STUDIES ON OXIDASE ACTIVITY IN POTATO TUBERS

I. o-PHENYLENEDIAMINE AS A COLORIMETRIC REAGENT *

by

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INTRODUCTION

The ability of certain plant extracts, (from mushrooms, potatoes, etc.) to oxidixe monophenols, such as cresol and tyrosine, as well as polyphenols, such as catechol, with the aid of molecular oxygen has been variously attributed to the action of a single enzyme (tyrosinase) or of multiple enzymes (monophenolase, polyphenolase). The reaction passes through a series of intermediate, more or less well-defined stages and leads eventually to insoluble, dark pigments designated as melanin. The subject has been reviewed by RAPER [1], and, more recently, by NELSON and DAWSON [2].

In the case of the potato, the reaction is apparently due to the action of a single enzyme tyrosinase, upon the substrate, tyrosine [2]. It is known that this amino acid occurs in free form in potato tubers [3].

In the enzymatic oxidation of monophenols, the first step presumably consists in the introduction of a second OH-group in the ortho position to the one already present, turning the mono into a polyphenol. From there on, further stages in the process are the same as in the polyphenol oxidation [1]. In the intact potato tuber, this reaction is prevented by the spatial separation of enzyme and substrate. When the substrate and tyrosinase are brought into contact, in the presence of molecular oxygen, through rupture of the tissue cells, as in peeling or dicing, or by freezing, bruising or bacterial infection, the oxidation of tyrosine is initiated, and a grey discoloration occurs on the potato surface.

In the industrial dehydration process, cut potato dices or slices are „blanched" in boiling water or steam to inactivate the tyrosinase prior to dehydration in the air tunnel. Sulfite has also been employed for the purpose. This blanching greatly improves the quality of the dehydrated vegetables [4]. Excessive blanching, however, impairs the quality of the products [5] so that a precise control of the blanching procedure is of considerable importance.

To test for the completeness of blanching, various test methods have been employed for determining residual enzymatic activity, involving the application of indicators susceptible to enzymatic oxidation on the cut surface of the potato slice

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or dice [6] e.g., guaiacol, benzidine, or gum guaiac for peroxidase ; catechol and p-cresol for oxidase activity.

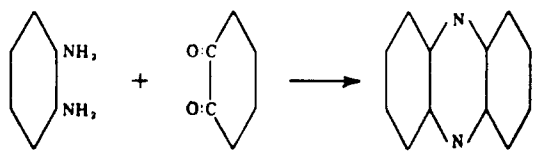
The enzymatic discoloration tendency of potatoes has also been determined photometrically by the degree of spontaneous color formation through the interaction of potato oxidase and potato substrate, presumably tyrosine, in macerated tuber tissue under standard conditions [7].

In the present experiments, the aim was to measure the colorforming capacity of a potato sample by trapping one of the intermediary products of the melanin formation and to form a stable complex which could readily be determined by colorimetric means.

Principle of Method

It was anticipated that the o-quinones formed in the course of the tyrosine melanin reaction [7] might react with *o-phenylenediamine* to form colored condensation products.

The relatively low p_H (6.0) of the potato maceration juice would favor a condensation of the following type [8] :



o-phenylene diamine + o-quinone → phenazine

It was indeed found that, when raw potato dices were mechanically dispersed in a medium containing o-phenylenediamine and in the presence of oxygen, a highly colored compound was formed which was water-soluble and, unlike the spontaneous coloration of the juice itself, was extractable with organic solvents. No coloration was produced in completely blanched potatoes, and the depth of color formed with any given sample was found to be an inverse function of the extent of blanching.

In aqueous solution, the coloration became first yellow then orange and finally deep orange-red on prolonged standing, but the greying or blackening that occurs when the potato extract alone is exposed to air over prolonged periods, and which is due to melanin formation, did not take place in the presence of sufficient o-phenylenediamine. The absence of melanin formation would be anticipated if one of the intermediates were fixed by the o-phenylenediamine. We propose provisionally to term the orange colored compound, „Tyrophenazine" on the assumption that it owes its formation to a condensation of the kind indicated above.

