase, and tubes in which colour appeared were repeated also without either tyrosine or *p*-cresol. In tubes 19 and 23 yellow colour appeared within 5 minutes, even in absence of tyrosine or *p*-cresol, and was evidently due to oxidation of ferrous to ferric

iron by hydrogen peroxide. After three days colour had appeared only in control tubes 11, 12, 15, 19, 20 and 23, which were very slightly greyish in series I, and slightly yellowish-pink in series II.

Salts tested in this way were (besides ferrous ammonium sulphate) ferrous sulphate, manganese sulphate, copper sulphate, cobalt chloride, calcium chloride, zinc chloride and cadmium chloride. The quantities used were approximately equimolecular with the quantities of ferrous ammonium sulphate stated above. Only the iron salts affected the rate of coloration of tyrosine and *p*-cresol by tyrosinase, with the possible doubtful exceptions of copper and cobalt. Ferrous sulphate in larger quantity (1 mg.), as used by Gortner [1910] and by Onslow [1917], was found to retard the action of tyrosinase, as reported by them.

Addition of the above salts at a dilution of about $M/5000$ to the bottles of Barcroft micro-respirometers in which tyrosinase was acting on tyrosine, *p*-cresol, or catechol, had no significant effect on the oxygen absorptions. The following mixtures were placed in the bottles of the first respirometer. Left: 1 cc. saturated tyrosine solution, or 0.5 % *p*-cresol or catechol solution in phosphate buffer p_H 6.5; 1 cc. buffer; 0.5 cc. water; 0.5 cc. tyrosinase from mealworms (dialysed against phosphate buffer p_H 8.0) diluted 1 in 8 for action on *p*-cresol and 1 in 20 for action on catechol. Right: 2 cc. buffer; 0.5 cc. water; 0.5 cc. enzyme preparation. In the other respirometers, 0.5 cc. water was replaced by 0.5 cc. metallic salt solution in both bottles. To each bottle a little solid thymol was added. Concentrated caustic potash was placed in the small tube fused into each bottle.

					H ₂ O	Fe	Cu	Co	Mn	Ca
Tyrosine.	mm. ³ O ₂ absorbed after 1 hr.				36	47	33	26	29	25
<i>p</i> -Cresol.	" " " "	$\frac{1}{2}$ hr.			249	221	203	223	233	205
Catechol.	" " " "	1 hr.			177	183	153	156	182	152

The effect of the ferrous salt was evidently too small to be measured by these means.

The effect of addition of metallic salts was also investigated using tyrosinase prepared from *L. turpis* juice as follows. The juice was treated with alcohol to 40 % and centrifuged; after addition of alcohol to 60 % the liquid was again centrifuged, and this last residue was dissolved in water to half the volume of the original juice. The juice acted not only on tyrosine but also quickly on guaiacol. The tyrosinase preparation did not act on guaiacol even after many days. In the following tests, tube 1 in every series contained 2 cc. phosphate buffer p_H 6.5. In series I and II tubes 2–10 contained 2 cc. saturated tyrosine solution in phosphate buffer p_H 6.5. In series III and IV tubes 2–10 contained 2 cc. 0.2 % *p*-cresol solution in phosphate buffer p_H 6.5. In series V tubes 1–10 contained 2 cc. phosphate buffer. Hydrogen peroxide and iron (as FeSO₄) were added to each series as stated in the following Table. 0.5 cc. enzyme preparation was added to series I and III, diluted 1 in 3 for

action on *p*-cresol. The contents of each tube were made up to 3 cc. with water. The time required for commencement of coloration was noted.

Tubes	0.5 cc. "perhydrol" %	Mg. Fe	Series I (mins.)	Series III (mins.)
2	Nil	Nil	70	35
3	0.05	"	12	10
4	0.005	"	25	20
5	Nil	0.05	15	8
6	"	0.005	16	10
7	0.05	0.05	10	5
8	"	0.005	12	8
9	0.005	0.05	12	8
10	"	0.005	13	10

Next day, in series II, IV and V, tubes 5, 7 and 9 were yellowish. In series IV, in addition, tubes 7 and 8 were pinkish.

Tubes 5-10 of all the above series were also repeated, using *A* manganese sulphate, *B* copper sulphate, *C* cobalt chloride, in place of ferrous sulphate, quantities being approximately equimolecular with the quantities of ferrous sulphate stated above.

