XIV. THE TYROSINASE-TYROSINE REACTION. III.

THE SUPPOSED DEAMINISING ACTION OF TYROSINASE ON AMINO ACIDS.

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FOR some years it has been generally accepted that the enzyme tyrosinase will bring about the oxidation of amino acids with the production of ammonia. This view was first suggested by Chodat and Schweizer [1913] to explain the production of formaldehyde and ammonia from glycine when it was submitted to the action of tyrosinase from potatoes. The process of deamination has been made the basis of a theory as to the components of the enzyme tyrosinase by Bach [1914], who accepts the view that tyrosinase has a deaminising action on tyrosine. Folpmers [1916] isolated a small amount of benzaldehyde as the *p*-nitrophenylhydrazone from the products of the action of tyrosinase on phenylaminoacetic acid, thus supporting the deamination hypothesis.

A study of the experimental evidence on which the deamination theory was based led us to the opinion, that certain of the experiments should be repeated and extended, and the results of this work are included in the present paper.

The tyrosinase used throughout this investigation was obtained by the dialysis of potato juice through moderately permeable collodion sacs. An attempt was made in the first instance to obtain from alanine, by the action of tyrosinase, the aldehyde corresponding to it, namely, acetaldehyde. This proved abortive although the enzyme preparations used were very active as shown by their action on tyrosine. It was then found that under the conditions of the experiments, in which free access of oxygen is a necessity, small traces of added acetaldehyde disappeared, probably by oxidation. Attempts to detect formaldehyde and benzaldehyde as products of the action of tyrosinase on glycine and phenylaminoacetic acid respectively were also negative. The search for aldehydes as products of the tyrosinase reaction was therefore discontinued and instead experiments were carried out in which the ammonia, if any, produced by the action of tyrosinase on amino acids could be estimated. The acids chosen were glycine, alanine, phenylaminoacetic, phenylalanine and tyrosine. The conditions were such that had any ammonia been produced it would have been estimated easily. These experiments also provided no evidence of deamination and in this respect agreed with the experiments of Raper and Wormall on tyrosine [1925] carried out by other methods.

Bach [1914] has suggested that after deamination the ammonia recombines with some other product of the enzyme reaction—possibly the aldehyde—and as this might conceivably lead to the non-detection of ammonia as a product of the reaction, a series of experiments was carried out to determine whether any change in the amino N content of the system took place when tyrosinase acted on glycine, alanine or phenylaminoacetic acid. The results were completely negative. In addition, measurements have been made of the oxygen taken up when tyrosinase acts on tyrosine, glycine and alanine under identical conditions. A considerable oxygen absorption was observed with tyrosine but none with the other two amino acids. We have thus come to the conclusion that potato tyrosinase, under the conditions in which the enzyme is very active, has no deaminising action on amino acids.

These results necessitated a re-examination of the experiments of Chodat and Schweizer in which they claimed to have obtained evidence of deamination of glycine by potato tyrosinase, by the detection of formaldehyde and ammonia as products of the reaction. On reading over the records of Chodat and Schweizer's experiments, it was noticeable that success was only achieved when p-cresol was added to the reacting system. This addition was made, according to Chodat and Schweizer, in order to prevent interaction of the ammonia and formaldehyde produced by the deamination process, since the uncertainty in detecting them when p-cresol was not present was attributed to their further reaction with each other.

On repetition of these experiments we have been able to confirm that when p-cresol is present along with glycine and tyrosinase, ammonia is liberated and there is a diminution in the amino-nitrogen content of the system.

The evidence in favour of formaldehyde production adduced by Chodat and Schweizer we have not been able to confirm. p-Cresol itself reduces ammoniacal silver nitrate solution so that in its presence a reduction of this reagent cannot be accepted as evidence of the presence of formaldehyde, a point seemingly overlooked by Chodat and Schweizer.

Schryver's reaction for formaldehyde—the second reaction which they used—has failed in our hands to show the presence of formaldehyde when tyrosinase acts on glycine. This reaction will detect with ease 1 part in 10,000 of formaldehyde so that if it is produced even in traces it should have been detected. We are led therefore to the conclusion that the deamination found by Chodat and Schweizer was due to the presence of p-cresol and tyrosinase and not to tyrosinase alone, and as Chodat has shown that the latter attacks the p-cresol, it is probable that the deamination is produced by some oxidation product of p-cresol acting on the amino acid.

It was of interest to compare the action of other phenolic and related substances with that of p-cresol, and this has been done with phenol, resorcinol, quinol, catechol, p-benzoquinone and o-benzoquinone.