Stabilization of the color formed at any stage of the reaction could be readily brought about by extraction with ethyl-acetate, or in the presence of high concentrations of acetone. The latter procedure proved to be more convenient, since the maceration juice, after adding acetone, could be readily clarified by simple filtration because of the precipitation of the colloids and fine particles by the acetone. The depth of color of the acetone-water solution is then measured photoelectrically.

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EXPERIMENTAL

The potato samples are peeled and cut into uniform dices of approximately 1 cubic centimeter volume and kept under water as much as possible. When a number of determinations are carried out on a given batch, the dices are washed in a beaker for one hour under running tap water to eliminate exposed matter from the cut cells.

In the blanching experiments, dices were cut to about 1 cubic centimeter size. A sieve was placed in a boiling water bath. The dices were dropped into the sieve which was withdrawn from the bath after a measured number of seconds. The volume of the bath must be sufficient so that the boiling temperature is maintained despite the addition of the cold dices.

O-phenylenediamine dihydrochloride (Eastman Kodak Company) was made up freshly in 1% solution. 10 ml of this reagent were intimately mixed with 50 g of potato dices, together with 240 ml of water, using a Waring Blendor. Blending was carried out for a period of two minutes, after which the mixture was strained through a cotton plug into a 500 ml Erlenmeyer flask. The temperature was maintained in the range of 25° C to 30° C.

After 15 minutes had passed from the beginning of the blending, 10 ml of the strained maceration extract were pipetted into 40 ml of acetone, which stops the enzyme reaction. The mixture is filtered through Whatman Z 5 filter paper by gravity into a colorimeter tube, and the color is determined in a Klett-Summerson photoelectric colorimeter with a blue No. 42 filter against a control consisting of 80% acetone and 20% water. The acetone extract is found to be stable with respect to color for at least four hours, and virtually stable even after 24 hours.

RESULTS

To determine the reproducibility of the method, seven 50 gm batches of washed potato dices were macerated in presence of o-phenylenediamine solution as described.

The results obtained are shown in Table I.

TABLE I

REPRODUCIBILITY OF COLOR FORMATION WITH O-PHENYLENEDIAMINE

Experiment	Color Reading of 80 % Acetone Solution	Deviation	% Deviation	Reading After 4 Hours
1	320	—13	—3.8	320
2	350	17	5.1	345
3	330	— 3	—0.9	332
4	345	12	3.6	340
5	325	— 8	—2.4	329
6	334	1	0.3	334
7	325	— 8	—2.4	319
Average	333	——	—.—	332

It will be seen that the method is reproducible within a variation of some 5% from the mean and that the color of the acetone extracts is stable over a period of at least 4 hours.

To determine the relationship between color formation and reaction time, a series of potato maceration extracts were pipetted into acetone after 3, 5, 10, 15, 20, 25 and 30 minutes had passed since addition of the o-phenylenediamine.

The comparative color formation in seven different samples of Green Mountain and California potatoes is illustrated in Figure 1.

It will be seen that the rate of color formation in all seven samples follows a closely

