LV. THE TYROSINASE-TYROSINE REACTION.

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Tyrosinase, an oxidising enzyme which is widely distributed in the vegetable kingdom and has also been met with in animal organisms, has formed the subject of many investigations since it was discovered by Bourquelot and Bertrand [1896]. The enzyme differs from other oxidation catalysts in that it is able to bring about the oxidation of tyrosine by atmospheric oxygen. Laccase, for instance, which is usually considered to contain an oxygenase-peroxidase system, and hydrogen peroxide and a peroxidase, are without effect on tyrosine solutions. The action of the enzyme on tyrosine is accompanied by the development of a red colour, which gradually becomes reddish brown and finally a black pigment (melanin) is deposited. The nature of the action has been a matter of dispute and has been attributed to a hydrolytic deaminase, a phenolase or oxygenase-peroxidase system, to an enzyme which splits the tyrosine by hydrolysis, and to the inorganic salts associated with the enzyme acting as condensing agents on the oxidation or hydrolytic products produced by the enzyme itself. Into the merits or demerits of these various theories we do not wish to enter at present. Our interest was directed to one important factor concerning the action of the enzyme by the observation that the reaction of the medium in which the enzyme acts has a very marked influence on the colour development both as regards the rate of its production and the tint obtained, and also, that if a partially oxidised tyrosine solution is heated, the red substance first formed is converted into one which is colourless. This colourless substance, while being stable for many hours in an acid solution, rapidly gives rise to a bluish-black pigment on being made alkaline with sodium carbonate. From these simple observations, it was inferred that any investigation of the action of tyrosinase must be carried out with careful regard to the p_H of the medium. This is especially true when work bearing on the activity as co-enzymes or inhibitors of various substances or extracts is concerned, and also when the action of tyrosinase on various substances other than tyrosine is being investigated. Our observations have led us to conclude that because of the neglect to work with buffered solutions, a large number of erroneous conclusions have been drawn regarding the enzyme and its action.

The work described in the present paper has defined the limits of p_H at which the enzyme is active, and the velocity of the action at three different values of p_H has been measured. It has also been shown that the accelerating effect of boiled potato juice on the enzyme action which has been ascribed by Haehn [1919] to the salts present, is not due to the salts but to some organic constituent of the juice, since the ash of the boiled juice does not show the same effect. The results arrived at by Haehn are undoubtedly due to working with unbuffered solutions, a conclusion arrived at independently by Chodat and Wyss [1922].

From what has been mentioned above, it is apparent that previous measurements of the velocity, in which the p_H was not kept constant or in which the measurements of the rate of oxidation have been carried out by colorimetric methods, are unreliable. The studies by von Fürth and Jerusalem [1907] and by Bach [1908] come into this category. Bach did not use a colour comparison for estimating the degree of oxidation but employed an empirical procedure by oxidising with permanganate in acid solution the pigment formed during the action of the enzyme. Whilst it is true that the pigment formation from tyrosine is a notable feature of the enzyme action, it is certainly true that more than one pigment is formed, and it is also possible that non-pigmented substances may first be formed from the tyrosine [Bach, 1914]. The permanganate method therefore appears to us to be too crude. The method used in the present work was to control the p_H rigidly by using well-buffered solutions, and to estimate the tyrosine at the various stages of the reaction by bromination. In this way, satisfactory curves have been obtained which indicate that as regards the disappearance of the tyrosine, the reaction is of the unimolecular type and that the enzyme is therefore a true catalyst. This observation is of special interest if an oxygenase is a constituent of the enzyme. The oxygenases are supposed to be autoxidisable substances which on exposure to air form peroxides. In the presence of a peroxidase these peroxides are decomposed and yield "active" oxygen. Substances such as aldehydes or unsaturated fatty acids are usually referred to as bodies which are similar to the oxygenases since they are known to form peroxides on exposure to air. They would not function as true catalysts, however, because the peroxides which they form, do not yield the original aldehyde or unsaturated acid on decomposition, but rather, oxidation products of these substances. Thus benzaldehyde gives rise to benzoic acid and active oxygen.

$$C_6H_5.CHO + O_2 \longrightarrow C_6H_5.\overset{\overset{O}{\parallel}}{C}—O—OH$$

Benzoyl hydrogen peroxide

$$C_6H_5.\overset{\overset{O}{\parallel}}{C}—O—OH \longrightarrow C_6H_5.COOH + O \text{ (active)}$$

Benzoic acid

If the oxygenases were of this type, their concentration in the enzyme system would gradually diminish as the reaction proceeded and the velocity

constant would diminish considerably. This we have not found to be the case with tyrosinase and it indicates that if an oxygenase is a component of the enzyme, it acts as a true catalyst and is not permanently altered in bringing about the transfer of atmospheric oxygen either to the tyrosine or to its decomposition products produced by some other component of the enzyme.

