XIII. THE TYROSINASE-TYROSINE REACTION. II. THE THEORY OF DEAMINATION.

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THE action of tyrosinase on tyrosine by which the latter is converted into pigmented substances has been studied by many, and various theories have been evolved to explain the mechanism of the oxidation processes. All these theories, however, are based on the assumption that deamination of the tyrosine takes place. Chodat and Schweizer [1913] investigated the action of tyrosinase on glycine and stated that among the end-products were formaldehyde, CO_2 and NH_3 . Bach [1914] applied this idea of deamination of the amino-acid to the action of the enzyme or mixture of enzymes on tyrosine and suggested that the first action of tyrosinase on tyrosine could be represented as

This reaction Bach attributed to an amino-acidase which splits up the tyrosine molecule into substances which are easily oxidised by the other enzyme component of tyrosinase, the phenolase (or peroxidase + H_2O_2). This action of the amino-acidase may be represented according to Bach as analogous to Strecker's reaction and results in deamination accompanied by or followed by decarboxylation.

Similar views have been expressed by other workers. Folpners [1916] carried out experiments which he interpreted as indicating that tyrosinase consists of two enzymes, a deaminising enzyme which converts tyrosine into *p*-hydroxyphenylacetaldehyde and a second enzyme which causes the further oxidation of the aldehyde (or its condensation product with ammonia) to form the black pigment melanin. Onslow [1923] accepts the view of Bach that the first step is the deamination of the tyrosine, followed by the further oxidation of these products and condensation with NH_3 to form melanin, and suggests that the reactions proceed as in equations (1) and (2). Haehn [1919] considers that tyrosinase consists of at least three different enzymes, one of which is a deaminase. Keeble, Armstrong and Jones [1913] have used the deamination theory in explaining pigment formation in plants. Abderhalden and Sickel

[1923] consider that the first stage in the tyrosinase-tyrosine reaction is a hydrolytic deamination accompanied by loss of CO_2 . Thus all these theories involve the preliminary deamination of the tyrosine to form products which are subsequently oxidised (in some cases with condensation with NH_3) to form melanin. The experimental evidence supporting this view of deamination does not appear to be very weighty and the theory rests on a few isolated observations. Chodat and Schweizer [1913] identified H.CHO and NH_3 as products of the action of tyrosinase on glycine, and state that benzaldehyde is formed from phenylglycine, and acetaldehyde from alanine by the action of tyrosinase. These experiments have been repeated by Happold and Raper with negative results [1925]. Bach [1914] to support his theory of deamination attempted to show that *p*-hydroxyphenylacetaldehyde in the presence of the requisite amount of ammonia is oxidised by tyrosinase to the same end-products as those obtained in the tyrosinase-tyrosine reaction. In view of the difficulty in obtaining this aldehyde, Bach substituted the next lower member of this series, *p*-hydroxybenzaldehyde and claimed that by the action of peroxidase and H_2O_2 on this aldehyde in the presence of ammonia, colour changes similar to those of the tyrosinase-tyrosine reaction were obtained. These results, the rapid reddening of the solution followed by darkening to a violet-black colour, were not obtained when a phenolase was substituted for the peroxidase and H_2O_2 system, and this is attributed to the greater inhibitory action of NH_3 on phenolase. The only other evidence in support of the deamination theory appears to be that offered by Folpmers [1916], who isolated the *p*-nitrophenylhydrazone of benzaldehyde from a reacting mixture containing phenylglycine and plant juices, and an analogous hydrazone in extremely small quantities (too small for identification) when tyrosine was used.

