IV. A NOTE ON PLANT OXIDATION: THE NATURE AND REACTIONS OF THE SUBSTANCE "TYRIN."

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In an investigation of the oxidising systems of the potato, Szent-Györgyi [1925] obtained a preparation which, he states, plays an important rôle in this plant oxidation. This preparation, to which he gave the name "tyrin," is capable of "oxidation" to a red compound ("oxytyrin") by *ortho*-quinone or by the system potato oxidase-catechol. Szent-Györgyi represents the changes occurring in the potato as follows:

Mol. O_2 + oxidase + catechol → *o*-diketoquinone.
o-Quinone + leuco-tyrin (reduced form) → catechol + oxytyrin (red compound).
Oxytyrin + "active" hydrogen → leuco-tyrin.

According to Szent-Györgyi, "tyrin" is concerned in the oxidations of the potato and possibly other plant and animal tissues, as similar preparations could be obtained from various mammalian and other tissues, and he concludes that "tyrin" can be classified as a member of Palladin's group of respiratory pigments.

Happold and Raper [1925] have shown that tyrosinase will act upon phenolic compounds in the presence of amino-acids to give deeply pigmented substances only when the phenol is likely to furnish an *ortho*-quinone on oxidation. In these cases, pigment formation is accompanied by deamination. *o*-Benzoquinone alone produced the same results as the system tyrosinase-catechol (phenol or *p*-cresol). On these grounds a satisfactory explanation was afforded of the liberation of ammonia and pigment formation in the Chodat *p*-cresol-tyrosinase and similar reactions.

In the light of the experiments of Happold and Raper it appeared possible that the properties and reactions of "tyrin" were partly or wholly due to amino-acids, peptides or compounds of these with carbohydrates [cf. Borsook and Wasteneys, 1925]. Preparations of "tyrin" made by Szent-Györgyi's method have all contained amino-nitrogen in appreciable quantity and it has been found that the "oxidation" reactions of these preparations can be accounted for by the presence of nitrogenous compounds. The reactions of "tyrin"—the formation of a red compound on treatment with *o*-quinone or the catechol-oxidase system, the reduction of this compound with NaHS

etc.—can all be produced to approximately the same extent with solutions of various amino-acids or peptone containing the same amount of nitrogen. Thus "tyrin" appears to be a mixture of nitrogenous compounds, especially amino-acids, and to have no other significance in oxidation processes.

Preparation and properties of "tyrin."

The method of preparation was that described by Szent-Györgyi [1925], the first product being a thick oily substance, from which, however, it has been found possible to prepare a white stable powder by precipitation from a methyl alcohol solution with ethyl alcohol. The powder obtained had all the properties of the original sticky mass. Szent-Györgyi records a negative Millon's test for tyrosine, but a small amount of this amino-acid has been detected in all preparations, when care has been taken to avoid excess of the reagent. The presence of a trace of tyrosine has been confirmed by the Folin and Denis colour reaction [1912].

Analysis.	Total nitrogen (micro-Kjeldahl)	5·7 %	3·6 %
	Free amino-nitrogen (formaldehyde titration)	1·4 %	1·3 %

Relationship between "tyrin" and amino-acids.

If the pigmentation of "tyrin" under the influence of *o*-quinone or the catechol-oxidase system is due to the amino-acids present, liberation of ammonia should occur as in the experiments of Happold and Raper [1925]. A definite increase in the ammonia content of the solution during this pigmentation was observed, the determinations being carried out wherever possible by two methods, the aeration method (after addition of potassium carbonate), and the permutite filtration method of Whitehorn [1923]. Experimental details have been described previously [Happold and Raper, 1925; Raper and Wormall, 1925].

Solutions of glycine, alanine and glutaminic acid containing the same amount of nitrogen as a solution of "tyrin" were prepared and the properties of all solutions compared in the following respects.

(*a*) Red pigment formation with *o*-quinone—this pigmentation was approximately equal in the case of "tyrin" and glycine and somewhat less in the other amino-acids.