Some of the observations recorded in the present paper point clearly to the existence of definite stages in the colour changes which occur when tyrosine is oxidised by the enzyme. These stages appear to be due to the formation of definite compounds which are intermediate between tyrosine and the final product, melanin. They are produced under definite conditions which have now been ascertained and it is hoped eventually to succeed in their isolation and identification. If the enzyme is allowed to act at p_H 6, the initial product is bright red in colour and with this reaction of the medium and in the cold, it is fairly stable, changing only slowly into the black pigment which is the end product of the reaction. If the oxidation is carried out at p_H 8·0, the red substance does not accumulate, but is rapidly transformed into the black melanin. The red substance, therefore, is to be regarded as the initial product of the reaction. It is not transformed directly into melanin, however, but rather into a colourless substance, which then undergoes oxidation to give melanin. This takes place rapidly in alkaline solutions. The evidence for this is that solutions of the red substance become colourless if allowed to stand in an oxygen-free atmosphere. The change takes place slowly in the cold, more rapidly on warming, and is almost instantaneous when the solution is boiled. If the colourless substance thus obtained be made alkaline, it rapidly darkens in the air, and finally deposits a black precipitate. Bach [1914] has also observed that the red substance is decolorised on heating or on standing in an inert atmosphere, but his conclusions about the change are very different from those we have reached. He attributes the decolorisation of the red substance to a process of reduction and suggests that the reducing agent concerned is a hydrolytic aminoacidase which he regards as a component of the enzyme. Although there appears to be satisfactory evidence that tyrosinase will deaminise amino-acids, the observations of Bach are the only ones which suggest that the action is a hydrolytic oxidation. The evidence submitted by him is, however, unconvincing, and in the present paper experiments are described which show that Bach's conclusions regarding the nature of the enzyme are erroneous. We have been unable to obtain any evidence that a hydrolytic oxidation is taking place. The red substance, it is true, becomes colourless on reduction by SO_2 as well as on simple standing in an inert atmosphere. It could therefore act as a hydrogen acceptor and be reduced if a hydrolytic oxidation catalyst were present. The only evidence submitted by Bach which is in favour of a reduction process is that the decolorisation of the red substance takes place in an inert atmosphere and that if the solution be then shaken in air, the colourless substance is reoxidised and becomes red. To show this, Bach takes a solution of tyrosine, adds tyrosinase and shakes

well for a few minutes at 35–40°. The tyrosine is oxidised and becomes red. The solution is then placed in an atmosphere of nitrogen and becomes colourless (according to Bach, by reduction). On readmitting air and shaking, it again becomes red. According to Bach, this is due to reoxidation of the colourless substance. It may, however, equally well be due to the further action of tyrosinase on tyrosine (both being present), which reaction can now proceed, oxygen having been admitted. That the latter is the true explanation is shown in the present paper. Solutions containing the red substance free from tyrosinase have been prepared. They become colourless on standing in an inert atmosphere, and do not give the red substance again on exposure to air; instead, melanin is produced; slowly if the solution be acid, quickly if it be alkaline. In this experiment, no enzyme is present when the red substance changes to the colourless compound, and the change, therefore, cannot be due to an enzymic reduction. Furthermore, tyrosinase in the presence of tyrosine will not reduce methylene blue in an inert atmosphere. If a hydrolytic oxidation catalyst were present, then the methylene blue ought to act as a hydrogen acceptor and be reduced. The most probable explanation of the above-mentioned colour changes is that the red substance is converted into the colourless compound by intramolecular change, this being slow in the cold, but rapid on heating.

In the light of the evidence presented in this paper, therefore, the action of tyrosinase on tyrosine is divisible into three stages.

1. The conversion of tyrosine into a red substance. This requires the presence of oxygen and the enzyme.

2. The conversion of the red substance into a colourless substance. This is spontaneous, and takes place rapidly on warming. It does not require the presence of tyrosinase and is probably an intramolecular change which the red substance undergoes.

3. The oxidation of the colourless substance by oxygen to melanin. This may take place spontaneously but may also be accelerated by the phenolase which is present in tyrosinase. It is rapid in alkaline solutions, but slow in acid solution.

Experimental Part.

The source of tyrosinase and its partial purification.

In certain reactions, it is essential that the tyrosinase should be free from some of the substances usually associated with the enzyme, and different methods of purification have been used according to the nature of the substances which were to be removed. In many cases, however, ordinary potato juice was found to give results identical with those obtained when the purified enzyme was used, and in these cases, untreated potato juice was used. This juice was obtained by washing ordinary market potatoes, mincing and then pressing in a meat press. The juice was allowed to stand for a short time and then centrifuged to remove starch.

The purified enzyme solutions most used in these experiments have been obtained by the dialysis of ordinary potato juice. The latter was contained in a very permeable collodion sac suspended in flowing water, and dialysed for four or five days under a slight pressure, this preventing undue dilution of the enzyme solution. The solution obtained in this manner does not give a reaction with tyrosine as good as that given by the original potato juice, but its activity can be increased by vigorous shaking after addition of sufficient sodium carbonate to make the solution 0·1 %.

