forms a compact mass of thick, irregular prisms from alcohol, melting at 187° (uncorr.). It is moderately soluble in alcohol, soluble in chloroform, somewhat soluble in hot benzene and very sparingly soluble in ether. With cold concentrated sulfuric acid it forms a golden solution, which becomes intensely red on adding a crystal of potassium nitrate. It dissolves in hydrochloric acid with a golden color and in hot acetic anhydride with an intense deep-green color. Luchini's reagent gives a red color; Wenzell's reagent, transient violet; Erdmann's reagent, orange red; Mandelin's reagent, blood red, becoming golden on heating; Fröhde's reagent, brownish, blood-red when heated.

Anal. Calcd. for $C_{20}H_{21}O_6N$: C, 67.54; H, 5.67. Found: C, 67.22; H, 5.94.

When warmed on the steam-bath with methyl alcoholic potash, the compound rapidly changes into papaveraldine, identified by comparison and mixed melting point determinations with the authentic compound and with the alkaloid xanthaline.

Summary

1,2-Dihydropapaverine, hitherto unknown, has been synthesized and its structure established. In the course of the work, papaverine and papaveraldine have been obtained by a new series of reactions.

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[CONTRIBUTION FROM BOYCE THOMPSON INSTITUTE FOR PLANT RESEARCH, INC., YONKERS, NEW YORK]

AN IODIMETRIC METHOD FOR DETERMINING OXIDASE ACTIVITY[1]

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The usual methods for estimating oxidase activity depend on either the production of a colored substance,[2] or the measurement of the volume of oxygen absorbed.[3] Recently an electrometric method has been suggested.[4] Colorimetric methods are often inapplicable on account of pigments or turbidity in the extracts to be tested. Methods measuring the oxygen uptake require special apparatus. The method to be described here requires no unusual equipment, is easy to use and reasonably accurate. With it as many as sixteen determinations have been made at the same time.

In a previous paper,[5] it was noted that potato juice contains a substance or substances that may be titrated with iodine in acid solution (trichloroacetic acid) and that this titration decreases on exposure to air. Ordinarily five cc. of juice reduces about 0.5 cc. of $N/100$ iodine. However, juice from one lot of potatoes was found to reduce 2.0 cc. of $N/100$ iodine. It was thought that this might be due to a low content of oxidase, the substance responsible for the iodine reaction not being oxidized in the

[1] Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 6.

[2] J. A. Dye, *Proc. Soc. Exptl. Biol. Med.*, **24**, 640–642 (1927).

[3] H. H. Bunzel, THIS JOURNAL, **34**, 303–316 (1912).

[4] A. E. Stearn and A. A. Day, *J. Biol. Chem.*, **85**, 299–306 (1929).

[5] F. E. Denny, L. P. Miller and J. D. Guthrie, *Am. J. Botany*, **17**, 483–509 (1930).

process of extraction for this reason. In order to test this point, juice of this lot of potatoes was boiled and filtered. It still reduced 2.0 cc. of $N/100$ iodine. To 50-cc. portions of this boiled, filtered juice, 1 cc. of fresh juice from the same lot of potatoes and also 1 cc. from a lot giving the usual iodine titration were added. On exposing these mixtures to air in a thin layer, the iodine titration of the one containing the juice suspected of being low in oxidase had decreased 0.8 cc. after forty-five minutes, while the titration of the one containing juice of the other lot had decreased 1.4 cc. A water blank decreased 0.2 cc.

These results suggested that if a substance could be found that would reduce iodine in acid solution and which would also be oxidized by the air in the presence of potato juice, a convenient method would be available for determining oxidase. Cysteine was first tried. Its oxidation is catalyzed by potato juice, but the reaction proved to be autocatalytic and therefore unsuitable for the purpose. The autocatalytic nature of the oxidation of cysteine has been previously noted.[6]

Szent Györgyi[7] has noted the iodine reaction of plant juices and has isolated a substance that reduces iodine in acid solution from the adrenal cortex, orange and cabbage. He finds it to be a hexuronic acid. For this reason glucose that had been warmed with dilute sodium hydroxide was tried, since this product is known to contain a great variety of carbohydrate derivatives and it was thought that some of these might reduce iodine in acid solution. It was found that the iodine titration was quite large. Tests showed that it decreased on exposure to air and that this oxidation was catalyzed by potato juice.

Preliminary Work.—During the first part of the work, the reaction was carried out in liter beakers, 25 cc. of the reacting mixture being exposed in a thin layer in the bottom. At intervals, 5-cc. aliquots were drawn, 10 cc. of 10% trichloro-acetic acid was added and titrated with $N/100$ iodine, using starch as an indicator. The first difficulty encountered was a high blank, but it was found that clearing the substrate with decolorizing charcoal obviated this. It was also found that the conditions for aeration were unsatisfactory, since the rate of oxidation ceased to be directly proportional to the concentration of enzyme when more than 1 cc. of potato juice was used. Carrying out the reaction in aeration tubes corrected this difficulty. This improvement necessitated the use of a foam breaker. Capryl alcohol was tried, but all samples available interfered with the end-point, probably due to some impurity. It also had a slight retarding effect. Amyl alcohol, while not affecting the end-point, was decidedly injurious to the enzyme. Paraffin oil, although not so efficient a foam breaker as the higher alcohols, was finally chosen. In

[6] M. Dixon and H. E. Tunnicliffe, *Proc. Roy. Soc.* (London), **94B**, 266–297 (1923).