Only those phenols which are likely to give an *ortho*-dihydric-phenol or *ortho*-quinone derivative on oxidation gave positive results in the deamination

experiments. Under the conditions in which the experiments were carried out, even in the absence of tyrosinase, o-quinone produced deamination. This suggests that the phenols which were found to be active display this activity because they yield o-quinone or a derivative of it (e.g. 3·4 toluquinone from p-cresol), on oxidation by tyrosinase and that this then reacts with the amino acid.

It should be noted that M. W. Onslow [1923] has suggested that tyrosinase is a mixture of enzymes; a water splitting enzyme (reductase or deaminase), a carboxylase and an oxydase (consisting of an oxygenase, a dihydroxy substance and a peroxydase), and that she suggests that p-cresol is oxidised by traces of active oxygen to 3·4-dihydroxytoluene, but there is no assumption of the formation of an o-quinone which reacts with the amino acid and causes deamination.

The observations recorded in this paper indicate that tyrosine is acted on by tyrosinase, not because it is an amino acid but because it possesses a phenolic hydroxyl group. Phenylalanine, which only differs from tyrosine in this particular, is not oxidised by tyrosinase. It seems likely therefore that a preliminary stage in the oxidation of tyrosine by tyrosinase may be the formation of an o-quinone derivative which then undergoes an intramolecular reaction with the side chain containing the amino group, or reacts with another molecule of tyrosine. The amino group is, however, not split off as ammonia, as it is when o-quinone attacks other amino acids.

EXPERIMENTAL.

In the experiments with tyrosinase described in this section, a phosphate buffer mixture adjusted to p_H 7 was used and toluene was added as an antiseptic.

I. *The Alleged Aldehyde Formation due to the Action of Tyrosinase on Amino Acids.*

(a) *From alanine.* For the detection of acetaldehyde, p-nitrophenylhydrazine was used in acetic acid solution. Two large flasks A and B were taken and into each was placed a mixture of 100 cc. dialysed potato juice, 100 cc. of the buffer solution and 10 cc. of toluene, in addition 1 g. of alanine was placed in A. The two flasks were left at room temperature for 24 hours, after which the contents were made just acid to Congo red by the addition of phosphoric acid and distilled.

The distillate was first caused to pass through a percolator packed with broken ice, after which it was collected in two separate portions, 25 cc. in volume, in a receiver, surrounded by melting ice, which contained the maximum theoretical amount of a p-nitrophenylhydrazine solution necessary for the conversion of any aldehyde formed into the p-nitrophenylhydrazone. A very faint turbidity was produced by the distillate obtained from A, while that from B produced no change.

This failure to detect acetaldehyde in the products of the reaction led to the consideration of the possibility under the conditions of the experiment of

        (1) condensation of the aldehyde with itself,

        (2) loss of aldehyde by oxidation and

        (3) a loss of aldehyde by volatilisation.

To test these possibilities the following experiments were undertaken:

(1) The above experiment was repeated with the phosphate buffer adjusted to p_H 6·5 so as to prevent the aldol condensation of acetaldehyde. Again no acetaldehyde was detected as a reaction product, thus ruling out autocondensation as an explanation of its possible disappearance from the system.

(2) Two 2-litre flasks were taken and into each was placed a mixture containing 50 cc. dialysed potato juice, 50 cc. buffer solution and 0·05 cc. acetaldehyde; the one was stoppered and the other unstoppered. Two similar flasks were set up at the same time as controls, water displacing the enzyme preparations previously used. After standing for 24 hours each flask was tested for acetaldehyde by the p-nitrophenylhydrazine reaction as previously described. There was a greater amount of hydrazone formation in the experiments in which water had taken the place of the enzyme and also in the case of the stoppered flasks as compared with the unstoppered; the greatest difference was, however, *small* and it was concluded from the results that while the loss of acetaldehyde due to possible oxidation was by no means negligible, that due to evaporation could be ignored.

(*b*) *From glycine.* With a view to testing whether formaldehyde was produced from glycine by the action of tyrosinase, 0·25 g. of glycine dissolved in 50 cc. of buffer solution was mixed with 50 cc. of dialysed potato juice and 10 cc. of toluene·and exposed to the air in a 2-litre flask for 24 hours side by side with a control which contained no glycine. At the end of the period portions tested with Schryver's reagent gave no formaldehyde reaction and no formaldehyde could be detected in the distillate collected over ice in the manner previously described for acetaldehyde.