Another method of purification depended on the digestion of the proteins of potato juice by pancreatic extracts. After four days' digestion in an incubator, the solution was acidified with dilute acetic acid until a definite and approximately maximum precipitate was obtained, care being taken to avoid excess of acetic acid. The precipitate was filtered off, washed with water, and suspended in 0·1 % sodium carbonate solution, in which it dispersed fairly well. It gave a good reaction with tyrosine, though only about half as intense as that given by a corresponding volume of the original potato juice.

Use has also been made of a fairly pure enzyme obtained by repeated precipitation with alcohol to 40 %, and suspension of the precipitate in 0·05 % or 0·1 % sodium carbonate solution. This method probably yields an enzyme preparation similar to that obtained by the method described by Chodat [1912].

The influence of the hydrion concentration on the action of the enzyme.

It has been found that the hydrogen ion concentration of the medium has a marked influence not only on the velocity of the enzyme action, but also on the nature of the pigments produced from tyrosine.

Buffered solutions containing 0·05 % of tyrosine were obtained from phthalate solutions, phosphate solutions, or boric acid and NaOH mixtures, according to the tables given in Cole's practical *Physiological Chemistry* and Clarke's *Determination of Hydrogen Ions*. 50 cc. of each solution were measured into a 200 cc. Erlenmeyer flask, 5 cc. of fresh potato juice added, and allowed to stand at the room temperature (15°). The solutions were shaken every half-hour, and at intervals were poured out into boiling tubes, any colour changes being noted.

p_H 4·0. No apparent oxidation, *i.e.* no colour changes.

p_H 5·0. Very little oxidation. Slight red colour after 5 or 6 hours.

p_H 6·0. Rapid oxidation. A deep red colour after one hour becoming very intense after 3 or 4 hours. At the end of 24 hours, the solution had become much darker, though the red phase was still very evident.

p_H 7·0. Rapid oxidation. A deep red colour after 1 hour, slightly blacker than that at p_H 6·0. The solution remained blacker than that at p_H 6·0 throughout the experiment and melanin formation was more rapid.

p_H 8·0. Rapid oxidation. After one hour, the solution had a red colour, which gradually became darker and more intense. The subsequent blackening and the formation of melanin were more rapid than in the solution at p_H 7·0.

p_H 9·0. Slight blackening with a little red colour after 2 hours. After 12 hours the solution was very dark, though much lighter than that at p_H 8·0.

p_H 9·5. Similar results to those at p_H 9·0, with corresponding colours of less intensity. Thus there is less visible oxidation than at p_H 9·0.

p_H 10·0. Very little oxidation. Slight darkening after 6 hours.

p_H 10·5, 11·0, 12·0. No apparent oxidation.

The solutions were also tested at intervals for the relative amounts of red substance present in each. This was carried out by withdrawing 5 cc. from each solution, adding one drop of 10 % acetic acid, boiling, filtering and adding sodium carbonate to the filtrate until it was definitely alkaline. By this treatment, the red chromogen is converted into the colourless substance by the process of boiling, which also destroys and coagulates the enzyme, while the addition of the alkali causes the rapid conversion of the colourless substance into melanin. This last reaction yields a bluish-black solution, and the degree of intensity can be regarded as an approximate measure of the amount of red substance present in the original sample.

The results show that the formation of this red chromogen is at a maximum at p_H 6·0 to 6·5, is less at p_H 7·0, and that the amount then decreases rapidly with increasing alkalinity. As the enzyme action takes place at p_H 8·0 with a velocity equal to, or slightly greater than, that at p_H 6·0, the small amount of red substance found at p_H 8·0 must be due to the rapid conversion of this substance into melanin.

The limits of oxidation of tyrosine by tyrosinase under the conditions given above, are p_H 5·0 and p_H 10·0 and the enzyme seems to be most active in neutral, slightly alkaline or slightly acid solutions, *i.e.* in the range p_H 6·0 to 8·0. Even over this range, however, the colour changes vary enormously.

Chodat and Wyss [1922] working with unbuffered solutions have obtained similar limits of p_H for the tyrosinase-tyrosine reaction, though their limit for the alkaline solutions (p_H 11·0) is somewhat higher than that observed in the experiments described above.

It is of interest that Mrs E. C. Venn [1920] working with a cheese organism which produced colour changes in tyrosine solutions found that the limits of p_H within which the colour was obtained were 3·23 and 9·7. The closeness of these limits to those observed by us suggests that the organism in question produced tyrosinase. The non-correspondence in the more acid media is possibly to be explained by the fact that the enzyme is precipitated in solutions at p_H 5·0, whereas it might still be active inside the bacteria.

THE NATURE OF THE CHANGES OCCURRING IN THE TYROSINASE-TYROSINE REACTION.

