[OFFICE OF DRUG-PLANT, POISONOUS-PLANT, PHYSIOLOGICAL, AND FERMENTATION INVESTIGATIONS.]

THE MEASUREMENT OF THE OXIDASE CONTENT OF PLANT JUICES.[1]

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Introduction.

The importance of the presence of oxidizing enzymes in plants is becoming more and more evident. The work of Palladin[2] and of others strongly emphasizes their fundamental role in the respiration of plants. The work of Woods[3] in this Bureau bears out their significance in diseases of plants. Furthermore their causal relationship to color production in plants,[4] their important part in the darkening of tea,[5] as well as in that of bread during its making[6] and in the production of the smooth black and hard lacquer of the Japanese from the white fluid soft secretion of the tree *Rhus vernicifera*[7] is well established.

[1] By permission of the Secretary of Agriculture. Communicated at the summer meeting of the American Chemical Society at San Francisco, 1910, and at the winter meeting of the same society at Minneapolis, 1910.

[2] Palladin, W., "Bildung der verschiedenen Atmungsenzyme in Abhängigkeit von dem Entwicklungs-stadium der Pflanzen," *Ber. Bot. Ges.*, **24**, 97–107 (1906). "Die Arbeit der Atmungsenzyme der Pflanzen unter verschiedenen Verhältnissen," *Z. physiol. Chem.*, **47**, 406–451 (1906). "Über die Wirkung von Giften auf die Atmung lebender und abgetöteter Pflanzen, sowie auf Atmungsenzyme," *Jahrbücher Wiss. Botanik*, **47**, 431–461 (1910).

[3] "The Destruction of Chlorophyll by Oxidizing Enzymes," *Centralbl. Bakteriologie*, **5**, 745–754 (1899). "Observations on the Mosaic Disease of Tobacco," U. S. Dept. of Agr., Bureau of Plant Ind., *Bull.* **18**, 17–22 (1902).

[4] Agulhon, H., "Influence de la réaction du milieu sur la formation des mélanines par oxydation diastasique," *Compt. rend.*, **150**, 1066–68 (1910). Palladin, W., "Synergin, das Prochromogen des Atmungspigmentes der Weizenkeime," *Biochem. Z.*, **27**, 442–449 (1910). "Die Verbreitung der Atmungschromogene bei den Pflanzen," *Ber. Bot. Ges.*, **26a**, 378–389 (1908). Bourquelot, E., and Bertrand, G., "Le bleuissement et le noircissement des champignons," *Compt. rend. soc. biol.*, [10] **2**, 582–584 (1895). Bailey, I. W., "Oxidizing Enzymes and Their Relation to 'Sap-stain' in Lumber," *Bot. Gaz.*, **50**, 142–147 (1910). Bourquelot, E., and Fichtenholz, A., "Nouvelles recherches sur la glucoside du poirier, son rôle dans la production des teintes automnales des feuilles," *Compt. rend. soc. biol.*, **69**, 605–607 (1910). Combes, R., "Du rôle de l'oxygène dans la formation et la destruction des pigments rouges anthocyaniques chez les végétaux," *Compt. rend.*, **150**, 1186–1189 (1910).

[5] Aso, K., "On the Role of Oxydase in the Preparation of Commercial Tea," *Bull. Coll. Agr. Tokyo*, **4**, 255–259 (1901).

[6] Boutroux, L., "Le pain et la panification," Paris, **1897**, p. 184. Bertrand, G., and Mutermilch, W., "Sur la Tyrosinase du son de Froment," *Bull. soc. chim.* [4] **1**, 837–841 (1907).

[7] Bertrand, G., "Sur le latex de l'arbre à laque," *Compt. rend.*, **118**, 1215–1218 (1894). "Recherches sur le latex de l'arbre a laque du Tonkin," *Bull. soc. chim.*, [3] **11**, 717–721 (1894).

These are only a few examples selected from the extensive literature on the subject,[1] but they fully demonstrate the great need of careful and thorough studies of this class of substances.

Nearly all of the experiments attempting to correlate the oxidase content with biological processes in plants have been of a qualitative nature.