[7] Szent Györgyi, *Biochem. J.*, **22**, 1387–1409 (1928).

order to test the effect of foaming, the addition of digitonin was tried. It greatly increased foaming but did not affect the results. As an additional improvement it was found that more accurate results could be obtained by adding a known quantity of iodine, allowing to stand and then titrating the excess with thiosulfate.

Preparation of Substrate.—Dissolve 40 g. of glucose in 400 cc. of *N* sodium hydroxide, place it in a 500-cc. Florence flask and immerse in a water-bath at 80° for fifteen minutes. Remove and neutralize at once by adding 10 cc. of 85% phosphoric acid. Add 25 g. of decolorizing charcoal (Norit A was used) and allow to stand overnight. Filter and add 25 g. of decolorizing charcoal to the filtrate. Allow to stand for fifteen minutes and filter. Dilute a small portion of the filtrate about one to five and determine the P_H value. If it is not close to P_H 6.5, adjust to this P_H with *N* sodium hydroxide or *N* hydrochloric acid. The addition of 2 cc. of either to 100 cc. of the filtrate shifts the acidity about 0.1 P_H. The iodine value for 25 cc. should be equal to about 60 cc. of *N*/50. Before using, dilute the filtrate with an equal volume of water.

The Method.—Pipet 25-cc. portions of the diluted substrate into Van Slyke–Cullen[8] aeration tubes. Add 2 cc. of the juice or extract containing the enzyme. For each determination run a blank, using 2 cc. of the boiled, filtered juice or extract. Add five drops of paraffin oil to each tube and aerate for one hour. Wash into 300-cc. Erlenmeyer flasks containing 25 cc. of 10% trichloro-acetic acid, adding in all about 50 cc. of water. Add 50 cc. of *N*/50 iodine in *N*/10 potassium iodide and allow to stand for thirty minutes. Titrate with *N*/100 sodium thiosulfate, using 1 cc. of 1% starch paste as an indicator. Titrate the blank first and the determination immediately afterward. The difference between these titrations is a measure of the oxidase activity of the sample.

Accuracy of the Method.—In order to test how nearly results could be duplicated, twelve determinations were made on the same lot of potato juice. The average value was 6.15 cc. with an average error of ±0.15 cc. To see how nearly the substrate could be duplicated, four batches were prepared and used with the same potato juice. The average value was 6.50 cc. with an average error of ±0.2 cc. This was repeated with four other batches of substrate and other potato juice. The average value was 6.8 cc. with an average error of ±0.15 cc.

Choice of P_H Value.—Acidity of the reacting medium greatly affects the activity of enzymes. Therefore, a series of determinations was made at various P_H values. These were obtained by the addition of *N* sodium hydroxide or *N* hydrochloric acid to the substrate. The quinhydrone electrode was used. Some difficulty was experienced, probably due to the interference of reducing substances. On the concentrated substrate values that were too alkaline were obtained. More acid values, which are believed to represent nearly the true P_H, were obtained by diluting with about five volumes of water and using a large amount of quinhydrone. This effect is probably brought about by diluting the interfering substances and thereby minimizing their effect. Several experiments were made to determine the effect of the P_H value of the substrate. A curve showing one of these, which is typical of the others, is shown in Fig. 1. Between P_H 6.0 and P_H 7.0 the oxidase activity is not greatly affected. Therefore, P_H 6.5 was chosen as the best for carrying out the determinations. Bunzell[9] has investigated the effect of hydrogen-ion concentration on oxidase activity and recommends approximate neutrality for making the determinations. His data, however, are insufficient to show the exact form of the P_H–oxidase curve, especially between P_H 6.0 and 7.0.

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[8] D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, **19**, 211–218 (1914).

[9] H. H. Bunzell, *ibid.*, **28**, 315–333 (1916).