The tyrosinase-tyrosine reaction may be represented as taking place in the following manner [Raper and Wormald, 1923]:

If the reaction is carried out in slightly acid solution (p_{H} 6.0), the red substance is the main pigmented product formed during the first 6 or 8 hours, during which time from 50 % to 75 % of the tyrosine may be oxidised. This red substance is the first visible sign of oxidation although it may not be the first intermediate product in the oxidation process. On the assumption that deamination takes place, according to equation (1), the oxidation of tyrosine by tyrosinase will result in the liberation of an equivalent amount of NH_3 which should pass into solution. If no increase in the ammonia content of the reacting mixture takes place as the reaction proceeds, such a result would admit of two possibilities: (a) that no deamination of tyrosine occurs or (b) that deamination takes place but is immediately followed by the recombination of the NH_3 with the products of deamination (and decarboxylation) to form

a substance which in this case must be the red pigment. The second conjecture appears improbable and would involve the assumption that the deaminised products in the presence of NH_3 are rapidly converted by tyrosinase into the red pigment. That this assumption is incorrect will be shown later and thus the non-liberation of NH_3 as the reaction proceeds is not in favour of the theory of deamination.

In the present work, tyrosine solutions buffered at p_{H} 6.0 were acted upon by tyrosinase from potatoes and the ammonia content of the reacting mixture determined at intervals during the oxidation of at least two-thirds of the tyrosine present. No increase in the ammonia content of these solutions took place at any time, although the amount of NH_3 which should have been liberated according to equation (1) is quite appreciable. Additions to special samples of the solution of one-quarter of the amount of NH_3 which should theoretically be produced, calculated from the amount of tyrosine oxidised, resulted in these added quantities being estimated almost quantitatively, and thus satisfactory conditions appear to have been obtained. These ammonia estimations were carried out by the permutite filtration method [Whitehorn, 1923] for this method is of special application to slightly acid solutions. The ordinary methods for estimating NH_3 involve adding an excess of alkali and driving off the NH_3 into an acid solution and could not be applied in this instance, as the addition of the alkali would result in the rapid blackening of the solution. Thus the objection could then have been made that the free NH_3 in the solution had condensed after the addition of the alkali, with the products of tyrosine deamination, to form melanin. This inability to obtain any increase in the ammonia content of the reacting tyrosinase-tyrosine solution is strong evidence against the theory of deamination.

The property of permutite in removing all ammonia from neutral or slightly acid solutions may be utilised in obtaining qualitative evidence against the theory of deamination. The view that the final product, melanin, contains nitrogen is generally accepted and has been confirmed in the present work, but according to Bach [1914] this product is formed as the result of oxidation of the deaminised tyrosine accompanied by condensation with NH_3 . If this is true, the removal of NH_3 from the reacting mixture should prevent the darkening of the solution, *i.e.* formation of melanin. It has been found however that no diminution in the production of melanin can be effected by shaking up the following solutions with permutite or filtration of these solutions through a layer of permutite: (1) the tyrosinase-tyrosine reaction mixture, (2) the clear red solution free from enzyme and (3) the colourless solution free from enzyme. Thus the removal of all the ammonia from these solutions does not inhibit the production of the final product melanin. It thus appears unlikely that ammonia is split off at any stage in the reaction.

From equation (1) it will be seen that *p*-hydroxyphenylpyruvic acid will be the first product or first non-nitrogenous product of the deamination of tyrosine and for this theory to hold good it is necessary that tyrosinase should

act on *p*-hydroxyphenylpyruvic acid in the presence of the requisite amount of ammonia to form the same end-products as the tyrosinase-tyrosine reaction. This acid has been prepared and it has been found that tyrosinase has no apparent action on it alone or with the addition of the calculated amount of NH_3 . In addition, no increase in the amount of red pigment, colourless substance or melanin could be produced by adding this acid, with or without the addition of the calculated quantity of NH_3 , to a reacting tyrosinase-tyrosine solution. This inability of tyrosinase to oxidise one of the intermediate products of the reaction according to the deamination theory, appears to be conclusive evidence against this theory.