The time has now come when mere qualitative study of enzymes is inadequate. This became particularly evident in the course of some biochemical investigations upon certain pathological conditions of important agricultural crops undertaken in this laboratory in coöperation with other divisions of the Bureau of Plant Industry. Among these conditions were the mosaic disease of tobacco, the curly-top of beets, and diseases of cabbage and spinach on the truck farms of Norfolk. For the first of these Woods[2] long ago demonstrated changes in the oxidase mechanism. Work in this laboratory has raised the question whether the other conditions mentioned may not also present symptoms of this general type. It was found impossible to settle this question without determining the oxidizing power of these tissues and extracts quantitatively. Unfortunately no sufficiently accurate quantitative methods suitable for the purpose exist. It therefore became necessary to devise such. This task has been undertaken by the writer, and the present report is the first step in the solution of this problem.

The various methods, which in the past have been used in investigations of this sort, are briefly reviewed in an article by Foà[3] and are discussed in detail in *Bulletin* **238,** Bureau of Plant Industry, Department of Agriculture. In addition to the criticisms made by Foà there are many other, at least as serious objections, to the use of colorimetric methods in the measurement of oxidizing enzymes. In the first place the tissue extracts available are rarely clear and colorless, but generally grayish and turbid, due among other things to the partial oxidation and subsequent precipitation of the chromogens contained in them. Since artificially prepared color standards are free from colored suspended

[1] Issajew, W., "Über die Malzoxydase," *Z. physiol. Chem.*, **45,** 331–350 (1905). Kelley, W. P., "The Influence of Manganese on the Growth of Pineapples," Hawaii Agr. Exp. Station, *Press Bull.* **23,** 14, Honolulu, 1909. Lagatu, H., "Sur la casse des vins; interprétation nouvelle basée sur le rôle du fer," *Compt. rend.*, **124,** 1461–1462 (1897). Bouffard, A., and Semichon, L., "Contribution à l'étude de l'oxydase des raisins. Son utilité dans la vinification," *Compt. rend.*, **126,** 423–426 (1898). Cazeneuve, P., "Sur le ferment soluble oxydant de la casse des vins," *Compt. rend.*, **124,** 406–408 (1897). Lépinois, E., "Note sur les ferments oxydant de l'aconit et de la belladone," *J. pharm. et chim.*, [6] **9,** 49–52 (1899). Lindet, L., "Sur l'oxydation du tannin de la pomme à cidre," *Bull. soc. chim.*, [3] **13,** 277–279 (1895).

[2] Woods, A. F., *loc. cit.*

[3] "Eine Methode graphischer Registrierung einiger Gährungsvorgänge," *Biochem. Z.*, **11,** 382–399 (1908).

matter, the comparison becomes very difficult and at the best inaccurate. On the other hand if the oxidase-containing solutions are freed from such disturbing constituents, or if only very small amounts of the juice to be studied are used, the methods based on color comparison become very unreliable. What is badly needed is a method applicable to the juice or extract freshly prepared from the plant tissue, the accuracy of which will be enhanced rather than impaired by the use of larger amounts of material. Fresh plant juices always contain appreciable amounts of protein in solution. It is well known that all proteins, being amphoteric colloids, are capable of combining with or absorbing colored compounds of all sorts. Since in the manipulations referred to in connection with the methods mentioned above no more than a small amount of the colored substance is formed, an appreciable error is introduced whenever fresh tissue juice is used, especially since the protein-dye combination is frequently insoluble.

Methods based on the measurement of the quantity of precipitate produced in the course of the oxidation of water-soluble substances to insoluble compounds are more reliable, but very limited in their applicability. Fürth and Jerusalem[1] measured the tyrosinase content of mushrooms by the volume of the melanin precipitate produced. The method of Bach and Chodat[2] which is based on the weighing of the purpurogallin formed in the presence of hydrogen peroxide and peroxidase is so well known that it does not require description. The first method here mentioned is inaccurate, the second tedious, owing to the number of weighings, and neither is of general applicability.

As Foà points out, the methods most satisfactory for the measurement of the rate of reactions involving oxygen absorption are those in which the quantities of oxygen absorbed are determined by measuring the changes of pressure within the reaction flask. The present publication deals with the description of such a method.

A manometric method has been devised and used successfully by A. P. Mathews in his work on the spontaneous oxidation of the cell constituents.[3] Similar methods have also been used by many other observers for the sake of obtaining a measure of the respiratory enzymes present, but none have observed all of the precautions necessary in such measurements. The precautions to be observed as well as the drawbacks of all methods of this type are described in detail in the bulletin.

[1] "Zur Kenntnis der melanotischen Pigmente und der fermentativen Melaninbildung," *Beitr. chem. Physiol. Path.*, 10, 131–173 (1907).