(*b*) Red pigment formation in the presence of catechol and potato oxidase at p_H 6·4—similar results to those in (*a*).

(*c*) Pigmentation with *o*-quinone after treatment with neutral formaldehyde—"tyrin" and amino-acid solutions were treated with varying amounts of neutral formaldehyde, and the solutions neutralised and tested with *o*-quinone after the addition of buffer solution at p_H 6·4. All solutions still retained the power of reacting with the quinone to form red compounds.

(*d*) Pigmentation with *o*-quinone after treatment with nitrous acid—"tyrin" solutions and amino-acid solutions behaved identically, the ability to react with *o*-quinone being destroyed almost completely in every case.

From these results the conclusion has been drawn that amino-acids, free and combined, present in the "tyrin" are solely responsible for the red pigment formation with *o*-quinone or with the catechol-oxidase system. Equivalent amounts of some of the more reactive amino-acids or peptone will give pigment formation to the same extent. "Tyrin" appears to contain, among other substances, a mixture of amino-acids, some simple peptides and compounds of these with reducing sugars.

THE CATECHOL-OXIDASE SYSTEM. *o*-QUINONE AND PEROXIDE FORMATION.

Szent-Györgyi [1925] produced evidence that the guaiacum reaction obtained with potato oxidase in the presence of catechol is due to the formation from the catechol of *o*-quinone and that the enzymes present take no further part in the blueing of guaiacum. These conclusions are based on the fact that if potato oxidase is allowed to act on catechol for 10 minutes at 37° and the enzymes precipitated with methyl alcohol, the enzyme-free filtrate will blue guaiacum. *o*-Quinone prepared by the method of Willstätter and Müller [1908] gave the same blueing of guaiacum, was inactivated by excess of catechol and showed the same general lability as the substance formed in the catechol-oxidase reaction. Proof of the identity of the reactive substance formed in the enzyme reaction was not furnished, but, recently, evidence of the formation of *o*-quinones from phenolic compounds under the action of the tyrosinase oxidase has been published [Happold and Raper, 1925; Raper, 1926]. Szent-Györgyi therefore divides the blueing of guaiacum by the catechol-oxidase system into two phases:

(*a*) catechol + oxidase + oxygen → *o*-quinone,

(*b*) oxidation of guaiacum by the oxide of catechol (*o*-quinone),

for which no enzyme is required.

The removal of enzymes from the solution was effected by Szent-Györgyi by the addition of methyl alcohol and filtration; but this does not preclude the possibility of small amounts of peroxidase or oxidase being present in the filtrate. These experiments, with additional controls, have therefore been repeated, using a method which is easy to carry out and which does not appear to be open to this objection. Potato oxidase was allowed to act on a catechol solution at p_H 6·4 for 10 minutes, the solution extracted with ether and the ethereal solution tested with guaiacum and benzidine. Positive results were obtained, while all the controls—catechol alone, enzyme alone and catechol + heated enzyme—gave negative results. Since these experiments were carried out, Onslow and Robinson [1926] have also confirmed the observations of Szent-Györgyi, a method involving precipitation of the enzymes with "dialysed iron" being used. As these authors admit, however, the use of "dialysed iron" in connection with guaiacum is open to objection, although their control tests gave negative results.

Experimental procedure. The enzyme used in these experiments was prepared by the method used by Szent-Györgyi [1925] which is based on that of Onslow [1920].

The following solutions *A*, *B*, *C* and *D* were prepared:

	0·1 % catechol cc.	Phosphate buffer (p_H 6·4) cc.	Distilled water cc.	Potato oxidase mg.
A	2	5	3	100
B	—	5	5	100
C	2	5	3	—
D	2	5	3	100 (heated)

The solutions were incubated at 37° for 5 minutes, each solution was shaken thoroughly with 15 cc. pure ether, and the ethereal extracts were pipetted off and filtered. These ether extracts were tested in the following manner with an alcoholic solution of guaiaconic acid and 1 % alcoholic benzidine.