The final product of the tyrosinase-tyrosine reaction, melanin, can be obtained in a comparatively pure state by the method described below, and prepared in this way, it is found to be a nitrogenous substance with a somewhat higher percentage of nitrogen than tyrosine. From the facts mentioned above, (a) the non-liberation of ammonia during the reaction, (b) the non-effect of removing any free ammonia from the reacting mixture on the production of red substance, colourless substance or melanin, and (c) the inability of tyrosinase to oxidise *p*-hydroxyphenylpyruvic acid in the presence of NH_3 , the conclusion must be drawn that no deamination takes place in the tyrosinase-tyrosine reaction. Further, since the final product, melanin, contains approximately the same percentage of nitrogen as tyrosine and as the intermediate products have certain properties which lead to the supposition that they are nitrogenous, it is suggested that all the intermediate products and final product contain nitrogen and that at no stage of the reaction is nitrogen split off in the form of ammonia.

EXPERIMENTAL.

In all the experiments described below except where the contrary is stated, dialysed potato juice prepared in the manner previously described [Raper and Wormall, 1923] was used as the source of tyrosinase. All the reactions were carried out in phosphate buffered solutions since the p_{H} of the solution has a very marked influence on the nature and quantity of the products obtained.

Ammonia content of the reacting tyrosine-tyrosinase mixture.

For this determination, the tyrosine solution was buffered at p_{H} 6.0, this reaction being considered most suitable for the purpose. In alkaline solutions and to a less extent at p_{H} 7.0 the red substance is rapidly converted into melanin, and according to the theory of Bach this melanin formation is due to the recombination of the deaminised fraction with ammonia and to condensation processes. Thus the best conditions for the detection and estimation of the liberated ammonia will be those in which the amount of melanin formed is at a minimum, while the amount of tyrosine oxidised is at or near the maximum. These conditions are best satisfied by allowing the enzyme action

to proceed in slightly acid solutions, and at p_H 6.0 the velocity of the oxidation is not greatly inferior to that at p_H 7.0 or 8.0 [Raper and Wormal, 1923].

Dialysed potato juice was added to the tyrosine solution, toluene added and a constant current of air drawn through this and a control solution containing no tyrosine, as previously described for the determination of the velocity coefficients. Four samples of 60 cc. each were withdrawn from each of the two solutions and half of these samples were heated to convert the red pigment where present into the colourless substance. The ammonia content of all these samples was then determined by the method of Whitehorn [1923], which involves the filtration of the solution through a layer of permutite, the liberation of the NH_3 by the addition of alkali to the permutite and nesslerisation. No increase in the ammonia present in the reacting tyrosine-tyrosinase solution could be observed at any time and since about two-thirds of the tyrosine present was oxidised during the time of observation, the amount of NH_3 liberated according to the deamination theory would have been appreciable.

Method of ammonia estimation.

Each sample (60 cc.) was filtered slowly through about 5 cc. of finely granular permutite contained in a $CaCl_2$ tube, the permutite having previously been washed with 2 % acetic acid to neutralise any alkali present and several times with distilled water. When the solution whose ammonia content was to be determined had filtered through, the permutite was washed five times with 10–20 cc. of distilled water and finally rinsed with the minimum quantity of water into a 100 cc. graduated flask. 5 cc. of 10 % NaOH were added to each suspension of permutite containing ammonia, the solution shaken for a minute and 15 cc. of modified Nessler's solution (Bock and Benedict formula) added. The solutions were allowed to stand for 15 minutes, centrifuged when necessary and estimated colorimetrically against a standard prepared as follows: 2 cc. of a standard NH_4Cl solution (5 cc. contain 1 mg. NH_3) + 20 cc. dist. H_2O + 5 cc. 10 % NaOH + 15 cc. mod. Nessler's solution. The solution was shaken, allowed to stand for 15 minutes and then made up to 100 cc.

Duplicate estimations were carried out after each interval of time and "test estimations" were also made by adding to special samples of the reaction mixture 2 cc. of the standard NH_4Cl solution (2 cc. contain 0.40 mg. NH_3). The procedure for these test estimations was the same as that for the ordinary samples, and these added amounts of NH_3 were estimated almost quantitatively.