[2] Chodat, R., "Darstellung von Oxydasen and Katalasen tierischer und pflanzlicher Herkunft. Methoden ihrer Anwendung." Abderhalden, E., "Handbuch der Biochemischen Arbeitsmethoden," Berlin, 1910, Vol. III, Pt. 1, pp. 42–74.

[3] *J. Biol. Chem.*, 6, 3–20 (1909). Mathews, A. P., and Walker, Sidney, *J. Biol. Chem.*, 6, 29–37 (1909).

In most of the experiments described in this paper, potatoes furnished the oxidase preparations. These were used for a number of reasons. They are easily obtainable at all times of the year, and can be readily grown for experimental purposes if it is found desirable to study the variation of oxidase content with varying conditions. Numerous experiments by other observers show that they are rich in oxidases. They seemed therefore the best test object for elaborating the method.

The potatoes used were rinsed off with cold water, and wiped dry with a clean towel. They were peeled, and the peelings ground up in a meat chopper. The juice was obtained by pressing the pulp through a piece of silk cloth. In all the experiments only fresh juice was used. The juice of beet leaves was obtained by pressing it from the cleansed leaves after grinding them in a meat chopper. Neither the potato nor the beet juice was filtered through paper. Since the activity of the juice undoubtedly depends to some extent on the mode of preparation, a method will be devised in the near future by which uniformity in this process can be assured.

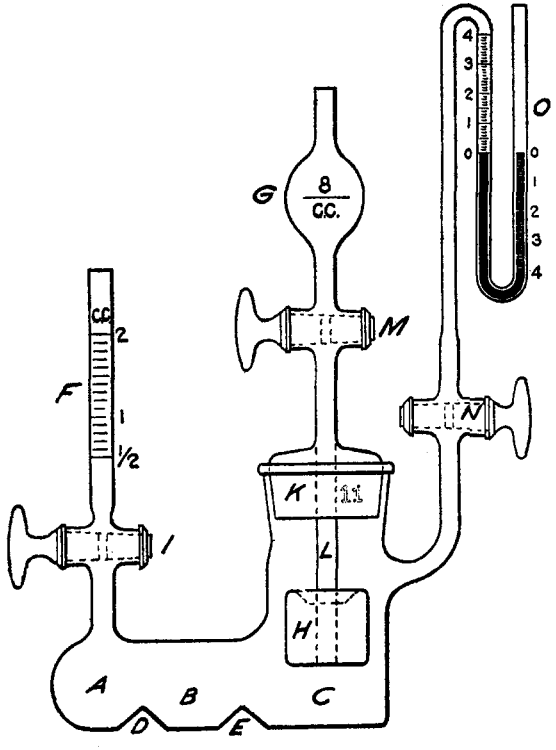

Fig. 1.

To start with it was decided to use pyrogallol as the substance to be oxidized.

It is obvious that all the experiments have to be carried out at a constant temperature. For this purpose an air thermostat was constructed by means of which the temperature could be maintained constant within 0.1–0.2°. A shaking machine was also made especially for the purpose.[1]

The procedure of the actual measurement is as follows: The oxidase apparatus (Fig. 1) is clamped on the carriage of the shaking machine in the thermostat.[1] Eight cc. of fresh 1% pyrogallol solution are measured into compartment C by means of bulb G. Two cc. of plant juice are measured into compartment A from buret F. Basket H is charged with 1 cc. normal sodium hydroxide solution. Only stopcock I is left closed. Then the interior of the thermostat is heated to the temperature desired and maintained there. About 30 minutes after the temperature of experimentation is reached, the windows of the thermostat are opened enough to allow the introduction of the arm and the stopcock M closed. Now the shaking machine is brought into action at an approximate rate of 5 complete excursions in 3.3 seconds.