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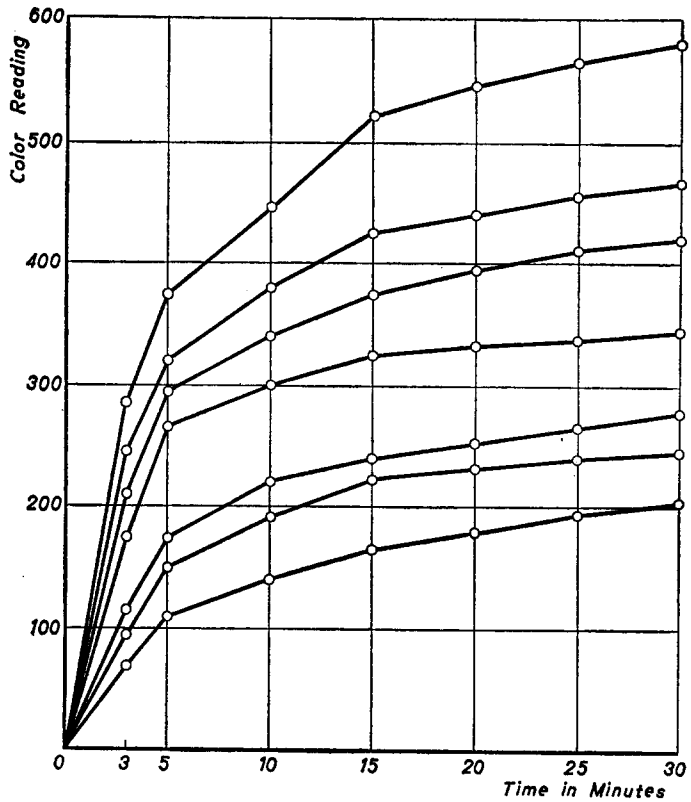

Fig. 1. Comparative Rate of „Tyrophenazine" Formation.

similar pattern, although the color depth varies with the individual samples used. This is presumably due to variations in enzymatic activity of the tubers.

Six different batches of potatoes were subjected to the previously described blanching procedure, the dices being held in boiling water for 10, 20, 30, 40, 50 and 60 seconds. In all cases, the reaction with the o-phenylenediamine was stopped after 15 minutes. The effect of blanching on the color formation is illustrated in Figure 2.

It will be seen that despite a variation of some two hundred and fifty percent in color formation by the original samples, complete inhibition of „Tyrophenazine" formation is achieved in all six samples by blanching for sixty seconds, and that the degree of color formation is in inverse relation to the blanching time.

To determine the effect of temperature on the rate of „Tyrophenazine" formation, the blending was carried out at temperatures ranging from 7° C to 65° C. The results are shown graphically in Figure 3. It appears that maximum color formation occurs in the vicinity of 40° C, but that not much lower values are obtained within the range of 25—30° C selected for the test, mainly for reasons of convenience.

The effect of the hydrogen ion concentration was determined by the addition of phosphoric acid and sodium hydroxide solution to the mixture in the Blendor prior to maceration, and the degree of color formed was compared with that produced in the unadjusted mixture. See Figure 4.

The hydrogen ion concentration was determined in the blended extracts by means of a BECKMANN p_H meter.

It will be seen that maximum color formation occurred near p_H 5.0, which was the p_H of the unadjusted mixture. This is more acid than the p_H of the potato juice alone which is approximately p_H 5.9.

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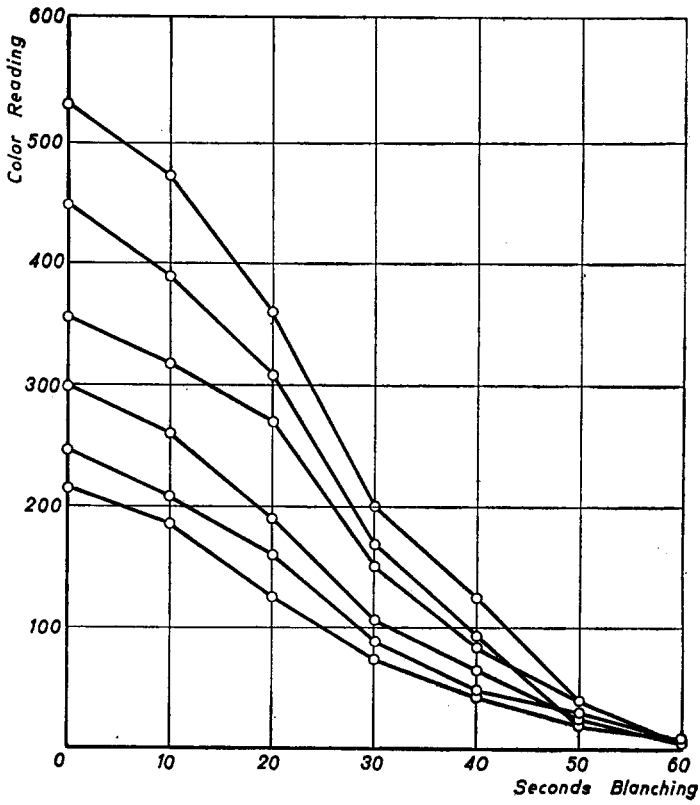

Fig. 2. Effect of Blanching on „Tyrophenazine" Reaction.