The first process in the oxidation of tyrosine by tyrosinase is the formation of a red pigment and this only takes place in the presence of tyrosinase and oxygen. The formation of this red substance can best be shown by the partial oxidation of a saturated tyrosine solution at p_H 6·0 by means of one-tenth its volume of fresh potato juice. This product is relatively stable in acid solutions, but in an alkaline medium it is rapidly converted into melanin, probably with intermediate formation of a colourless substance.

If a partially oxidised tyrosine solution at p_H 6·0 is treated with colloidal ferric hydroxide to remove the enzyme and other colloidal substances, a clear deep red solution is obtained which is fairly stable, but on standing several hours it deposits a black pigment, melanin. This blackening, which is a process of oxidation, continues until all the red colour disappears. At higher temperatures such as 35–40°, the decolorisation of the red substance is more rapid, but no blackening takes place unless the solution has free access to oxygen. If the red solution is boiled, immediate decolorisation takes place and subsequent exposure to the air causes the precipitation of melanin. In this way, by the use of colloidal ferric hydroxide to remove tyrosinase from the coloured solutions, Bach's theory [1914, p. 222] that tyrosinase contains a hydrolytic oxidation enzyme and that the conversion of the red substance into the colourless compound is an enzymic reduction process is disproved. It has also been found that the decolorisation of the red substance takes place spontaneously, though it may be accelerated by the enzyme, possibly by a process of adsorption. It takes place in the absence of oxygen, and can be quickened by raising the temperature, taking place extremely rapidly at 100°. These, and certain other facts suggest to us that the process is due to an intramolecular change.

In repeating the experiment of Bach [1914, p. 222) the red solution obtained by the action of 1 vol. of potato juice on 10 vols. of a saturated tyrosine solution at p_H 6·0, which contained tyrosine, tyrosinase, the red substance, and possibly some of the colourless compound, was divided into two parts. One half was treated with colloidal ferric hydroxide and filtered. A clear red solution was obtained which contained no enzymes. This filtrate and the other half of the original red solution containing tyrosinase were placed in flasks, the latter evacuated to remove all the oxygen, and then filled with CO_2. They were then made airtight with rubber stoppers and placed in an incubator at 37°. After some time both solutions became colourless, but on shaking with air, only that containing tyrosinase became red again. Thus the subsequent reddening obtained by Bach, and attributed by him to the reoxidation of the colourless substance, is really due to the renewal of the action of tyrosinase on tyrosine, both of which are still present in the solution. This experiment shows clearly that the decolorisation of the red substance is not a process of reduction by the enzyme, for it takes place in the absence of the latter, and

also the colourless substance cannot be oxidised to the red compound again by shaking with air or oxygen.

Other evidence pointing to the non-existence of a hydrolytic oxidation enzyme in potato juice is our inability to obtain any oxidation of tyrosine by tyrosinase in the absence of oxygen and in the presence of methylene blue as a hydrogen acceptor. Buffered 0·05 % tyrosine solutions at p_H 6·0 and 8·0 with one-tenth their volume of potato juice or dialysed potato juice do not undergo any visible oxidation in an atmosphere of hydrogen or *in vacuo*, even if varying amounts of methylene blue are previously added. No reddening or darkening of the solutions occurs, even after 48 hours in an incubator, but subsequent admission of air causes fairly rapid oxidation, showing that the enzyme has not lost its activity towards tyrosine.

The kinetics of the tyrosinase-tyrosine reaction.

In the following experiments, an attempt has been made to study this oxidation process on quantitative lines, to determine the "order" or type of the reaction, and also to make a careful study of the effects produced by adding to the enzyme certain constituents of potato juice which by themselves have no action on tyrosine. The results obtained indicate that the reaction is of the ordinary uni-molecular type and that the activity of the enzyme decreases very little during the reaction. Further, it is shown that in order to make any useful comparison, it is essential that the solutions used should be at the same hydrogen ion concentration, and should have an efficient buffering capacity. Thus we can confirm the statement of Chodat and Wyss [1922], that the results previously obtained by Haehn [1920] were simply due to alterations in the hydrogen ion concentration of the solution. In one respect, however, we have confirmed Haehn's results and find that potato juice rendered almost inactive by dialysis can be activated by the addition of certain mineral salts. This activation is quite different from that brought about by boiled potato juice, and our experiments show that the latter effect is not due to inorganic salts. It is found that certain samples of boiled potato juice, which are themselves inactive towards tyrosine, are able to activate the tyrosinase of potato juice, but the nature of this "co-enzyme" or activator is uncertain. This "co-enzyme" is relatively heat stable, but is not in the nature of an inorganic salt, because the ash of the same boiled potato juice has no activating power.