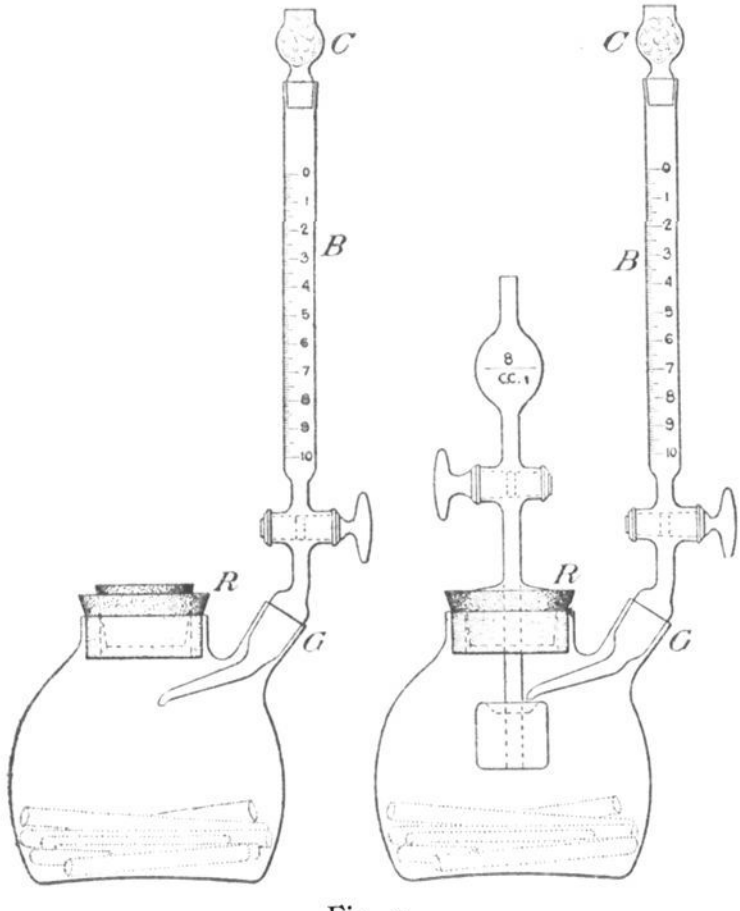

Fig. 2.

[1] Illustrations and descriptions of these apparatus are to be found in *Bull.* 238, Bureau of Plant Industry.

At 15-minute intervals the shaking is interrupted long enough to read the manometers. When the oxygen absorption has come to an end, as indicated by the identity of successive manometric readings, the experiment is considered completed.

If it is desired to determine approximately the carbon dioxide formed during the experiment, the ground joint *K* with the basket *H* is lifted off, a drop of phenolphthalein added and the basket *H* placed in the titration flask shown in Fig. 2. By rotating the buret *B* about the ground joint *G*, its tip is brought just above the basket. 0.1 *N* sulfuric acid is run into the basket during slow agitation until the appearance of the red color. The buret is then read; three drops of Congo red solution are placed in the basket and the titration continued until the bright red color disappears. From the difference between the two end points the amount of carbon dioxide absorbed may be calculated. In this fashion it is possible to carry out the titration in an atmosphere practically free from carbon dioxide, since the titration flask contains solid potassium hydroxide.

Experiments on the Effect of the Variable Factors Involved in the Method on the Total Oxygen Absorption.

In all of the experiments to be described in this publication the pressure readings on the manometer are given and these values reduced to the arbitrary volume of 150 cc. An absolute unit of oxidase content will be discussed at the end of the paper, but will not be made use of in the results given since the latter are only relative and have value only in proving the efficiency of the method. The juice used was freshly prepared for each experiment and, as it differed in each, different values were necessarily obtained from experiment to experiment.

TABLE I.—THE EFFECT OF VARYING THE CONCENTRATION OF PYROGALLOL.

Number of apparatus.	Volume of pyrogallol solution. cc.	Concentration of pyrogallol solution. Per cent.	Volume of potato juice. cc.	Content of glass basket.
1.....	8	10	2	1 cc. *N* NaOH
4.....	8	10	2	1 cc. *N* NaOH
5.....	8	5	2	1 cc. *N* NaOH
7.....	8	5	2	1 cc. *N* NaOH
11.....	8	2.5	2	1 cc. *N* NaOH
12.....	8	2.5	2	1 cc. *N* NaOH

11.20 A.M., put into thermostat; 11.45 A.M., began to shake. Rate of shaking—5 complete excursions, 3.1 seconds.

Table II shows that the absorption of oxygen comes to an end in the course of about 2 hours. In the following tables only this end result will be given. The full details have been included in the tables of the Bulletin.

TABLE II.