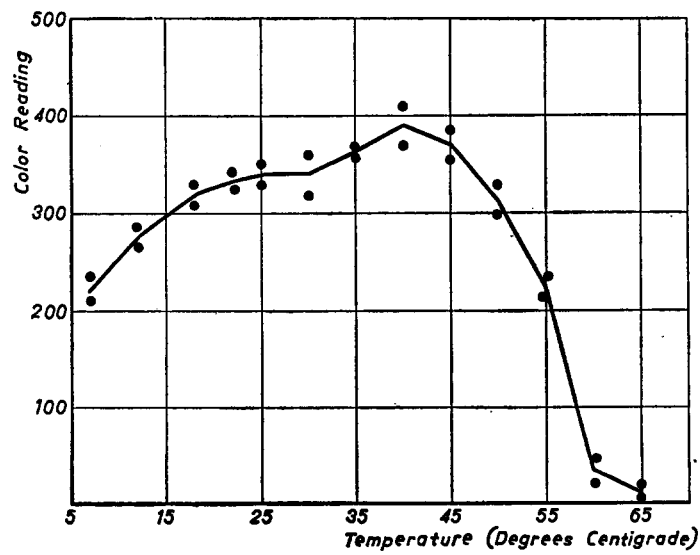

Fig. 3. Influence of Temperature on „Tyrophenazine" Formation.

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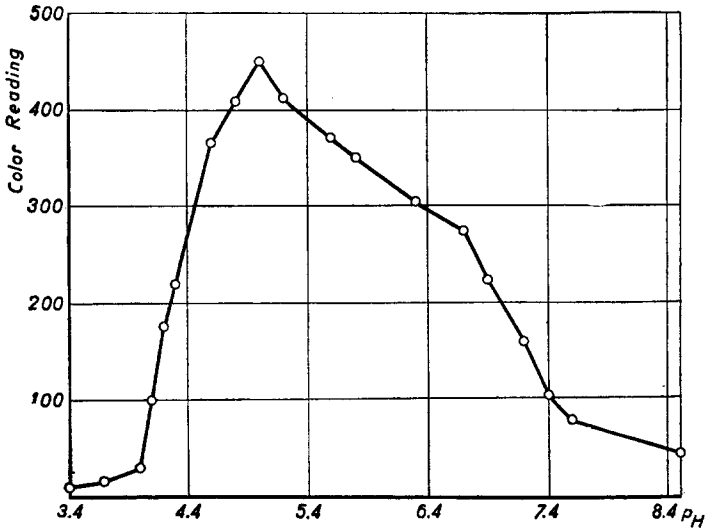


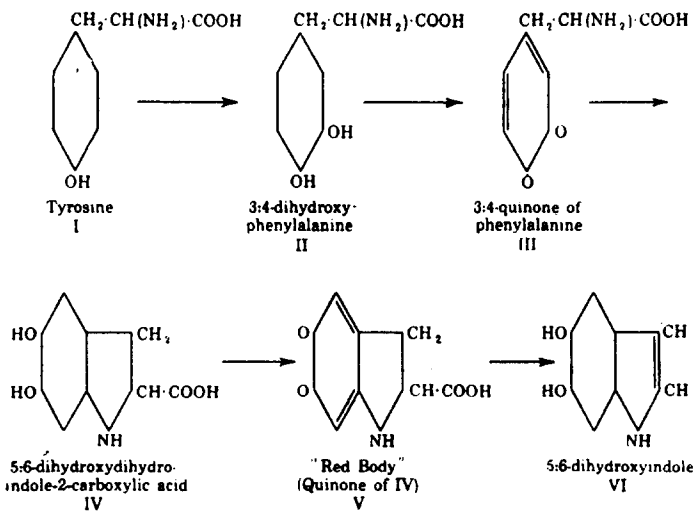
Fig. 4. Comparative Formation of „Tyrophenazine" at Varying Hydrogen-Ion Concentrations.