Tubes	<i>A</i> I (mins.)	<i>A</i> III (mins.)	<i>B</i> I (mins.)	<i>B</i> III (mins.)	<i>C</i> I (mins.)	<i>C</i> III (mins.)
5	80	35	35	35	75	35
6	90	35	70	35	70	35
7	12	10	30	12	8	10
8	12	12	12	15	10	20
9	25	20	30	23	25	25
10	25	20	20	25	25	25

The colour produced in series *B* I tubes 5, 6, 7 and 9 was not reddish but greenish-grey. In series *B* II, IV and V, tubes 5 and 9 were bluish, and, in addition, in series II tube 7 became grey; series IV tube 7 orange-pink, tube 8 pink; series V tube 7 was bluish. No essential difference was noted between the use of tyrosinase from mealworms and from fungi.

Using as metallic salts ferrous sulphate and manganese sulphate, series I and III were also repeated with crude *L. turpis* juice in place of tyrosinase preparation.

Tubes	Iron I (mins.)	Iron III (mins.)	Manganese I (mins.)	Manganese III (mins.)
2	30	35	—	—
3	4	5	—	—
4	10	15	—	—
5	5	5	30	35
6	8	8	33	35
7	1	3	4	5
8	6	5	4	5
9	1½	4	10	15
10	7	6	10	15

Although the first appearance of colour was not hastened, the colour produced from *p*-cresol by the crude fungus juice deepened more rapidly in presence of manganese salt than in its absence; this may possibly be connected with the presence of laccase in the crude juice.

The coloration of tyrosine and of *p*-cresol by hydrogen peroxide with ferrous salt was evident in the experiments described above. The following tests were carried out to find the smallest amount of ferrous salt necessary to make this reaction apparent. In one series of test-tubes were placed 2 cc. water, in another series 2 cc. saturated tyrosine in water. To the first tube of either series was added 0.5 cc. containing 0.5 mg. Fe in the form of ferrous sulphate, to the second pair 0.05 mg. Fe, to the third pair 0.005 mg. Fe, and so on, finally a pair with water in place of iron salt solution. To each tube was added 0.25 cc. 1 % hydrogen peroxide. The first three tubes without tyrosine showed a yellow colour owing to the action of hydrogen peroxide on the ferrous salt. The first and second tyrosine tubes became rapidly green, then brown. The green colour first produced is evidently due to the reaction of *o*-dihydric phenol with ferric salt. The third tyrosine tube became pinkish, later brown; this corresponds to an added iron content of 0.005 mg. Effect in further tubes was not immediate, but after several days effect was detectable as far as an added iron content of 0.00005 mg. The above brown colours in presence of hydrogen peroxide tended to fade. The above tests carried out on tyrosine in phosphate buffer p_H 6.5 instead of aqueous solution of tyrosine yielded a detectable effect only to an added iron content of 0.005 mg., iron being precipitated as phosphate.

Attempts were made to imitate the action of tyrosinase by means of mixtures of peroxidase from horse-radish with hydrogen peroxide and ferrous salt. To 2 cc. saturated tyrosine in phosphate buffer p_H 7.0 were added 1 cc. 0.05 % hydrogen peroxide, 2 cc. peroxidase preparation, and 1 cc. ferrous sulphate solution containing 0.1 mg. Fe. A comparison tube contained buffer in place of tyrosine solution; in this a slight yellow colour appeared. The liquid in the tyrosine tube became slightly yellow, then pink, but the pink colour faded, and no darkening was noticeable. Variations were made in the quantities used, and aqueous solution was tried in place of buffer, but darkening was observed only when it took place likewise in absence of peroxidase. Continuous catalytic action, such as is shown by tyrosinase, was not successfully imitated. In the preceding experiment the iron salt solution was also replaced by 0.5 cc. 0.002 % catechol in buffer. A deep pink colour appeared, which faded without darkening. This pink colour is evidently due to the reaction shown by Pugh [1930] to occur between tyrosine and *ortho*quinone, produced from catechol by peroxidase with hydrogen peroxide.