Time of reading of manometer.	Time elapsed since beginning of experiment expressed in minutes.	Temperature at the time of measurement expressed in degrees centigrade.	Manometer readings expressed in centimeters of mercury in apparatus. No. 1.	No. 4.	No. 5.	No. 7.	No. 11.	No. 12.
11.45	0	36.4	0.00	0.00	0.00	0.00	0.00	0.00
12.00	15	36.4	0.50	0.60	0.52	0.55	0.70	0.65
12.15	30	36.4	0.62	0.80	0.90	0.80	1.00	1.05
12.30	45	36.4	0.85	0.95	0.98	0.80	1.20	1.10
1.30	105	36.4	1.25	1.40	1.32	1.60	1.70	1.30
1.45	120	36.4	1.40	1.60	1.40	1.60	2.20	1.40
2.00	135	36.5	1.50	1.58	1.45	1.65	3.20	1.50
2.15	150	36.4	1.58	1.80	1.60	1.80	Pyr. sol. splash into bulb.	1.60
2.30	165	36.5	1.70	1.80	1.70	1.80		1.60
Final readings corrected to a volume of 150 cc.[1]			1.62	1.83	1.72	1.87		1.42

TABLE III.

Volume of pyrogallol solution used 8 cc.
Volume of potato juice used .....2 cc.
Content of glass basket.....1 cc. *N* NaOH.

Concentration of pyrogallol solution used. Per cent.	Manometer readings expressed in centimeters of mercury in apparatus. corrected to a volume of 150 cc.
16.0	2.11
8.0	2.48
4.0	2.64
2.0	2.60
1.0	2.86
0.5	2.76

In the following series fresh juice was used, different from that in the experiment just described.

0.80	1.39
0.40	1.46
0.20	1.22
0.10	0.31

The results shown in Tables I–III show definitely that the concentra-

[1] The apparatus used in all the experiments described in this paper (Fig. 1) have the approximate volume of 150 cc. They fluctuate in actual volume from 136 cc. to 156 cc. and all the corrections have to be made in order to make the volumes comparable. The apparatus of the latest form have the volume of 87 cc. and are all of the same size within 1 cc. This particular volume was chosen so that the actual volume of gas in the apparatus during the experiment would be 76 cc. and a change in pressure of 1 cm. of mercury under these conditions would correspond to the absorption of 1 cc. of oxygen. These apparatus may be obtained from Machlett & Son, 143 East 23d St., New York City.

tion of the pyrogallol solution has no appreciable effect on the end result, provided the concentration is above a certain lower limit, which in the experiments cited is a little more than 0.20%. It appears that a certain quantity of potato juice is capable of bringing about the oxidation of a very definite quantity of pyrogallol; quantities of pyrogallol present beyond this amount remain unoxidized. No attempt was made in the course of the work here described to determine exactly the smallest quantity of pyrogallol required; the experiments were carried out solely for the purpose of finding the concentration of pyrogallol solution necessary to obtain comparable results.

From Experiment 3 it is apparent that very great concentrations of pyrogallol, such as 16%, have a slight retarding action on the oxidation. This is especially noticeable in the rate with which the end point is reached.

On the strength of the results of these experiments it was decided to use, at least for the present, a 1% pyrogallol solution in all of the experiments to be made.

TABLE IV.—THE COMPARATIVE EFFECTIVENESS OF FRESH AND OF OLD PYROGALLOL SOLUTIONS.

Volume of pyrogallol solution used in each experiment 8.0 cc.
Volume of potato juice ..... 2.0 cc.
Volume of *N* NaOH solution used in basket ..... 1.0 cc.

Strength and age of pyrogallol solution used.	Final pressure in apparatus expressed in terms of cm. of mercury, corrected to a volume of 150 cc.
1.0% old solution	1.82
1.0% fresh solution	1.92
0.1% old solution	0.79
0.1% fresh solution	0.83

This experiment was undertaken to determine whether it is necessary to prepare a fresh pyrogallol solution at the beginning of each experiment. As the experiment shows, the very old solution gives the same result as that freshly prepared and therefore no precautions need be taken in this respect. The difference between the results of the first pair and the second pair of the series is due to the low concentration of the pyrogallol in the latter (see Table III).

The fact that a definit quantity of juice is capable of bringing about the oxidation of a definit quantity of pyrogallol by a definit quantity of oxygen, led to the supposition that the total quantity of oxygen consumed in the oxidation of the pyrogallol in the presence of excess of the latter is directly proportional to the concentration of the oxidase in the juice. To test this hypothesis fresh potato juice was diluted with varying volumes of water and the activities compared. The results are given in Table V.

TABLE V.

Volume of 1 % pyrogallol solution..... 8.0 cc.
Volume of *N* NaOH used in basket.... 1.0 cc.