Preliminary experiments indicated that the extent of color formation increases somewhat with increasing oxygen supply.

Purified potato phenolase, prepared essentially according to KUBOWITZ [11] showing an activity of 66 catecholase units/ml when tested by DAWSONS's chronometric method[11], failed to give rise to appreciable color formation when added to an o-phenylenediamine solution of the same concentration as employed in the present study. The addition of a maceration extract from fully blanched potatoes to the system resulted in the formation of color, indicating that the production of „Tyrophenazine" is not simply due to a direct oxidation of the o-phenylenediamine. That a substrate present in the potato tissue is involved in the reaction is further supported by the observation that the formation of „tyrophenazine" is markedly increased by the addition of blanched potato extract to the mixture of raw potato extract and o-phenylenediamine.

DISCUSSION

The sequence of events leading from tyrosine to melanin has been pictured by RAPER [1]:



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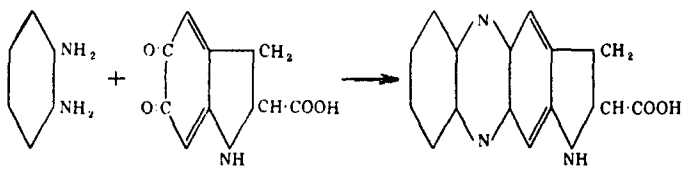
The 5 : 6 dihydroxyindole (VI) through loss of hydrogen, further uptake of oxygen, and condensation yields melanin, the end-product of the process.

Of the various intermediates shown in the schema, the orthoquinoid forms (III and V) would seem to be the most likely to react with the reagent employed in this study.

The tendency of compounds containing two adjacent CO groups to form condensation products with o-phenylenediamine, has been utilized in a number of reactions. Thus, the coupling of alloxan with o-phenylenediamine in neutral or weakly acid solution to yield alloxazine was described by KUEHLING [8]. A number of alloxazines, substituted in the benzene and the alloxan ring of the molecule, have been prepared in an analogous manner by K. G. STERN and his co-workers [9].

In the instance of o-quinones as reaction partners, phenazines are formed as shown in page 3. The condensation of o-phenylenediamine with the „Red Body" isolated by RAPER as intermediate in the tyrosine-tyrosinase reaction (5 : 6-dioxydihydroindole-2-carboxylic acid), would be expected to yield the following four-ring heterocyclic compound:



o-phenylene diamine | "Red Body" of Tyrosine Oxidation | "Tyrophenazine"

The actual chemical structure of the orange-colored compound here observed is at present under investigation. Its behavior on reduction with hydrosulfite and reoxidation with air, both in mineral-acid, weakly acid, and neutral solutions, indicates that it represents a *reversible* oxidation-reduction system as would be expected of a compound of the type cited above.

It should be mentioned that the action of purified horse radish peroxidase and H_2O_2 on o-phenylenediamine hydrochloride produces a strongly *red* colored compound which may also be reduced and reoxidized reversibly, but which in detail shows some differences in behavior with „Tyrophenazine". The direct oxidation of o-phenylenediamine would be expected to yield as a primary product o-phenylenediamine which might then condense with unchanged o-phenylenediamine to form a phenazine derivative. Thus it is known that $FeCl_3$ oxidizes o-phenylenediamine in weakly acid solution to 2 : 3-diaminophenazine [12].

SUMMARY

1. When an aqueous solution of o-phenylenediamine reacts with macerated potato tissue in presence of air, a pronounced coloration occurs which is at first yellow and then orange and finally deep orange-red. The color is extractable in various organic solvents. It may be conveniently stabilized in 80% acetone which also permits ready filtration and photometric determination of the clarified extract.