A few observations have been made concerning the action of iron salts on certain phenols. It is well known that with dilute ferric chloride solution phenol or *p*-cresol gives a violet colour and *o*-dihydric phenols give a green colour followed by darkening. If, however, a drop of 1 % hydrogen peroxide is added to the violet liquid, after a few seconds' delay it becomes green. This appears to indicate that not only ferrous but also ferric iron may be able to bring about the oxidation of these monohydric phenols to *o*-dihydric phenols by hydrogen peroxide. Ferrous sulphate solution and hydrogen peroxide added to phenol or *p*-cresol solution produce a green colour forthwith. Ferrous iron is converted

into ferric iron by hydrogen peroxide, but ferric chloride alone does not oxidise phenol or *p*-cresol to *o*-dihydric phenol, since solutions of phenol or *p*-cresol with ferric chloride are violet, and do not become green when left to stand. Ferrous sulphate and hydrogen peroxide added to guaiacol solution in water produce a green colour followed by darkening.

Loss in activity on dialysis.

The loss in activity of tyrosinase from mealworms on dialysis was investigated as follows. Dialysis in a collodion sac was tried first into ammoniacal water; this lost in alkalinity, and the preparation lost in activity, even when its alkalinity was restored. Dialysis was then tried into dilute sodium hydroxide solution, p_H 8.0; this also lost in alkalinity, presumably owing to the buffering action of the proteins in the preparation. Dialysis was next tried into phosphate buffer of various p_H values. At p_H 8.5 there was no appreciable loss in activity on dialysis. In these experiments the ammoniacal extract of tyrosinase from mealworms, prepared by the centrifuge method, was centrifuged, and 10 cc. were dialysed into 500 cc. phosphate buffer at *A* p_H 7.0, *B* p_H 8.0, *C* p_H 8.5; *D* was kept undialysed. After two days each was made up to 12 cc. and centrifuged. There was much precipitate in *A*. Each was diluted 1 in 10 for action on *p*-cresol, 1 in 100 for action on catechol. 0.5 cc. was added to 2 cc. 0.2 % *p*-cresol or catechol at p_H 6.5, and to buffer for comparison. Times required for appearance of colour were as follows:

<i>A</i>	<i>p</i> -Cresol	10 mins.	Catechol	1 min.
<i>B</i>	"	5 "	"	$\frac{1}{2}$ "
<i>C</i>	"	4 "	"	20 secs.
<i>D</i>	"	3 $\frac{1}{2}$ "	"	20 "

Loss in activity on dialysis is therefore probably due to some irreversible change resulting from loss in alkalinity.

The presence of phosphate was found not to affect the time required for appearance of colour by the action of tyrosinase on its substrates, but it did affect the final intensity of colour. The following mixtures were placed in test-tubes (1) 4 cc. water, (2) 2 cc. water and 2 cc. phosphate buffer of the same p_H as the water, (3) 2 cc. water and 2 cc. 0.2 % *p*-cresol in water, (4) 2 cc. water and 2 cc. 0.2 % *p*-cresol in the same phosphate buffer. To each was added 0.5 cc. tyrosinase from mealworms dialysed into water made faintly alkaline with ammonia, then centrifuged. In both (3) and (4) a yellow colour appeared after 18 mins. When *p*-cresol was replaced by catechol, the enzyme preparation being diluted 1 in 10, colour appeared in both (3) and (4) after 3 mins. Next day, however, coloration was much further advanced in the tubes containing phosphate buffer than in those without it. This is apparently due to the maintenance of a p_H near the optimum for tyrosinase by the buffering action of the phosphate.

DISCUSSION.

An attempt has been made to discover whether the action of tyrosinase depends in any way on the presence of iron. The iron estimations described in this paper have not revealed any relationship between the iron content and the oxidase activity of various preparations of tyrosinase. They therefore provide no evidence that tyrosinase necessarily contains iron. Tyrosinase in the fungus juices used was shown to be contaminated with impurities which do contain iron. If the smallest iron content found in an active tyrosinase preparation is used as a basis to calculate the minimum molecular weight of tyrosinase, on the assumption that each molecule contains one atom of iron, the result, about 100,000, is by no means more than is to be expected. It was shown that a tyrosinase preparation from mealworms contains non-diffusible iron. This may or may not be part of the tyrosinase. There is therefore no evidence that tyrosinase does not necessarily contain iron. The limits of direct estimability of iron have been approached, and it is evident that direct estimation of iron in tyrosinase preparations will not provide an answer to the problem.