(*c*) *From phenylaminoacetic acid.* Folpmers' experiment on the formation of benzaldehyde has also been carefully repeated with negative results. 0·2 g. of phenylaminoacetic acid was placed in a mixture of 25 cc. dialysed potato juice, 25 cc. buffer solution and 10 cc. toluene; a similar control with water replacing the enzyme was set up. After 24 hours' exposure to the air no benzaldehyde formation could be demonstrated in either case. The phenylaminoacetic acid used in this experiment was prepared in the usual way by the hydrolysis of its nitrile hydrochloride and was carefully purified by recrystallisation from dilute acetic acid. It seemed possible that a contamination of the phenylaminoacetic acid with its nitrile as a result of incomplete hydrolysis might have accounted for Folpmers' results, so to test this 1 g. of the nitrile hydrochloride was subjected to the action of tyrosinase for 24 hours in the presence of air and the solution subsequently acidified and distilled.

From the distillate 0·42 g. of the *p*-nitrophenylhydrazone of benzaldehyde was obtained which was practically pure after one crystallisation. It appears therefore that the apparent deamination of phenylaminoacetic acid demonstrated by Folpmers may possibly have been due to the contamination of the phenylaminoacetic acid with its nitrile. In his paper, however, Folpmers does not state the source of the phenylaminoacetic acid which he used.

II. *The Alleged Ammonia Formation.*

The method of detecting ammonia formation was based on Folin's method of estimating ammonia in urine. The apparatus, which was in duplicate, consisted of three bottles, *A*, *B* and *C*, which were connected in series and air was aspirated through both sets by the same pump; *A* contained 20 % H_2SO_4 so that any air drawn through it would be ammonia free, *B* was the reaction bottle in which the enzyme-buffer mixture was placed and *C* the absorption bottle, contained 100 cc. of $N/50$ H_2SO_4.

The whole having been connected up, 10 cc. of toluene, 25 cc. of dialysed potato juice and 25 cc. of the buffer solution containing a definite amount of the amino acid were added to the reaction bottle *B*; a control mixture without the amino acid being placed in the reaction bottle of the duplicate series.

By means of a water pump the whole was aerated gently for 24 hours. At the end of this time about 4 g. of Na_2CO_3 and a few drops of capryl alcohol were added to each reaction bottle, and any free ammonia in the reaction bottle was drawn over into the respective absorption bottles.

The figures represent the cc. of $N/10$ NaOH used in the titration of the acid in the absorption bottles. Any formation of ammonia in the direct experiment would cause this figure to be lower than the figure from the control bottle. In no case was deamination observed.

	Experimental Readings	Average	Control Readings	Average
Alanine, 0·25 g.	19·90 19·94 19·95 19·95	19·93	20·01 19·99 19·95 19·99	19·98
Glycine. 0·5 g.	19·96 19·89	19·93	20·00 20·01	20·01
Phenylalanine, 0·25 g.	19·98 19·98	19·98	19·90 20·04	19·97
Tyrosine, sat. sol.	19·95 19·97	19·96	20·02 20·04	20·03
Phenylaminoacetic acid, sat. sol.	19·85 19·95	19·90	19·85 19·95	19·90

The small differences observed are within the experimental error.

Study of the Amino Nitrogen Content of the System during the Period of Aeration.

The reaction and control mixtures were aerated in the same apparatus as that used in the ammonia determination, samples were withdrawn at intervals from both mixtures and the amino nitrogen content of these estimated by means of the micro van Slyke method.

The amino acids studied were alanine, glycine and phenylaminoacetic acid; in the case of the first two, 0·5 cc. of the enzyme-buffer mixture was used for each determination, in the case of the last 3·5 cc. were taken. The figures given are in cc. of nitrogen at N.T.P. The amino acid was dissolved in a mixture of equal parts, enzyme and buffer solutions.

Amino acid	Time hours	Experiment cc.	Control cc.	Difference cc.
Alanine: 0·4645 g. in 50 cc.	0	1·33	0·26	1·07
enzyme-buffer solution	24	1·39	0·26	1·13
	30	1·41	0·29	1·12
Glycine: 0·45 g. in 60 cc.	0	1·50	0·36	1·14
enzyme-buffer solution	2	1·51	0·37	1·14
	26	1·55	0·41	1·14
Phenylaminoacetic: 0·0349	0	0·53	0·26	0·27
g. in 60 cc. enzyme-buffer	24	0·62	0·35	0·27
solution	26	0·62	0·35	0·27

These experiments show that the amount of amino nitrogen in the system in each case was unchanged after 24 hours or more.