Amount of potato juice used. Cc.	Amount of water added to potato juice. Cc.	Manometer readings expressed in centimeters of mercury in apparatus.
2.0	0	1.73
2.0	0	1.89
1.0	1.0	0.97
0.5	1.5	0.38
0.5	1.5	0.48

This experiment shows that the total oxygen absorption is at least approximately proportional to the quantity of potato juice present. What the exact relationship is between the concentration of potato juice and the quantity of oxygen absorbed will be determined later.

Effect of Concentration of Alkali in the Absorption Basket on the Result.—It was necessary to determine the strength of the alkali solution required to insure prompt and complete absorption of the carbon dioxide produced during the experiments. For this purpose a number of experiments were carried out with varying concentrations of alkali in the basket, all other conditions being uniform.

TABLE VI.

Volume of 1 % pyrogallol solution. 8.0 cc.
Volume of fresh potato juice..... 2.0 cc.

Strength of NaOH used.	Manometer readings expressed in centimeters of mercury in apparatus.
2.50 *N*	2.10
1.00 *N*	1.73
0.50 *N*	2.08
0.25 *N*	1.98
0.10 *N*	1.73
0.00	0.80

As these results show, it is necessary to use at least 0.25 normal solution of sodium hydroxide to make sure of the complete removal of the carbon dioxide formed during the oxidation of the pyrogallol. To be certain of an excess of alkali, 1 cc. of a normal solution was used in all these experiments. There seems to be no doubt about the fact that under these conditions the absorption of the carbon dioxide from the atmosphere of the flasks is practically complete. This is borne out by the fact that in all of the experiments the reaction comes to completion within a few hours. If a measurable amount of carbon dioxide were unabsorbed in the oxidase apparatus, the pressure as indicated by the manometer would diminish as the shaking is continued until practically all of the carbon dioxide is absorbed.

Effect of the Rate of Shaking.—In the experiments cited, the oxidase apparatus were shaken at a rate of 5 complete excursions of the

machine in 3.0 seconds. Under these conditions in some cases a small amount of pyrogallol splashed into the alkali in the small glass basket (Table II). This became noticeable at once by an increased rate of oxygen absorption and the failure of the absorption to come to completion. It is impossible to overlook such an error for the following reasons: no experiment is taken into account unless the diminution of pressure comes to a definite end in the course of a few hours. To avoid accidents due to the splashing of pyrogallol into the basket the rate of shaking henceforth was reduced to 5 complete excursions in 3.3 seconds. Under these conditions, as will be seen, no difficulty due to splashing was ever experienced.

Effect of Temperature.—The temperature in the thermostat varied, as experiments given in Table VII shows, no more than 0.1° and it is certain that the maximal variations within the oxidase apparatus were less than that. Since the pressure is directly proportional to the absolute temperature, a rise of 0.1° at 36.4° will involve an increase of pressure of $\frac{76.0}{309.4 \times 10}$, *i. e.*, 0.025 cm. of mercury. This is not greater than the errors involved in the measurements of the pressure existing within the oxidase apparatus.

TABLE VII.

Time of reading of manometer.	Time elapsed since beginning of experiment expressed in minutes.	Temperature at the time of measurement expressed in degrees centigrade.	Manometer readings expressed in centimeters of mercury in apparatus					
			No. 1.	No. 4.	No. 5.	No. 7.	No. 11.	No. 12.
10.00	0	36.4	0	0	0	0	0	0
			run in					
10.30	30	36.5	1.80	0	0	0	0	0
				run in				
11.00	60	36.4	2.10	1.25	0	0	0	0
					run in			
11.30	90	36.5	2.35	1.65	1.10	0		
						run in		
12.00	120	36.5	2.42	1.70	1.40	1.00	0	
							run in	
12.30	150	36.4	2.70	1.90	1.50	1.35	1.20	
								run in
1.00	180	36.4	2.80	2.00	1.80	1.50	1.50	1.40
1.30	210	36.4	2.75	2.10	1.70	1.50	1.50	1.70
2.00	240	36.4	2.80	2.20	1.90	1.75	1.80	1.80
2.30	270	36.4	2.80	2.20	1.90	1.75	1.80	1.90
Final readings. Corrected to a volume of 150 cc.....			2.67	2.24	1.93	1.82	1.73	1.68

9.40 put into box; 10.00 began to shake. Rate of shaking—5 complete excursions, 3.4 seconds.