In order to follow quantitatively the relations existing between the enzyme and the substrate, only one of the two usual procedures has been used. Tyrosine being only slightly soluble in water, it is not possible to allow varying amounts of the enzyme to act upon a tyrosine solution and to assume that the concentration of the substrate remains constant during the experiment. In consequence, the rate of disappearance of tyrosine in the presence of a constant amount of enzyme has been followed. The method is laborious and time consuming, since the intermediate products of the oxidation as well as

the final product, melanin, have to be removed before the tyrosine can be estimated. A colorimetric estimation involving the use of a phosphotungstic and phosphomolybdic acid reagent, was found to have no advantage in this respect over the older method of bromination, so the latter was adopted. Errors due to bromine-absorbing compounds other than tyrosine, which were present in the enzyme solution, were eliminated by the use of controls.

General method of procedure. The method consisted of placing the tyrosine and control solutions contained in 500 cc. round-bottomed flasks in a thermostat at 20° and drawing through each solution a constant current of air saturated with water vapour and toluene. All the solutions were buffered by means of mixtures of NaOH and KH_2PO_4 solutions, and, by the addition of distilled water when necessary, it was arranged that all the solutions in comparative experiments should have the same total volume. In the case of the tyrosine solutions, 300 cc. of a phosphate buffered 0·05 % solution of tyrosine were used in each experiment, and for each of these solutions a control was set up, using the same quantity of distilled water and buffering solutions. Equal amounts of potato juice were added to all the solutions, together with 10 cc. of toluene, the latter preventing frothing and bacterial decomposition. The potato juice used was obtained by the process described above (p. 457). This juice was not purified in any way since the bromine absorbing capacity did not prove to be very high.

Samples (20 cc.) were withdrawn from each solution at the commencement of the reaction, and also after certain intervals. Each sample was immediately treated with 0·5 cc. of 10 % acetic acid and boiled to destroy the enzyme and coagulate the protein matter. After being plugged with cotton-wool, the flask containing the sample was allowed to stand for two days. The solution was then filtered, the precipitate of melanin and protein coagulum being washed several times with hot water to remove all the tyrosine adhering to the precipitate, and the filtrate made alkaline by the addition of 1 cc. of 10 % sodium carbonate. The solution was then boiled, the flask plugged with cotton-wool, and allowed to stand 24 hours. 1 cc. of 10 % acetic acid was then added, the solution boiled, allowed to stand for a few hours, and finally filtered. The tyrosine in each sample was then estimated by the method described below. This treatment of the samples was the one found most satisfactory in removing the intermediate products of oxidation together with most of the substances present in potato juice which absorb bromine. In spite of this very drastic treatment, however, the samples from the controls, *i.e.* the solutions containing only the buffering salts and potato juice, absorbed a small amount of bromine, but this remained almost constant throughout the experiment. Thus, by subtracting the "tyrosine figure" of the control from that of the corresponding tyrosine solution, a true "tyrosine figure" has been obtained for each sample and these enabled us to calculate the concentration of tyrosine in each solution after the various intervals of time.

These figures of tyrosine content have been used directly to draw curves

showing the relationship between the concentration of tyrosine and the time of reaction, and have also been used to calculate the values of k, the velocity coefficient, on the assumption that the reaction is of the uni-molecular type. The constancy of k throughout the experiments indicates that this assumption is correct, and thus the velocity of the reaction at any particular time is directly proportional to the concentration of tyrosine at that moment.

The estimation of tyrosine. The method, which is a modification of that used by Millar [1903], consists of adding to each tyrosine solution a standard solution of bromine containing more bromine than can be absorbed by the tyrosine and other bromine-absorbing substances present and estimating the excess.

The tyrosine solution was treated with 10 cc. of standard $NaBrO_3$ (0·8502 g. per litre; 1 cc. = 0·001527 g. tyrosine) solution, 2 cc. of 50 % KBr solution, and 7·5 cc. of 20 % HCl solution, the flask corked and allowed to stand 20 minutes. 2 cc. of 10 % KI solution were then added, and the free iodine titrated with $N/50$ $Na_2S_2O_3$ solution. After making allowance for the bromine absorption of the control, the true tyrosine content was calculated as below, but in some cases the differences between the sodium thiosulphate titration figures of each sample and the corresponding control were used to represent the relative concentrations of tyrosine.

The $Na_2S_2O_3$ solution was first standardised by liberating a known amount of bromine from solutions of $NaBrO_3$, KBr and HCl, replacing the free bromine by iodine, and titrating the latter with the thiosulphate solution.

All the following experiments were carried out in a thermostat regulated at 20°. A qualitative experiment showed that the rate of oxidation increased with rise of temperature.

Comparison of the influence of air and oxygen on the rate of oxidation. In all the experiments, a constant current of air was drawn through each solution during the whole of the reaction under observation, and thus it was first necessary to prove that the oxygen supply was adequate. If this were not so, the reaction followed would merely be the enzyme action controlled by a limited oxygen supply, and if the enzyme is of the ordinary oxidase type, that reaction would be the rate of formation of the peroxide.