As a useful comparison, figures were obtained showing the amount of tyrosine oxidised under the above conditions. The method adopted for the estimation was the modification of Miller's method devised by Raper and Wormall [1923]. A mixture of 60 cc. of the buffer solution containing 0·0264 g. of tyrosine and 60 cc. of dialysed potato juice having been divided into two parts 10 cc. of toluene were added to each, both portions being then aerated as in previous experiments. After 24 hours 0·0146 g. tyrosine was left, so that 0·0118 g. of tyrosine out of a possible 0·0264 g., or 44·7 %, had been oxidised.

Oxygen Absorption in Tyrosinase-Amino-Acid Mixtures.

If the alleged deamination of amino acids by tyrosinase is carried out by the same chemical processes which are involved when this enzyme acts on tyrosine then oxygen should be absorbed as it certainly is when tyrosine is the substrate. To investigate this experiments have been carried out in a Barcroft apparatus. In one bottle a buffered solution of the amino acid was used and in the other the buffer solution only. Toluene was present in each bottle and 0·2 cc. of enzyme was placed in the cup of each stopper. When equilibrium had been obtained with the bottles open to the air, they were connected to the manometer and the enzyme solution spilt into the bottles. Experiments were carried out with tyrosine (saturated solution in the buffer mixture), glycine and alanine, the amino acids all being used in approximately equimolecular quantities. No oxygen absorption was observed in 6 hours with glycine or alanine. There was, in fact, a slight increase in the case of alanine (12 mm.[3]). With tyrosine a considerable oxygen absorption occurred, which at the end of 18 hours measured 0·1053 cc. These experiments indicate therefore that no oxidation of glycine or alanine takes place under conditions in which a considerable oxidation of tyrosine can be demonstrated. They do not support the view that tyrosinase exerts an oxidative deamination on amino acids in general.

III. *The Effect of the Addition of Phenols and Related Substances to the System Tyrosinase-Amino-Acid.*

Preliminary experiments showed that deamination and a diminution in the amino N content of the system took place when tyrosinase acted upon glycine in the presence of p-cresol. A systematic study was therefore made of the influence of p-cresol on other amino acids in the presence of tyrosinase and the investigation was extended to include other phenols and allied substances. The phenols, etc., used were phenol, catechol, resorcinol, quinol and p-benzoquinone. Of these only p-cresol, phenol and catechol produced deamination. The results are tabulated below. The method of experiment was as follows. The amino acid was dissolved in 50 cc. of the phosphate buffer solution, 50 cc. of dialysed potato juice were added and the solution divided into two equal portions. To one 10 cc. of a solution of the phenol or related substance were added and to the other, which served as a control, 10 cc. of water. Toluene was added as an antiseptic. The amino N was determined in a small sample from each at the beginning of the experiment and usually after 24 and 48 hours' gentle aeration at room temperature. Finally, any ammonia produced was liberated by adding solid sodium carbonate and blowing it over into $N/50$ sulphuric acid. The titration figures given in the last column but one give the amount of $N/10$ NaOH used in the titration of the excess of acid not neutralised by ammonia.

Phenol etc. used	Amino acid used		Amino N in cc.			cc. $N/10$ NaOH	NH_3 liberated mg.	Deamination
			0 hr.	24 hr.	48 hr.			
p-Cresol, 0·4 %	Alanine, 0·5 g.	Exp.	2·36	2·08	1·72	18·10	4·5	Positive
		Cont.	2·34	—	2·33	20·75		
	Phenylalanine, 0·3 g.	Exp.	0·93	0·83	0·73	18·50	3·8	Positive
		Cont.	0·94	—	0·86	20·75		
	Leucine, 0·5 g.	Exp.	1·63	—	0·91	17·67	5·0	Positive
		Cont.	1·59	—	1·60	20·65		
Phenol, 1·0 %	Glycine, 0·4 g.	Exp.	2·27	1·81	1·33	15·28	8·8	Positive
		Cont.	2·22	—	2·21	20·48		
	Alanine, 0·5 g.	Exp.	2·26	1·85	—	18·65	3·0	Positive
		Cont.	2·29	2·29	—	20·42		
Resorcinol, 0·5 %	Glycine, 0·3 g.	Exp.	1·90	1·95	—	19·81	0	Negative
		Cont.	1·78	1·76	—	19·76		
	Alanine, 0·5 g.	Exp.	2·51	2·46	2·43	19·71	0	Negative
		Cont.	2·52	2·49	2·52	19·71		
Quinol, 1·0 %	Glycine, 0·45 g.	Exp.	2·46	2·49	—	19·86	0	Negative
		Cont.	2·45	2·41	—	19·71		
	Alanine, 0·55 g.	Exp.	2·82	—	2·82	19·61	0	Negative
		Cont.	2·85	2·83	2·80	19·61		
	Phenylalanine, 0·3 g.	Exp.	1·11	1·16	—	19·99	0·17*	Negative
		Cont.	1·04	1·10	—	20·09		
p-Benzoquinone, 1·0 %	Glycine, 0·25 g.	Exp.	1·62	1·59	—	19·48	0·1*	Negative
		Cont.	1·61	1·60	—	19·54		
	Alanine, 0·5 g.	Exp.	2·64	2·66	—	19·95	0·17*	Negative
		Cont.	2·64	2·67	—	20·05		
	Phenylalanine, 0·25 g.	Exp.	0·99	0·98	—	19·49	0	Negative
		Cont.	0·98	1·00	—	19·50		
Catechol, 0·5 %	Glycine, 0·25 g.	Exp.	1·84	1·73	—	18·36	2·4	Positive
		Cont.	1·85	1·87	—	19·78		
	Alanine, 0·55 g.	Exp.	2·84	2·37	—	18·28	2·3	Positive
		Cont.	2·84	2·83	—	19·62		