Tyrosine estimations.

These were carried out on 20 cc. samples withdrawn from the solutions at the commencement of the reaction and after $7\frac{1}{2}$ hours and the method used was the bromination method previously described. The results show that approximately two-thirds of the tyrosine was oxidised during the experiment.

Solution A. 1000 cc. phosphate buffered 0.05 % tyrosine solution (p_H 6.0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution B. 1000 cc. phosphate buffered water (p_H 6.0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution A (red) refers to the samples of the reacting mixture which were used unchanged for the estimations.

Solution A (colourless) refers to samples which had been boiled to convert the red pigment into the colourless substance. This conversion was brought about by adding to the sample 1 cc. $N/10$ HCl, boiling, filtering and finally adding 1 cc. $N/10$ NaOH.

Results. Ammonia content of the samples in mg. per 60 cc.

Solution A (red)	0.16	0.17	0.17	0.16
Solution A (colourless) ...	0.17	0.18	0.20	0.18
Solution B (unboiled) ...	0.15	0.17	0.16	0.17
Solution B (boiled) ...	0.15	0.18	0.16	0.16
Tyrosine in Solution A (%)	0.045	—	—	0.013
Time (hrs)	0	3	5	7½

The amount of NH_3 which would have been liberated by the deamination of the tyrosine which was oxidised in 7½ hours is 1.80 mg. per 60 cc., and as less than a quarter of this amount, if added to a sample, was estimated in full, it appears inconceivable that deamination or the liberation of ammonia takes place during the action of tyrosinase on tyrosine.

The action of tyrosinase on p-hydroxyphenylpyruvic acid.

This acid has been prepared by the method used by Neubauer [1909], but the filtrate from the benzoic acid was immediately extracted with successive quantities of ether until almost all of the keto acid had been extracted, as shown by the inability of the neutralised aqueous solution to give more than a faint green colour with dil. ferric chloride solution. The ethereal solution containing the *p*-hydroxyphenylpyruvic acid was distilled under reduced pressure to remove the solvent, the residue dissolved in the minimum quantity of hot alcohol and poured into cold water. A white crystalline ppt. of the keto acid separated, was filtered off, washed with a little benzene and dried *in vacuo*. In some cases the acid was practically pure but in other cases had to be recrystallised from hot water (m.p. 220°). A further quantity of the acid can be recovered from the mother liquor. Solutions containing 0.05 % of this keto acid were then prepared and buffered at p_H 6.0, 7.0 and 8.0. These solutions were then acted upon by potato juice both with and without the amount of NH_3 (as NH_4Cl) theoretically required.

Controls were also set up at each p_H , using only the phosphate buffers and potato juice. The whole of the experiment was then repeated using dialysed potato juice as the source of tyrosinase and the results were exactly the same as those quoted below.

Experiments were then carried out to see whether the addition of *p*-hydroxyphenylpyruvic acid together with the theoretical amount of NH_3 to

reacting tyrosine-tyrosinase solutions would increase the yield of red pigment, colourless substance or melanin. No increase in the amount of either of the intermediate compounds or of the final product took place.

Observations.

Solutions used: I. 0.05 % phosphate buffered tyrosine solution (p_H 6.0, 7.0 and 8.0).

II. 0.05 % phosphate buffered *p*-hydroxyphenylpyruvic acid solutions (p_H 6.0, 7.0 and 8.0).
1.5 % NH_4Cl solution. Fresh and dialysed potato juice.