2. For a given sample of potatoes, the amount of coloration formed is in inverse relation to the blanching time and is completely inhibited in fully blanched potatoes.

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Maximum color formation was found to occur when the reaction is carried out at 40° C and at a hydrogen concentration in the vicinity of p_H 5.0.

3. The method was found to be reproducible within about 5%, under the conditions described. Various samples of potatoes differing widely in their final coloration showed a similar pattern of color increase.

4. The possible mechanism of the underlying reaction is discussed in terms of a condensation of the o-phenylenediamine with an orthoquinoid intermediate of the tyrosinase-tyrosine reaction to form a phenazine-like compound. For this reason, the provisional name *„Tyrophenazine"* is suggested for the orange-red compound observed in the present experiments.

RÉSUMÉ

1. Quand une solution aqueuse de o-phénylène-diamine réagit sous l'action de tissus de pommes de terre macérés en présence de l'air une coloration prononcée se produit. Cette coloration passe du jaune à l'orangé et finalement devient rouge-orange. Le colorant peut être extrait au moyen de divers solvants organiques. Il peut être stabilisé dans l'acétone à 80 %, ce qui permet également la filtration et la détermination photométrique rapides de l'extrait clarifié.

2. Pour un échantillon donné de pommes de terre, le degré de coloration obtenu est inversement proportionnel à la durée de blanchiment et la coloration est complètement inhibée dans les pommes de terre entièrement blanchies. On a trouvé que la coloration atteint son degré maximum quand la réaction a lieu à 40° C et quand la concentration d'ions de l'hydrogène, est voisine de p_H 5.0.

3. On a trouvé que la méthode est reproductible dans les limites de 5% environ dans les conditions décrites. Divers échantillons de pommes de terre, dont la coloration finale différait notablement, présentaient la même gamme de coloration.

4. Le mécanisme que l'on suppose être à la base de la réaction est une condensation de la o-phénylène-diamine avec un intermédiaire orthoquinoïde de la réaction tyrosinase-tyrosine formant un composé semblable à la phénazine. C'est pourquoi le terme *„tyrophénazine"* a été proposé pour désigner provisoirement le composé rouge-orange observé dans les expériences en question.

ZUSAMMENFASSUNG

1. Wenn eine wässerige o-Phenylendiaminlösung mit maceriertem Kartoffelgewebe in Anwesenheit von Luft reagiert, tritt eine starke Färbung auf, die zunächst gelb ist, dann orange und schliesslich tief orange-rot wird. Der Farbstoff kann durch verschiedene organische Lösungsmittel extrahiert werden. Er kann in 80-prozentigem Aceton stabilisiert werden, wodurch auch eine schnelle Filtrierung und photometrische Bestimmung des geklärten Extraktes ermöglicht wird.

2. Für eine gegebene Kartoffelprobe steht der Färbungsgrad in umgekehrtem Verhältnis zur Bleichdauer ; bei vollkommen gebleichten Kartoffeln wird die Färbung vollständig verhindert. Maximale Färbung tritt ein, wenn die Reaktion bei 40° und einer Wasserstoffionenkonzentration von p_H ca. 5.0 stattfindet.

3. Unter den beschriebenen Bedingungen ist die Methode innerhalb einer Grenze von 5% reproduzierbar. Verschiedene Kartoffelproben, deren Endfärbung grosse Unterschiede aufwies, zeigten trotzdem einen ähnlichen Anstieg der Färbung.

4. Der möglicherweise der Reaktion zugrunde liegende Mechanismus wird als Kondensation des o-Phenylendiamins mit einem orthochinoiden Zwischenprodukt der Tyrosinase-Tyrosin-Reaktion diskutiert, unter Bildung einer phenazinähnlichen Verbindung. Aus diesem Grunde wurde vorläufig der Name *„Tyrophenazin"* für die in den vorliegenden Experimenten beobachtete orangerote Verbindung vorgeschlagen.

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