Effect of Concentration of Enzyme and Time of Aeration.—In order to test the effect of the concentration of enzyme and decide on the time of

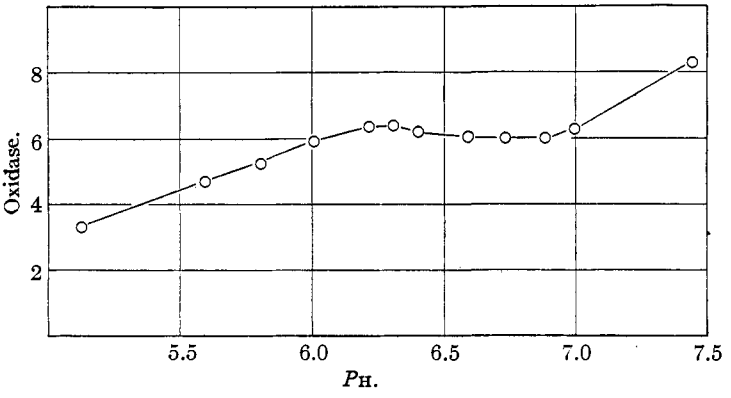
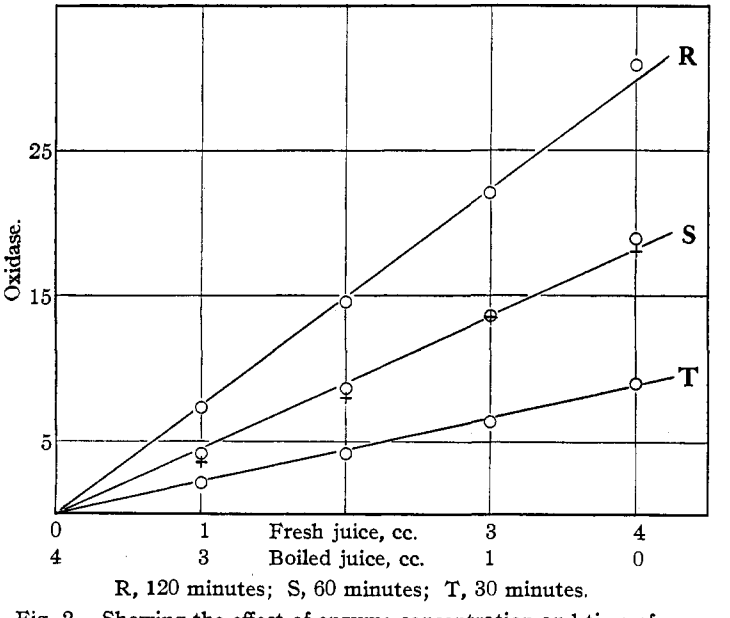


Fig. 1.—Showing the effect of hydrogen-ion concentration on the oxidase activity of potato juice. Aerated for one hour; two cc. of juice used.

aeration, experiments were made using one, two, three and four cc. of potato juice and aerating for different periods. In the first experiments



R, 120 minutes; S, 60 minutes; T, 30 minutes.

Fig. 2.—Showing the effect of enzyme concentration and time of aeration. Circles determined with usual substrate concentration, crosses with half this concentration.

which were aerated for two hours the oxidase activity was not linear with the concentration, but curved upward. This tendency was negligible

when the aeration was only for one hour. It was found that the reason for this was the protective action of the juice, the enzyme in the tubes containing the larger amounts of juice being better protected. This protective action is present in the boiled, filtered juice. Therefore, an experiment was made in which the tube with 1 cc. of fresh juice contained also 3 cc. of boiled juice, the tube with 2 cc. of fresh juice, 2 cc. of boiled juice, and so on. Thus each tube contained 4 cc. of juice, but different amounts of enzyme in each. The results are shown in Fig. 2. It will be seen that the oxidase activity is almost linear with the concentration of enzyme even with two hours' aerating. Up to one hour the reaction is linear with time. In the second hour the reaction goes more slowly, showing that some enzyme is being destroyed. Therefore, one hour has been chosen for the time of aeration. Points are also given for the one-hour aeration curve in which half the usual concentration of substrate was used. Very little difference is noted, showing that in this range substrate concentration is not an important factor.

Results with Other Plants.—In order to see if the methods could be used on other materials besides potato juice, several other plant juices were tried. The results are shown in Table I. Qualitative tests were also made with the indophenol reagent.[10] The quantitative results correlate well with the qualitative.

TABLE I

APPLICATION OF METHOD TO VARIOUS PLANTS. TWO CC. OF JUICE USED UNLESS OTHERWISE NOTED

Plant	Iodimetric oxidase		Indophenol oxidase
Onion (bulb)	0.6	0.4	−
Turnip (root)	0.3	0.3	−
Beet (root)	7.7	8.3	++
Beet (leaves)	7.6	7.7	++
Carrot (root)	2.3	2.5	+
Apple (fruit)	2.6	2.2	++
Tomato (leaves) 0.5 cc.	11.8		+++++
Tomato (stems) 0.5 cc.	1.3		++
Tobacco (healthy leaves) 0.5 cc.	4.4		+++
Tobacco (mosaic leaves) 0.5 cc.	12.8		+++++

Summary

An iodimetric method is given for the estimation of oxidase activity. Oxidase activity as measured by the method is a linear function of the enzyme concentration. The effect of hydrogen-ion concentration on potato oxidase has been studied. The method is applicable to a variety of plants.

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[10] H. M. Vernon, *J. Physiol.*, **42**, 402–432 (1911).