			1 hour	4½ hours	36 hours
1.	100 cc. Solution I (p_H 6.0)	+ 10 cc. potato juice	Red	Red	Black
2.	100 " " II (p_H 6.0)	+ 10 " " "	No change	No change	No change
3.	100 " " II (p_H 6.0)	+ 10 " " " + 1 cc. 1.5 % NH_4Cl	No change	No change	No change
4.	100 " " I (p_H 7.0)	+ 10 cc. potato juice	Red	Black-red	Black
5.	100 " " II (p_H 7.0)	+ 10 " " "	No change	No change	No change
6.	100 " " II (p_H 7.0)	+ 10 " " " + 1 cc. 1.5 % NH_4Cl	No change	No change	No change
7.	100 " " I (p_H 8.0)	+ 10 cc. potato juice	Red-black	Black	Black
8.	100 " " II (p_H 8.0)	+ 10 " " "	No change	No change	No change
9.	100 " " II (p_H 8.0)	+ 10 " " " + 1 cc. 1.5 % NH_4Cl	No change	No change	No change

These results lead to the conclusion that *p*-hydroxyphenylpyruvic acid is not an intermediate product in the oxidation of tyrosine by tyrosinase and thus the view that deamination occurs according to equations (1) and (2) appears to be untenable.

The preparation and nitrogen content of the melanin.

2 g. of tyrosine were dissolved in 4 litres of water, 400 cc. of fresh potato juice added and the reaction of the solution adjusted to p_H 6.0. The solution was then warmed to 37° and stirred vigorously in a wide vessel. When the intensity of the red colour reached a maximum and the solution was beginning to darken slightly, usually after 5–6 hours, the stirring was stopped. The solution was transferred to a 5-litre flask, 40 cc. of 10 % acetic acid added and the liquid boiled as quickly as possible. After being thoroughly cooled, the solution containing the coagulated proteins was filtered, and the filtrate allowed to stand overnight after being made definitely alkaline with 100 cc. 10 % Na_2CO_3 . On the addition of the alkali the colourless solution rapidly darkened and the formation of the melanin was accelerated by shaking with air. The melanin obtained in this way could not be filtered off, but was obtained in the precipitated condition by adding 200 cc. 10 % acetic acid and boiling the solution. The melanin was filtered off, leaving a clear solution and was purified as far as possible by boiling with dil. HCl, then with successive quantities of water, and washing with hot water. It was dried on porous plates *in vacuo* over H_2SO_4 . Examined under the microscope it appeared to be amorphous and was hard enough to be powdered finely. Nitrogen was present in considerable quantity according to the qualitative test (fusion with Na, etc.), but no sulphur. The absence of sulphur is evidence in favour of the view that the melanin is free from nitrogenous substances derived from the

potato juice, for the proteins of this juice have been found to contain sulphur. Four different samples of melanin were prepared by the above method, the yield being 0.5 g. to 0.8 g. melanin from 2 g. tyrosine, and the nitrogen content determined by the Kjeldahl method. The results showed that 8.43 %, 8.30 % and 8.47 % of nitrogen were present in three of the preparations by the Kjeldahl method and 8.65 % by the Dumas method. This is rather higher than the percentage of N in tyrosine (7.73 %), and is probably due to the fact that although oxygen has been absorbed by the tyrosine, molecules of water have been removed in the condensation processes, with resulting slight increase in the percentage of nitrogen present.

SUMMARY AND CONCLUSIONS.

1. Evidence against Bach's theory of deamination of the tyrosine has been obtained in the following manner and appears to be a conclusive refutation of this view.

(a) A reacting tyrosinase-tyrosine solution shows no increase in ammonia content during the oxidation of at least two-thirds of the tyrosine present.

(b) Removal of all the ammonia initially present in the solutions or formed by any part of the oxidation process does not affect in any way the formation of the intermediate products, *i.e.* the red and colourless substances, or the final product melanin.

(c) Tyrosinase has no action on solutions of *p*-hydroxyphenylpyruvic acid either with or without the necessary ammonia for recondensation.

2. The final product of the tyrosinase-tyrosine reaction, melanin, is a nitrogenous compound with a slightly higher percentage of nitrogen than tyrosine (8.40 % against 7.73 % N in tyrosine).

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