Effect of Shaking on the Activity of the Potato Juice.—Since it is known from the work of Meltzer, Schmidt-Nielssen and others that many of the enzymes lose their activity on vigorous shaking, it seemed advisable to see whether the potato juice loses its activity to any extent on account of the shaking during the experiments. This was hardly to be expected, since the rate of shaking employed was never very rapid.

To test the point in question three experiments were carried out as follows: Oxidase apparatus were clamped to the carriage of the shaking machine and the baskets charged with 1 cc. of normal sodium hydroxide. Into each apparatus were put 2 cc. of potato juice and 6 cc. of water. Two cc. of a 4% pyrogallol solution were placed in each of the graduated pipettes. In this fashion, after mixing, the usual dilutions were obtained. The pyrogallol solution was run into one apparatus just before the shaking was begun, into another some time later, into a third still later, and so on.

These results bring out a very remarkable fact. If the potato juice is shaken for 15–30 minutes before the addition of the oxidizable substance, its oxidizing power is reduced to about half its original value. On longer shaking no further effect is noticeable. Potato juice shaken for $2^1/_2$ hours gives approximately the same result as that shaken only one hour. Whatever change the juice suffers takes place in the first hour of the experiment. The activity after this period is still quite appreciable and does not suffer any measurable loss on further shaking for 2–3 hours, which is the maximum duration of the measurements. This indicates that two phases of the process are dealt with, each one of which may be measured separately. In order to get the total oxidizing effect of the plant juice it is necessary to make the measurements right from the beginning when the shaking is begun.

These experiments very clearly point out the conditions under which the experiments must be carried out in order to obtain comparable results. The details of the method are based on these experiments and are described in an earlier part of this paper. The experiments also show that by means of this method it is possible to obtain quite accurate and reliable results, as shown by numerous parallel experiments carried out. It is true that in some of the duplicate experiments, especially in the beginning of the investigation, there are differences in the end results of from 2–3 mm. or even more (Experiments 11 and 12, Table II), but these differences become smaller as the work advances and the writer's experience with the apparatus grows.

It is intended to take up the results obtained and discuss their significance in a later paper on the mode of action of the oxidases in plant juices.

Practical Application of the Method to the Study of the Curly-Top Disease of Beets.

The Division of Cotton and Truck Diseases of the Bureau of Plant Industry, Department of Agriculture, has for some years been investigating the curly-top disease of sugar beets.[1] Through the courtesy of Mr. W. A. Orton and Mr. H. B. Shaw, the writer was able to obtain for experimental purposes fresh samples of sugar-beet leaves affected by this disease to a striking degree and also samples of normal beet leaves. All of the beets, of which the leaves were examined, were grown in a greenhouse, and therefore were subjected to practically uniform conditions. The leaves were treated in the same way as the potato peelings.

TABLE VIII.

	Juice used.	Manometer readings expressed in centimeters of mercury in apparatus.	Grams of CO_2 absorbed by alkali.
1.	Juice of normal beet leaves	1.16	0.0015
2.	Juice of normal beet leaves	1.07	.....
3.	Juice of diseased beet leaves	5.61	0.0050
4.	Juice of diseased beet leaves	4.30	0.0040
5.	Juice of normal beet leaves	1.10	0.0014
6.	Juice of normal beet leaves	1.17	0.0016
7.	Juice of diseased beet leaves	2.72	0.0031
8.	Juice of normal beet leaves	1.19	0.0018
9.	Juice of normal beet leaves	1.21	
10.	Juice of diseased beet leaves (showing only slight symptoms)	1.51	

Experiments given in Table VIII show a very striking difference between the juice of the normal and that of the diseased beet leaves. In all of the experiments the oxidase content as indicated by the oxygen absorption of the pyrogallol in the presence of the juice is markedly greater in the diseased than in the healthy leaves. The oxidase content of the normal leaves seems to be fairly constant, while the juice of the curly-top beet leaves shows wide variations. The leaves used in Experiment 3 give about 5 times as high a figure as normal leaves, while the leaves chosen in Experiment 10 show a variation of only 25% from the normal. It is very interesting to note that the deviation in oxidase content of the pathological leaves, as measured by the method described, runs parallel with the appearance of the leaves. The plants used in Experiment 3 showed very marked signs of curly-top, the leaves being small and shriveled, and the hairy roots abundant, while the diseased beet used in Experiment 10, which showed a relatively low oxidase content, but still higher than normal, had only a slight curling of the leaves.