The method adopted to prove this point before passing on to the more important investigations, was to compare the rate of oxidation under these conditions with the rate when the oxygen supply was increased five-fold by replacing the air current by an oxygen current approximately equal in volume.

Solution *A*. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
„ *B*. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
Control *A*. 300 cc. phosphate buffered water (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
„ *B*. 300 cc. „ „ „ „ „
Air was passed through *A* and Control *A*. *Oxygen* through *B* and Control *B*.

Samples (20 cc.) of each of the four solutions were then taken at the commencement of the reaction, and after certain intervals, and after being treated

as indicated previously, the tyrosine in each was estimated by the bromination method.

Results. Amount of tyrosine (g.) present in the original volume of solution (330 cc.):

A (air)	0·1437	0·1275	0·0954	0·0716	0·0573
B (oxygen)	0·1437	0·1272	0·0935	0·0748	0·0574
Time (mins.)	0	67	160	250	315

The control figures remained constant, or the variations were within the limits of experimental error, and both controls gave the same titration figures.

The above results prove conclusively that the oxygen supply is adequate if a current of air similar to that used in this experiment is drawn through each solution and if this supply is maintained throughout the experiment. It may also be noted that this current of air also keeps the solution well agitated and so prevents differences in temperature or local concentration of enzyme.

Influence of hydrion concentration on the velocity of oxidation. In this experiment, three tyrosine solutions (*D*, *C* and *B*) were prepared, buffered by phosphate solutions at p_H 6·0, p_H 7·0 and p_H 8·0 respectively, and a control (*A*) was set up, using the same amount of phosphate solution at p_H 6·0. A preliminary experiment proved that controls at the three different hydrion concentrations gave equal thiosulphate titration figures, and so only one control was necessary for this comparative experiment. To 300 cc. of each of the solutions *B*, *C*, and *D* and solution *A* 30 cc. of potato juice were added and 10 cc. of toluene, and they were then placed in the thermostat at 20°. The same current of air was passed through all four solutions. Duplicate samples were withdrawn at intervals and treated as described above.

Results. Grams of tyrosine in 330 cc.:

B. p_H 8·0	0·1198	0·0911	0·0781	0·0706	0·0629	0·0561	0·0148
C. p_H 7·0	0·1179	0·0973	0·0821	0·0772	0·0688	0·0603	0·0214
D. p_H 6·0	0·1168	0·0963	0·0855	0·0796	0·0730	0·0622	0·0339
Time (mins.)	0	135	233	313	418	485	1420

Velocity coefficients. These were calculated on the assumption that the reaction is uni-molecular, *i.e.* $k = \frac{1}{t}\log_e\left(\frac{a}{a-x}\right)$ or $k = \frac{2{\cdot}302}{t}\log_{10}\left(\frac{a}{a-x}\right)$, where a = the original concentration of tyrosine and $(a - x)$ the concentration after time t.

Velocity coefficients (k).

							Average
B. p_H 8·0	0·0020	0·0018	0·0017	0·00155	0·0016	0·0015	0·0017
C. p_H 7·0	0·0014	0·0015	0·0014	0·0013	0·0014	0·0012	0·0014
D. p_H 6·0	0·0014	0·0013	0·0012	0·0011	0·0013	0·0009	0·0012
Time (mins.)	135	233	313	418	485	1420	—

This experiment was repeated later with another enzyme preparation, and the following figures obtained:

Velocity coefficients (k).

					Average
p_H 8·0	0·0023	0·0023	0·0022	0·0019	0·0022
p_H 7·0	0·0015	0·0017	0·0020	0·0019	0·0018
p_H 6·0	0·0015	0·0015	0·0015	0·0013	0·0015
Time (mins.)	66	188	375	1440	—

The curves obtained by plotting the concentration of tyrosine against the time are of the true logarithmic type, indicating that the amount of tyrosine oxidised during any short interval of time is directly proportional to the average concentration of tyrosine during that interval. In Fig. 1 the curve at p_H 8·0 was obtained by substituting for k in the formula $k = \frac{1}{t} \log_e \frac{a}{a-x}$, the average value for k found in the experiment at p_H 8·0, and calculating from this the corresponding values of $(a - x)$ or x for different intervals of time. Points are also marked on the graph which represent the concentrations of tyrosine actually found by estimation, and it will be seen that these are in close agreement with the average curve. Similar curves are also drawn giving the oxidations at p_H 6·0 and p_H 7·0, and it will be observed that the velocity is greatest at p_H 8·0 and least at p_H 6·0.