* These small amounts are within the experimental error.

Since only those phenols which were likely to give an *ortho*-quinone on oxidation caused deamination to take place, experiments were carried out with *o*-quinone itself and it was found that this substance attacked amino acids, even in the absence of tyrosinase, causing a liberation of ammonia and diminution in the amino N content of the reacting system. The *o*-quinone was prepared by Willstätter and Müller's method [1908]. The quinone is very unstable and two methods were used to demonstrate its action. In the first the quinone obtained from 0·25 g. catechol was thrown down from its solution in ether by the addition of light petroleum, washed with the latter solvent and then redissolved in ether. This solution was added to the solution of the amino acid (0·175 g. glycine in 60 cc. of the buffer mixture) and a similar solution to which no quinone was added served as a control. Aeration was carried out for 24 hours. The initial amino N figures were 2·01 and 1·99 cc. for the experiment and 2·02 and 1·99 for the control; the final figures were 1·91 and 1·90 cc. for the experiment and 2·00 and 1·99 cc. for the control. The $N/10$ NaOH used for the acid in the ammonia absorption bottle was 18·88 cc. for the experiment and 19·58 cc. for the control. Both these results therefore provided satisfactory evidence of deamination. In the second experiment, the quinone from 0·5 g. catechol was precipitated directly from the ether in the reaction bottle and was not further purified, hence it would contain a small amount of catechol. The amino acid solution was added directly to this and 0·25 g. catechol was added to the control solution in order to allow for any possible deamination due to catechol alone. An experiment with 0·2 g. of glycine gave the following results. Initial amino N figures: experiment 2·38 and 2·40 cc., control 2·43 and 2·45 cc.; final amino N: experiment 1·96 and 1·95 cc., control 2·49 and 2·51 cc. The titration figures were: experiment 15·32 cc., control 19·84 cc. An experiment with alanine (0·25 g.) carried out in the same way with the quinone from 0·25 g. catechol gave initial figures of 2·45 cc. and 2·44 for the experiment and control respectively and final figures of 2·285 cc. and 2·50 cc. The titration figures were: experiment 19·30 cc., control 20·00 cc.

The control solution containing catechol in these experiments coloured slightly to a faint pink during the 24 hours' aeration but it did not produce any deamination, consequently the assumption is justified that the observed deamination in the presence of catechol and *o*-quinone was due to the latter.

SUMMARY AND CONCLUSIONS.

1. No evidence of aldehyde formation, liberation of ammonia or a diminution in amino nitrogen has been found when potato tyrosinase acts upon glycine, alanine or phenylaminoacetic acid.

2. When tyrosinase acts upon tyrosine to form melanin or its precursors oxygen is absorbed, but no oxygen absorption occurs when equivalent molecular solutions of glycine and alanine are exposed to the action of the enzyme.

3. The ammonia formation observed by Chodat and Schweizer when tyrosinase acts upon amino acids is due to the *p*-cresol which was added to the reacting system. Phenol and catechol exert the same action but not resorcinol, quinol or *p*-benzoquinone.

4. *o*-Benzoquinone alone is capable of causing deamination of amino acids in the same way as the system *p*-cresol-tyrosinase.

5. It is suggested that the deamination which takes place when tyrosinase acts upon certain phenols in the presence of amino acids is due to the preliminary formation of an *ortho*-quinone derivative which then attacks the amino acid, with the liberation of ammonia and the formation of deeply pigmented substances (Chodat's *p*-cresol-tyrosinase reaction).

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