It is fully realized that these experiments are subject to the criticism

[1] Shaw, Harry B., "The Curly-Top Disease of Beets," U. S. Dept. of Agr., Bureau of Plant Industry, *Bull.* 181, 1910.

that the juices of the two sets of leaves as prepared for the experiments may not be comparable. It may be merely an expression of the fact that one set of leaves is richer in cells than the other, or it might be that one is richer in water or in cellulose than the other. It is hoped to settle these questions in a future communication by paralleling the oxidase determinations with determinations of water, nitrogen, etc. Since the pathological leaves had in some cases more than three times the oxygen-absorbing power of the controls, it seems hardly possible, however, that this symptom can depend upon mere differences in the composition of the leaves.

Discussion of Results.

The main object of this paper is to describe a new method for the estimation of oxidases in plant juices. The method has been tested upon a number of samples of potato juice and found to give results in good agreement. There are still slight deviations between the results of duplicate determinations and efforts are being made at present to reduce these to a minimum. The main source of error lies in the rise of pressure within the oxidase apparatus, as soon as the shaking is begun.[1]

A number of experiments have been carried out in which the influence of the variable factors of the method on the end result has been studied. Incidentally several very interesting facts came to light in the course of these experiments. The most important of these perhaps is the fact that only a very definite and limited quantity of oxygen is absorbed by pyrogallol in the presence of a definite quantity of potato juice within a short period of time, say 2 or 3 hours. Oxidation of the pyrogallol will proceed after that time but at a rate which is not measurable under the conditions of the experiment.

The concentration and total quantity of pyrogallol present is without effect on the end result, provided the pyrogallol is in excess. Within the limits of the experiments, the amount of the chemical change is directly proportional to the concentration of the oxidase present, all other factors remaining the same; doubling the volume of potato juice added doubles the volume of oxygen absorbed.

Chodat[2] working with Lactarius juice could not confirm the law of direct proportionality which he and Bach propounded for the action of peroxidase, but has experimental indications that the discrepancy is due to the inadequacy of his technique.

These facts are in contradiction to our conception of enzyme action in general. We are accustomed to look at enzymes as catalytic agents, quite analogous in their mode of action to the inorganic catalysts. If

[1] At present this difficulty is nearly entirely overcome by allowing the apparatus and the contents to stay at the temperature of experimentation for at least 30 minutes.

[2] "Mode de l'action de l'oxydase," *Arch. sci. phys. nat.*, 19, 501.

the substances in the potato juice which are responsible for the rapid absorption of oxygen by the pyrogallol were enzymes in the accepted sense of the word one would expect small quantities of the juice to bring about the oxidation of relatively large quantities of pyrogallol and that the oxidation would continue as long as pyrogallol and free oxygen are present, or until the activity of the juice is lost by deterioration. In the reaction discussed in this article, the process comes to completion when only a small definite portion of the pyrogallol is oxidized and while there is still an abundance of oxygen.

It seems therefore that the oxidase in potato juice accelerating the oxidation of pyrogallol by atmospheric oxygen is not an enzyme in the customary sense of the word, but rather a substance entering directly into the reaction and destroyed in the course of the same.

With the exception of a few isolated cases there exists no conception of what the composition of the so-called oxidases is; there are only theories as to their mode of action, and, on account of the diversity of the reactions they accelerate or bring about, as the case may be, we have not even a satisfactory definition to cover all of them. A starting point in their exact study must be made and it seemed to the writer necessary to take one type of reaction after another and correlate them, if possible, at the end. In this paper only the oxidation of pyrogallol by atmospheric oxygen has been considered and the method here worked out serves simply as a measure of the weight of oxygen that pyrogallol is capable of taking up in neutral aqueous solutions, due to the interaction of a certain volume of plant juice.

After the study of pyrogallol from this point of view has been exhausted, other compounds, such as hydroquinone, thymol, tannic acid, various sugars, etc., will be used. Then the reaction of the medium will be varied. It is hoped that on the basis of the experiments the oxidases may be classified.

Since it is desirable to express the strength of the juice in terms of some standard, the writer proposes as a unit for future experiments an oxidase solution of such strength that one liter of it will be capable of bringing about the consumption by pyrogallol of the equivalent of 1 gram of hydrogen, *i. e.*, of 8 grams of oxygen. This unit of "strength" may not have any relation to the rate of the absorption as it refers here explicitly only to the total amount absorbed. It is customary to let the "activity" of an enzyme be measured by the rate of action. It is an interesting question for future investigation whether the strength of an oxidase solution as expressed by this proposed standard is proportional to the rate at which the absorption takes place.