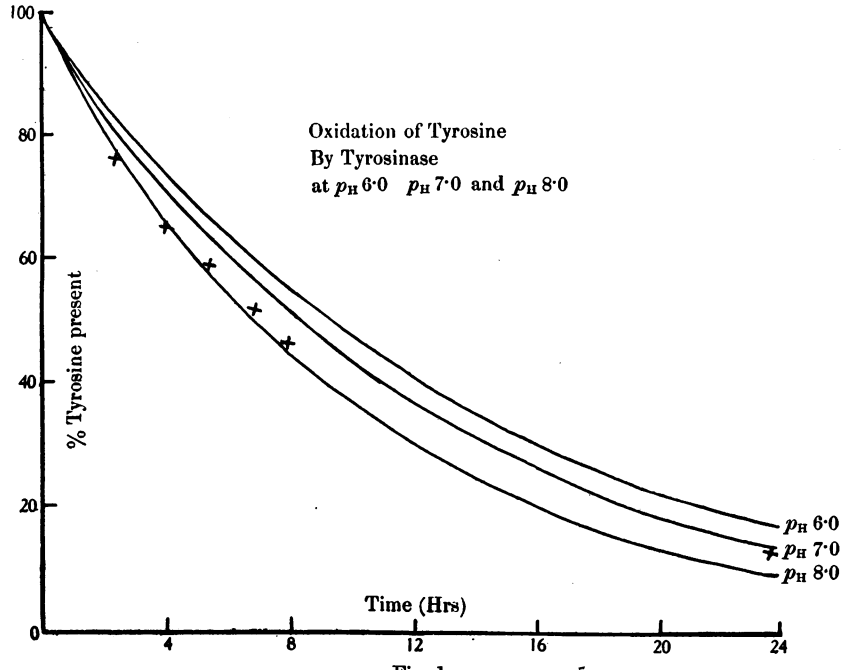

Fig. 1.

× — Experimental points for p_H 8·0.

The approximate constancy of the values of the velocity coefficient during the whole of the reaction proves that the reaction is of the uni-molecular type and is therefore comparable with other enzyme actions. In this constancy of the values of k, however, the reaction seems to deviate somewhat from those followed by other workers. Thus in the case of the hydrolysis of milk-sugar by lactase [Armstrong, 1904] the value of the velocity coefficient falls rapidly from 0·0640 for the first hour to 0·0310 for 5 hours and 0·0129 for 24 hours. In the case of the hydrolysis of cane-sugar by invertase [Henri, 1903] the velocity coefficient steadily increases from 0·00058 during the first

hour to 0·00085 during 11 hours. This constancy in the case of the tyrosinase-tyrosine reaction is probably due to the absence or elimination of certain factors which would have an accelerating or retarding effect on the reaction. Thus the nature of the reaction seems to preclude the possibility of a reverse reaction playing any part in the process, while effects due to changes in hydrion concentration may be ruled out on account of the buffering of the solution. With regard to the destruction or change in the nature of the enzyme, our observations lead to the conclusion that tyrosinase is not very unstable at these hydrion concentrations.

The values obtained for the velocity coefficient fluctuate during the experiment or show a slight decrease during the oxidation of half the tyrosine, followed by a larger decrease during the oxidation of the remainder. These divergences during the first six or eight hours are not very great, except perhaps at p_H 8·0 and in view of the small amount of tyrosine present in each sample (0·0075 g. in the first samples and about 0·0010 g. in the last) and the rigorous treatment to which these samples must be submitted, these divergences are not greater than the experimental error involved in their determination. With reference to the "slowing" which occurs during the second part of the reaction, it is interesting to note that this is less marked in the experiments carried out at p_H 7·0 and this indicates certain factors which may be the cause of retardation. In acid solutions, the enzyme is precipitated, and although this is not very marked at p_H 6·0, it will have an appreciable effect on reactions proceeding for 24 hours, and will cause a decrease in velocity similar to that observed. With regard to the decrease at p_H 8·0, no destruction or precipitation of the enzyme should take place, because tyrosinase seems to be very stable and well dispersed at this hydrion concentration, and the determining factor appears likely to be the adsorption of the catalyst by the melanin produced, this being the main product of oxidation under these conditions.

The results obtained indicate that slight alkalinity favours the action of the enzyme, for the velocity at p_H 8·0 is greater than that at p_H 7·0, and this in turn is greater than that at p_H 6·0. The exact conditions determining this greater velocity at p_H 8·0 are not known, but it is suggested that there are two factors which probably play a very important part in this phenomenon. Firstly, the dispersion of the enzyme, as judged by the naked eye, is best at this hydrion concentration, and secondly, if the first reaction, the conversion of tyrosine into the red pigment, tends to be at all reversible, this will have least effect at p_H 8·0, because of the rapid conversion of the intermediate products into the final product, melanin.

The effect of adding boiled potato juice.

Some ordinary potato juice was boiled in a small Erlenmeyer flask for 2–3 minutes, cooled and filtered. When tested with a tyrosine solution, it did not give the characteristic tyrosinase reaction, showing that the enzyme present had been destroyed.

A. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

B. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

Control *A*. 300 cc. phosphate buffered water (p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

„ *B*. 300 cc. phosphate buffered water (p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

Results.