	A	*B*	*C*	*D*
3 cc. ether extract + 3 drops alcoholic benzidine	Blue	—	—	—
3 cc. ether extract + 3 drops alcoholic guaiaconic acid + 2 cc. distilled water	„	—	—	—

These experiments have been repeated a large number of times and in no case did the controls give any colour. Specially purified ether has been used for the extractions, but this does not appear to be essential, for negative controls are obtained with once distilled ether. The blueing of guaiacum and benzidine in ethereal solutions appears to preclude absolutely the possibility of enzyme interference in this phase of the reaction.

In the course of an investigation of the production of peroxides from catechol and other phenols, solutions containing catechol and potato oxidase have been examined for hydrogen peroxide, with positive results. Onslow and Robinson [1926] have also demonstrated the production of hydrogen peroxide in such solutions and in solutions of catechol which had been allowed to oxidise by exposure to the air for several days.

This formation of hydrogen peroxide in the catechol-oxidase reaction, although slight, for most of that formed will quickly be decomposed by the catalase present or utilised by the peroxidase for oxidation purposes, can readily be demonstrated by the following method, which does not necessarily involve the use of ether. This solvent usually contains a peroxide which will give the titanium sulphate test for hydrogen peroxide and therefore has to be specially purified if used in connection with these tests.

Experimental procedure.

	1·0 % catechol drops	Phosphate buffer (p_H 6·4) cc.	Distilled water drops	Potato oxidase mg.
A	5	5	—	50
B	5	5	—	—
C	5	5	—	50 (heated)
D	—	5	5	50

These solutions were dialysed in parchment sacs against 20 cc. distilled water in each case. Samples (2 cc.) of each dialysate were withdrawn after 5, 10 and 30 minutes and tested for hydrogen peroxide by the addition of 0·5 cc. of 25 % H_2SO_4 and varying amounts of titanium sulphate in 5 % H_2SO_4. The presence of peroxide, presumably H_2O_2, in the dialysate from solution *A* only was indicated by a slight yellow coloration. A stronger reaction with the titanium sulphate reagent was obtained by carrying out the reactions on a larger scale, acidifying the dialysate with H_2SO_4 and concentrating under reduced pressure. The concentrated dialysates gave a yellow coloration only in the case of solution *A*. The slight yellow colour obtained with some of these controls was apparently due to the catechol, since no colour was produced if the solutions had been extracted previously with purified ether, as suggested by Onslow and Robinson [1926]. This treatment, however, in no way affected the positive peroxide tests with solution *A*.

Conclusions.

1. The "tyrin" preparation of Szent-Györgyi has been shown to contain appreciable amounts of amino-acids.

2. All the oxidative properties attributed to this preparation can be explained by the presence in it of free or combined amino-acids and can be imitated exactly by a mixture of amino-acids. "Tyrin" therefore plays no significant rôle as a respiratory pigment.

3. The suggestion made by Szent-Györgyi as to the mechanism of the blueing of guaiacum by the catechol-oxidase system has been confirmed by a method which precludes the possibility of enzymic action in the second part of the reaction. The production of hydrogen peroxide in addition to this oxidation product (presumably *o*-quinone), which blues guaiacum directly, has also been demonstrated.

REFERENCES.

Borsook and Wasteneys (1925). *Biochem. J.* **19**, 1128.
Folin and Denis (1912). *J. Biol. Chem.* **12**, 245.
Happold and Raper (1925). *Biochem. J.* **19**, 92.
Onslow (1920). *Biochem. J.* **14**, 535.
Onslow and Robinson (1926). *Biochem. J.* **20**, 1138.
Raper (1926). *Biochem. J.* **20**, 735.
Raper and Wormall (1925). *Biochem. J.* **19**, 84.
Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.
Whitehorn (1923). *J. Biol. Chem.* **56**, 751.
Willstätter and Müller (1908). *Ber. deutsch. chem. Ges.* **41**, 2580.