Small quantities of ferrous salts hasten the appearance of colour in mixtures of tyrosinase with the monohydric phenols, tyrosine or *p*-cresol, although larger quantities inhibit the action of the enzyme. Since hydrogen peroxide with ferrous salt is able to oxidise certain monohydric to *o*-dihydric phenols, it is reasonable to suppose that the activating effect of ferrous salts on the action of tyrosinase depends on this reaction. This would involve the assumption that hydrogen peroxide is produced by the action of tyrosinase on its substrate, which is very probable; the apparent hastening of the action of tyrosinase on monohydric phenols by ferrous salts, in fact, appears to be indirect evidence of the production of hydrogen peroxide.

The essential action of tyrosinase is to bring about the oxidation of certain monohydric to the corresponding *o*-dihydric phenols, and this action is hastened by some product or products of action of tyrosinase. Nevertheless, the fact that hydrogen peroxide with ferrous salt is able to imitate the characteristic action of tyrosinase is no real evidence that tyrosinase contains iron. The action of this reagent (Fenton's reagent) as an oxidising agent is well known. It is less specific than the action of tyrosinase. In particular, tyrosinase does not cause oxidation of guaiacol, but there is indication that guaiacol may be oxidised by hydrogen peroxide with ferrous salt to an *o*-dihydric phenol.

It is interesting to compare the smallest concentration of iron which, as ferrous salt with hydrogen peroxide, will bring about immediate coloration of tyrosine with the amount of iron in a similar tyrosinase preparation. The smallest effective concentration of inorganic iron was found to be about 0.002 mg. per cc., which is of the same order as the amount of iron in a similarly effective tyrosinase preparation, *L. turpis* A, containing the smallest percentage of iron of any of the present preparations. More highly purified preparations might contain less iron, since the crude tyrosinase is associated

with impurities which contain iron, but on the other hand it is probable, as Shibata and others have suggested, that the activity of iron in appropriate organic combination would be greater than that of inorganic iron. It is by no means impossible that the activity of tyrosinase is due to iron in the tyrosinase molecule. It is evident, however, that other methods must be devised before the essential nature of tyrosinase can be elucidated.

The effect of iron in causing coloration of phenols has been known for many years. Walter [1898] showed that pure solid phenol remains uncoloured for months in paraffin-coated vessels; whereas addition of a little ferrous sulphate produced coloration in three days, and of hydrogen peroxide in addition, in two days. The action of iron without hydrogen peroxide was ascribed to the formation of hydrogen peroxide from air and moisture by the iron.

The mechanism by which iron salts bring about oxidations by means of hydrogen peroxide has been a subject of some speculation. Although hydrogen peroxide oxidises ferrous to ferric salt, ferric salt alone does not bring about oxidation of monohydric phenols. Some indication is, however, given in this paper that both ferrous and ferric iron will catalyse the oxidation of monohydric phenols by hydrogen peroxide. This would be in agreement with the theory put forward by Mummery [1913] of the oxidising action of iron salts with hydrogen peroxide. He supposed that hydrogen peroxide forms with iron salts a ferrous perhydrol formulated $\text{FeX}(\text{O.OH})$, or a ferric perhydrol $\text{FeX}_2(\text{O.OH})$, both of these being able to act as oxidising agents, with reformation of ferrous or ferric salt; the former, however, being much the more effective oxidising agent.

Inorganic iron salts do not appear to play any essential part in the ordinary action of tyrosinase. The loss in activity of tyrosinase preparations on ordinary dialysis can be prevented by carrying out the dialysis into alkaline buffer solution, and is therefore due apparently to some irreversible change resulting from loss in alkalinity, and not to loss in iron or any other specific salts.

SUMMARY.

1. The iron contents of some tyrosinase preparations from mealworms and from fungi have been estimated. Results of such estimations, however, can provide no conclusive evidence with regard to the nature of tyrosinase.
2. The coloration of tyrosine and of *p*-cresol solutions with tyrosinase is hastened by ferrous salts in small quantity. This provides indirect evidence that hydrogen peroxide is produced during the action of tyrosinase.
3. The oxidation of monohydric to *o*-dihydric phenols by hydrogen peroxide with ferrous salt is less specific than the action of tyrosinase, and provides only a very imperfect imitation of the action of tyrosinase.
4. Means must be devised along other lines to determine whether tyrosinase contains iron or not.
5. The loss in activity of tyrosinase from mealworms on dialysis may be

prevented by carrying out the dialysis into alkaline buffer solution, and is therefore not due to loss of iron or other specific salts.

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