Tyrosine present in g. per 330 *cc.* (*Average of two estimations.*)

	1	2	3	4	5
A. Ordinary potato juice	0·1427	0·1269	0·1062	0·0905	0·0321
B. Potato juice and boiled potato juice	0·1442	0·1185	0·0980	0·0804	0·0287
Time (mins.)	0	130	220	360	1380

The comparison is easier if the amount of tyrosine oxidised is represented as a percentage of the amount originally present, *i.e.* % of tyrosine oxidised.

	1	2	3	4	5
A.	0	11·08	25·58	36·59	77·50
B.	0	17·82	32·03	44·24	80·09

Average velocity coefficient for $A = 0{\cdot}00114$.
„ „ $B = 0{\cdot}00151$.

This activation has been noticed by Haehn [1919] who used boiled potato juice and tyrosine freed from the non-colloidal matter of potato juice by ultrafiltration, but this author erroneously attributes it to the presence of certain salts (calcium salts, phosphates, etc.) in the potato juice which act as co-enzymes. This activator or co-enzyme is not an ordinary inorganic salt, but is probably of organic nature, since it is not present in the ash of boiled potato juice, as shown below. It is interesting to note in this connection, that this activator is nearly always present in the boiled juice of new potatoes, but rarely, if ever, present in that from old potatoes.

Effect of adding the ash of boiled potato juice.

Boiled potato juice containing the activating agent referred to in the previous experiment, but having no tyrosinase reaction, was evaporated down to dryness and ashed. The ash was dissolved in a little warm dilute HCl, neutralised and made up to the original volume, a precipitate of the hydroxides of aluminium and iron remaining in suspension. To prove the activating power of the original boiled potato juice, an experiment was included to show that the oxidation of tyrosine by the unboiled potato juice used was accelerated by the addition of some of this boiled juice.

Solution *A*. 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

„ *B*. 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

„ *C*. 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. solution of ashed juice + 10 cc. toluene.

Controls *D*, *E* and *F* for solutions *A*, *B* and *C* respectively were set up under exactly the same conditions, except that the buffered solutions in these cases contained no tyrosine.

Results.

Time (mins.)	0	128	243	385							
	Tyrosine (g.) present in 330 cc. of solution. Average of 2 estimations				% tyrosine oxidised			Velocity coefficients			
	1	2	3	4	2	3	4	2	3	4	Average
A. (normal)	0·1415	0·1237	0·1050	0·0878	12·58	25·80	37·95	0·0011	0·0012	0·0012	0·0012
B. (+boiled juice)	0·1415	0·1144	0·0938	0·0747	19·19	33·69	47·23	0·0017	0·0017	0·0017	0·0017
C. (+ashed boiled juice)	0·1415	0·1248	0·1066	0·0896	11·73	24·64	36·67	0·0010	0·0012	0·0012	0·0011

Summary and conclusions.

1. Tyrosinase first produces from tyrosine a red substance. This process requires the presence of the enzyme and only takes place in the presence of oxygen. This red substance becomes colourless spontaneously and is then oxidised to form melanin. These last two processes take place in the absence of tyrosinase, but may be accelerated by it or by other oxidases found in potato juice.

2. The hydrion concentration of the medium has a marked influence on the velocity of the oxidation of tyrosine by tyrosinase and an even greater influence on the nature of the pigments produced. The limits of p_H between which potato tyrosinase acts upon tyrosine are 5 and 10. In the neutral and acid solutions, the main product during the first six hours is the red substance, but in alkaline solutions, the conversion of this into melanin is so rapid that the solutions quickly darken, and the preliminary reddening is not very marked.

3. Evidence is presented which is opposed to the view of Bach that tyrosinase contains a hydrolytic oxidation catalyst.

4. Haehn's experiment showing that tyrosinase, which has been rendered relatively inactive by dialysis, can be activated by the addition of certain mineral salts has been confirmed. But the acceleration of the enzyme action brought about by the addition of boiled potato juice is not due to inorganic salts but to some organic constituent or constituents of the potato juice. This activator is not always present in boiled potato juice, but is usually to be found in the boiled juice of new potatoes.

5. In order to obtain any true comparison between the oxidations of tyrosine by tyrosinase under different conditions, it is necessary to have all solutions well buffered at a definite hydrion concentration. In addition, the temperature must be kept constant and a satisfactory supply of oxygen must be maintained.

6. The results obtained show that the reaction is of the uni-molecular type, giving a true logarithmic curve when values of the concentration of tyrosine are plotted against the time, and also giving values for the velocity coefficient, which are approximately constant throughout the reaction. Tyrosinase is thus a true catalyst.

7. The oxidation of tyrosine by tyrosinase in a buffered solution at p_H 8·0 has a greater velocity than that at p_H 7·0, and the velocity at p_H 7·0 is greater than that at p_H 6·0.

8. The acceleration of the enzyme action by the addition of boiled potato juice has been definitely proved by following the rate of disappearance of tyrosine and it has also been shown that the ash of the same boiled juice produces no acceleration of the action.

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